Disruption of endothelial adherens junction by invasive breast cancer cells is mediated by reactive oxygen species and is attenuated by AHCC

Mehran Haidari a,b,⁎, Wei Zhang b, Koji Wakame c

a Department of Internal Medicine, Division of Cardiology, The University of Texas Health Science Center at Houston, USA
b Texas Heart Institute at St. Luke’s Episcopal Hospital, Houston, TX, USA
c Research and Development Division, Amino Up Chemical Co., Ltd., Sapporo, Japan

A R T I C L E   I N F O

Article history:
Received 12 July 2013
Accepted 25 October 2013

Keywords:
Adherens junction
VE-cadherin
Active hexose correlated compound (AHCC)
Breast cancer cells
Reactive oxygen species

A B S T R A C T

Aims: The effect of antioxidants on treatment of cancer is still controversial. Previously, we demonstrated that interaction of breast cancer cells with endothelial cells leads to tyrosine phosphorylation of VE-cadherin and disruption of endothelial adherens junction (EAJ). The molecular mechanism underlying the anti-metastatic effects of mushroom-derived active hexode correlated compound (AHCC) remains elusive.

Main methods: Several cellular and biochemical techniques were used to determine the contribution of oxidative stress in the disruption of EAJ and to test this hypothesis that AHCC inhibits the breast cancer cell-induced disruption of EAJ.

Key findings: Interaction of breast cancer cells (MDA-MB-231 cells) with human umbilical vein endothelial cells (HUVECs) leads to an increase in generation of reactive oxygen species (ROS). Treatment of HUVECs with H2O2 or phorbol myristate acetate (PMA) led to tyrosine phosphorylation of VE-cadherin, dissociation of β-catenin from VE-cadherin complex and increased transendothelial migration (TEM) of MDA-MB-231 cells. Induction of VE-cadherin tyrosine phosphorylation by PMA or by interaction of MDA-MB-231 cells with HUVECs was mediated by HRas and protein kinase C-α signaling pathways. Disruption of EAJ and phosphorylation of VE-cadherin induced by interaction of MDA-MB-231 cells with HUVECs were attenuated when HUVECs were pretreated with an antioxidant, N-acetylcysteine (NAC) or AHCC. AHCC inhibited TEM of MDA-MB-231 cells and generation of ROS induced by interaction of MDA-MB-231 cells with HUVECs.

Significance: Our studies suggest that ROS contributes to disruption of EAJ induced by interaction of MDA-MB-231 cells with HUVECs and AHCC attenuates this alteration.

⁎ Corresponding author at: 6770 Bertner Ave, C1000, Houston, TX 77030, USA. Tel.: +1 832 355 9077; fax: +1 832 355 9333.
E-mail address: Mehran.Haidari@uth.tmc.edu (M. Haidari).

Introduction

Extensive research has revealed the mechanisms by which continued oxidative stress can lead to cancer (Reuter et al., 2010). Reactive oxygen species (ROS) has been reported to play a major role in tumor initiation and survival induced by a variety of agents both in animal models and humans (Trush and Kensler, 1991; Cerutti, 1985; Slaga et al., 1981) by mediating cellular signal transduction pathways. Transendothelial migration (TEM) of melanoma cells is enhanced by ROS (Cheng et al., 2004). Although there is considerable evidence of anticancer effects of antioxidants from cell culture and animal studies, the results from observational studies and interventional trials are inconsistent (Nagel et al., 2010; Kimmick et al., 1997; Lin et al., 2009).

The molecular mechanisms regulating tumor cell migration across endothelial cells are poorly understood, but clearly depend on the invasive capacity of tumor cells and their ability to breach the endothelial cell barrier. The molecular mechanisms that regulate invasiveness of cancer cells were the focuses of the majority of previous studies that explore metastasis of cancer cells. Therefore, the protective role of endothelial cells in prevention of migration of cancer cells through endothelial cells remained underappreciated. In this study we focused on the integrity of endothelial barrier function as a protective factor in cancer metastasis. Selective barrier function of vascular endothelial cells is accomplished by a number of transmembrane cell–cell adhesion proteins that, when coupled with their binding partners, contribute to the adhesion of one endothelial cell to another. The adherens junction complex, comprised of cadherins and the catenins, is a major adhesion structure in endothelial cells that connects to the actin cytoskeleton (Xiao et al., 2003; Hartsock and Nelson, 2008). Vascular endothelial-cadherin (VE-cadherin) is found specifically in the endothelial adherens junction (EAJ) and has been implicated in playing a fundamental role in controlling the transport across the endothelial barrier and in regulating angiogenesis (Iyer et al., 2004; Vestweber, 2008). Tyrosine phosphorylation of VE-cadherin has been implicated in the disruption of EAJ, and desis of leukocytes/metastatic cancer cells (Alcaide et al., 2008; Potter...
et al., 2005; Weis et al., 2004). In our recent study we demonstrated that interaction of invasive breast cancer cells with HUVECs leads to disruption of EAJ through an increase in VE-cadherin tyrosine phosphorylation (Haidari et al., 2012a). In the present study we sought to determine if oxidative stress contributes to disruption of EAJ induced by interaction of invasive breast cancer cells with endothelial cells.

Active hexose correlated compound (AHCC) is an extract prepared from cocultured mycelia of the Lentinula edodes mushroom. During long-term culture, saccharolytic and proteolytic enzymes are produced by basidiomycete. The majority of previous studies that explored the mechanism of action of AHCC referred to its immunomodulatory function as the mechanisms behind its benefits (Shah et al., 2011).

In this study we tested this hypothesis that AHCC alters the endothelial signal transduction in a way that protects the integrity of endothelial barrier against disruption of EAJ induced by invasive breast cancer cells.

Materials and methods

Reagents and antibodies

Phospho-specific and nonphospho-specific antibodies against Src (pY416), and β-catenin, were purchased from Abcam (Cambridge, MA, USA). Phospho-specific antibodies and nonphospho-specific antibodies against VE-cadherin (Y731) were purchased from Invitrogen (Camarillo, CA, USA). AHCC was provided by AminoUp Chemical Co., Ltd., Sapporo, Japan. Hydrogen peroxide (H2O2) and [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay kit, phorbol myristate acetate (PMA), N-acetylcysteine (NAC), and Src inhibitor, 1-tert-Butyl-3-(4-chlorophenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP2) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pre-made recombinant Ras N17 (dominant negative [DN]), protein kinase C-α (PKC-α), null control and GFP adenoviruses, and ViraDuctin adenovirus transduction reagents were purchased from Cell Biolabs, Inc. (San Diego, CA, USA).

Cells

Human umbilical vein endothelial cells (HUVECs), human aortic endothelial cells (HAEC), human breast cancer cells (MDA-MB-231), human ovarian and prostatic cancer cells (SKOV3, PC-3), were purchased from ATCC. HUVECs and HAEC were grown in Lonza’s EGM-2-MV medium on collagen-coated (20 μg/ml) tissue culture dishes. HUVECs and HAEC from fewer than 4 generations were used for all experiments. The cancer cells were maintained in DMEM medium with 10% heat-inactivated FCS.

Interaction of breast cancer cells and HUVECs

To study the signal transductions in HUVECs that is induced after interaction with cancer cells, cancer cells were detached from culture flask with trypsin, were washed and then added to HUVECs (3 × 10^6 cancer cells per 1 × 10^6 HUVECs). To detach cancer cells from endothelial cells, HUVECs were washed four times with cold PBS. The microscopic examination showed that breast cancer cells were firmly attached to HUVECs 10 min after their addition and could not be removed by vigorous washing. Therefore, to avoid the contamination of HUVEC lysates with cancer cells a maximum time of 7 min was chosen for the Western blot experiments of HUVECs.

Transduction of adenovirus

The conditions used for the transduction of recombinant adenoviruses were optimized by using adenovirus encoding GFP. All reagents and kits, including transduction reagents, an adenovirus purification kit, and an adenovirus titration kit, were purchased from Cell Biolabs, Inc. After purification, the titration of each recombinant adenovirus was determined by an ELISA titrating kit. HUVECs were seeded into 6-well plates for 24 h until they reached 80% confluence. According to the manufacturers’ protocol, adenovirus was transduced into cells by using ViraDuctin (Cell Biolabs, Inc.). HUVECs were infected with adenoviral vectors with a multiplicity of infection (MOI) of 100 plaque-forming units per cell in the presence of ViraDuctin. After incubation with viral particles for 48 h, the cells were assessed for the expression of the transduced genes. The efficacy of all recombinant adenoviruses was previously tested (Haidari et al., 2011, 2012a,b).

Intracellular ROS measurement

Intracellular ROS generation in HUVECs was examined by flow cytometry using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR). DCFHDA is a lipid permeable nonfluorescent compound and is oxidized by intracellular ROS to form the lipid impermeable and fluorescent compound DCF. The HUVECs were cultured in six-well plates until 90–100% confluence. HUVECs were pre-incubated with 10 μM of DCFHDA for 30 min then MDA-MB-231 cells were added to HUVECs for 5 min. HUVECs were washed four times with PBS, trypsinized (0.05% trypsin) and then resuspended in phosphate buffer (1 ml) to a final concentration of 5 × 10^7 cells/ml. ROS generation of these cells were determined by flow cytometry (FACscan, Becton-Dickinson, CA) using 488 nm for excitation and 525 nm for emission.

Cell viability assay

The effect of AHCC and other chemicals on the viability of HUVECs was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay as described (Haidari et al., 2009).

Western blotting

HUVECs were grown to confluence in 35-mmol/l dishes or 6-well plates. Cells were extracted in radioimmunoprecipitation assay (RIPA) buffer, which contained 0.1% sodium dodecyl sulfate (SDS), 1% deoxycholate, 1% NP-40, 10 mmol/l sodium phosphate, 150 mmol/l NaCl, 2 mmol/l EDTA, 50 mmol/l NaF, 5 mmol/l sodium pyrophosphate, 0.1 mmol/l sodium vanadate, 2 mmol/l PMSF, 0.1 mg/ml leupeptin, and 100 μl/ml aprotinin. Samples were loaded onto an SDS-polyacrylamide electrophoresis (PAGE) gel and run at 150 V for 1 h. The proteins were then transferred onto nitrocellulose paper at 300 mA for 1.5 h, followed by Western blot analysis. Blots were blocked with 5% dry milk in 0.1% Tween 20 in phosphate-buffered saline (PBS) for 1 h at room temperature. The primary antibodies were used at a dilution of 1:500 to 1:1000. All antibodies were added for 1 h at room temperature or overnight at 4 °C. After washing, the appropriate secondary antibodies (Pierce) were added at a dilution of 1:10,000 for 1 h at room temperature. After extensive washing, blots were developed with the Super Signal enhanced chemiluminescence kit (Pierce) and visualized on Kodak-AR film.

Immunoprecipitation

Cells were grown to confluence, washed twice gently with ice-cold PBS, and lysed in 1 ml of RIPA lysis buffer. After 10 min on ice, cell lysates were collected and preclarced for 30 min at 4 °C with protein A-agarose. After centrifugation (14,000 × g, 15 s at 4 °C), the supernatants were incubated with primary antibodies (1 μg/ml of IgG) overnight at 4 °C with continuous mixing. Protein A agarose (40 μl) was added to the lysate, and, after 2 h at 4 °C, the beads were washed 3 times in lysis buffer, and proteins were eluted by boiling in SDS-sample.
buffer containing 4% 2-mercaptoethanol (Bio-Rad, Hercules, CA). The samples were analyzed by SDS-PAGE.

**Transendothelial migration (TEM) assay**

A kit from Cell Biolabs, Inc. was used for TEM assays according to the manufacturer’s instructions. MDA-MB-231 cells (25 × 10⁶ each) were resuspended in 1 ml of complete medium and incubated for 1 h at 37 °C in the presence of 50 μg/ml calcein-AM. After the cells were labeled, they were resuspended at a concentration of 3 × 10⁵ cell/ml in DMEM. MDA-MB-231 or (3 × 10⁵ in 100 μl) was added to the upper compartment of transwell chambers with 6.5-mm diameter and 8-μm pores for 4 h. To remove non-migrating cells, the apical side of the filter was scraped gently with cotton wool and discarded; only cells that attached to the bottom side of the filter or migrating tumor cells were quantified with an Ultra384 plate reader (Tecan) by using 485 and 535 nm excitation and emission filters, respectively.

**Results**

High level of reactive oxygen species (ROS) increases tyrosine phosphorylation of VE-cadherin and dissociates β-catenin from VE-cadherin complex

Tyrosine phosphorylation of VE-cadherin has been implicated in the disassembly of EAJ components, and increased transendothelial migration of monocytes/tumor cells (Alcaide et al., 2008; Potter et al., 2005; Weis et al., 2004). Previously our group demonstrated that interaction of highly invasive human breast cancer cells (MDA-MB-231) with HUVECs leads to tyrosine phosphorylation of VE-cadherin. This was accompanied by activation/phosphorylation of Src tyrosine kinase, which is a regulator of VE-cadherin tyrosine phosphorylation (Haidari et al., 2011, 2012a,b). The integrity of EAJ depends on the association of VE-cadherin with β-catenin (Potter et al., 2005). The interaction of MDA-MB-231 cells with HUVECs also led to dissociation of β-catenin from VE-cadherin (Haidari et al., 2012a).

In this study we sought to test this hypothesis that reactive oxygen species (ROS) mediates disruption of EAJ induced by interaction of...
invasive breast cancer cells with endothelial cells. Our study demonstrated that interaction of MDA-MB-231 cells with HUVECs for 5 min increases ROS generation in the endothelial cells in a dose-dependent manner (Fig. 1A). In this experiment MDA-MB-231 cells were not attached to HUVECs after 5 min. In addition, to avoid contamination of HUVECs with MDA-MB-231 cells, HUVECs were washed vigorously (after 5 min of interaction with MDA-MB-231 cells) and then used for the flow cytometric measurement of ROS. To determine if increased levels of ROS result in tyrosine phosphorylation of VE-cadherin hydrogen peroxide (H2O2) and phorbol myristate acetate (PMA), a tumor promoter that increases intracellular ROS generation were added to HUVECs. Treatment of HUVECs with 500 μM of H2O2 or 50 nM of PMA for 1 h did not show any significant toxicity on endothelial cells, using MTT assay (data no shown). The effect of PMA on generation of ROS in HUVECs at the concentration that was used in our study has been previously demonstrated (Elgini et al., 2005; Görlach et al., 2000). As indicated in Fig. 1B,C H2O2 increased tyrosine phosphorylation of VE-cadherin and Src in a dose dependent manner. When HUVECs were treated with increasing concentrations of PMA for 30 min tyrosine phosphorylation of VE-cadherin was increased (Fig. 1D). In addition, treatment of HUVECs with 50 nM of PMA for 30 min disrupted structure of EJA by dissociating β-catenin from VE-cadherin (Fig. 2A). Furthermore, treatment of HUVECs with 50 nM of PMA for 30 min increased transendothelial migration of MDA-MB-231 cells (Fig. 2B). To determine TEM of MDA-MB-231 cells in this experiment HUVECs were pretreated with PMA for 30 min then PMA was removed from HUVECs and MDA-MB-231 cells were added to HUVECs. In our previous studies we demonstrated that H-Ras mediates tyrosine phosphorylation of VE-cadherin induced by interaction of MDA-MB-231 cells with HUVECs (Haidari et al., 2012a). PMA is known as an activator of protein kinase C (PKC) family. We compared the role of HRas and PKC-α, as a representative of PKC classical isoforms in tyrosine phosphorylation of VE-cadherin induced by interaction of MDA-MB-231 cells with HUVECs. Our experiment demonstrates that inhibition of PKC-α by the dominant negative adenovirus recombinant blocked PMA-induced tyrosine phosphorylation of VE-cadherin (Fig. 2C). To a lesser extent, inhibition of HRas by the dominant negative adenovirus recombinant attenuated tyrosine phosphorylation of VE-cadherin induced by PMA (Fig. 2C). Next

**Fig. 2.** PMA dissociates β-catenin from VE-cadherin complex and increases transendothelial migration of MDA-MB-231 cells. A. Addition of PMA to HUVECs led to the dissociation of β-catenin from VE-cadherin. PMA (50 nM) was added to HUVECs, and after 30 min, HUVECs were washed and used for the immunoprecipitation assay. mlgG, mouse non-immune IgG. B. Transendothelial migration of MDA-MB-231 cells was increased after addition of the indicated concentrations of PMA for 30 min, using transwell chamber assay. After addition of MDA-MB-231 cells (3 x 10^5 cells per well for 4 h) the migrating tumor cells were quantified with a plate reader by using 485 and 535 nm excitation and emission filters, respectively. Data are expressed as the mean ± SD from triplicate experiments. C. PMA-induced tyrosine phosphorylation of VE-cadherin was attenuated when HRas, and PKC-α were inhibited. HUVECs were transduced with the indicated adenovirus, and after 48 h, 50 nM PMA was added to HUVECs for 30 min. D. Induction of VE-cadherin tyrosine phosphorylation by MDA-MB-231 cells was attenuated after inhibition of HRas and PKC-α by the dominant negative adenovirus recombinant forms. HUVECs were transduced with the indicated adenovirus, and after 48 h, MDA-MB-231 cells (3 x 10^6 MDA-MB-231 cells per 1 x 10^6 HUVECs) were added to HUVECs for 5 min. BCC, breast cancer cells, MDA-MB-231 cells. *P < 0.05, **P < 0.01, ***P < 0.001, vs control. Each experiment was independently performed 3 to 4 times.
we compared the contribution of HRas and PKC-α in tyrosine phosphorylation of VE-cadherin induced by interaction of MDA-MB-231 cells with HUVECs. As shown in Fig. 2D inhibition of HRas blocked tyrosine phosphorylation of VE-cadherin, while inhibition of PKC-α reduced but not blocked tyrosine phosphorylation of VE-cadherin. The inhibitory effects of adenovirus dominant-negative forms of HRas, and PKC-α were confirmed as described previously (Haidari et al., 2011, 2012a,b).

Inhibition of Src blocks disruption of endothelial adherens junction induced by PMA

VE-cadherin tyrosine phosphorylation is regulated by Src tyrosine kinase (Haidari et al., 2011, 2012a). To determine if induction of tyrosine phosphorylation of VE-cadherin by PMA is mediated by Src, we used an inhibitor of Src, PP2. Inhibition of Src by pretreatment of endothelial cells with PP2 diminished VE-cadherin tyrosine phosphorylation induced by PMA (Fig. 3A). In addition, inhibition of VE-cadherin tyrosine phosphorylation by PP2 attenuated dissociation of β-catenin from VE-cadherin complex induced by PMA (Fig. 3B). In addition, treatment of HUVECs with PP2 attenuated H2O2-induced tyrosine phosphorylation of VE-cadherin (Fig. 3C). As shown in Fig. 3D pretreatment of HUVECs with PP2 attenuated PMA-induced TEM of MDA-MB-231 cells. Treatment of HUVECs with H2O2, PMA and PP2 has no significant effect on expression of β-catenin (Fig. 3E). Treatment of endothelial cells with 50 μM of PP2 for 2 h did not show any significant toxicity on endothelial cells, using MTT assay (data not shown). These results suggest that induction of VE-cadherin tyrosine phosphorylation by PMA or H2O2 is mediated by Src tyrosine kinase.

Treatment of endothelial cells with an antioxidant, N-acetylcysteine (NAC) diminished MDA-MB-231 cells-induced disruption of endothelial adherens junction

To determine if ROS mediate MDA-MB-231 cells-induced tyrosine phosphorylation of VE-cadherin HUVECs were pretreated with an antioxidant N-acetylcysteine (NAC). As shown in Fig. 4A,B NAC attenuated induction of VE-cadherin and Src tyrosine phosphorylation by MDA-MB-231 cells. In addition, pretreatment of HUVECs with NAC for 2 h inhibited transendothelial migration of MDA-MB-231 cells in a dose-dependent manner (Fig. 4C). To determine if the attenuating effect of NAC on TEM of MDA-MB-231 cells is due to the effect of NAC on HUVECs or MDA-MB-231 cells, HUVECs were pretreated with NAC and after 2 h were washed. Then we added MDA-MB-231 cells and studied the migrating tumor cells after 30 min, 1 h, 2 h and 4 h and compared it with untreated cells. The results showed that in all time points there was a reduction in TEM of MDA-MB-231 cells. However, attenuating effect

Fig. 3. Inhibition of Src attenuates PMA-induced disruption of endothelial adherens junction. A. Pretreatment of HUVECs with 10 μM of Src inhibitor, PP2 for 2 h, attenuated tyrosine phosphorylation of VE-cadherin induced by treatment of HUVECs with PMA (50 nM for 30 min). B. Pretreatment of HUVECs with 10 μM of PP2 for 2 h, attenuated dissociation of β-catenin from VE-cadherin complex induced by PMA (50 nM for 30 min). C. Pretreatment of HUVECs with 10 μM of Src inhibitor, PP2 for 2 h, attenuated tyrosine phosphorylation of VE-cadherin induced by treatment of HUVECs with H2O2 (100 μM for 30 min). D. TEM of MDA-MB-231 cells after stimulation with PMA was attenuated by pretreatment of HUVECs with PP2 (10 μM, 2 h), using transwell chamber assay. E. Treatment of HUVECs with H2O2 (100 μM, 30 min), PMA (50 nM, 30 min) and PP2 (10 μM, 2 h) did not show significant effect on expression of β-catenin. mlgG, mouse non-immune IgG. *P < 0.05, **P < 0.001, vs control. Each experiment was independently performed 3 to 4 times.
of NAC reduced with longer incubation time with MDA-MB-231 cells (65, 49, 34 and 20% relative reduction in number of migrating cells after 30 min, 1 h, 2 h and 4 h of incubation, respectively).

AHCC attenuates MDA-MB-231 cells-induced tyrosine phosphorylation of VE-cadherin and inhibits dissociation of $\beta$-catenin from VE-cadherin complex

To determine the molecular mechanism(s) underlying the anticancer effects of AHCC we determine the effect of AHCC on MDA-MB-231 cells-induced tyrosine phosphorylation of VE-cadherin. We found that when HUVECs are pretreated with 1 mg/l of AHCC overnight the induction of VE-cadherin tyrosine phosphorylation by MDA-MB-231 cells is attenuated (Fig. 5A). Pretreatment of HUVECs with AHCC also attenuated the activation/phosphorylation of Src tyrosine kinase (Fig. 5B). In addition, addition of AHCC to HUVECs inhibited dissociation of $\beta$-catenin from VE-cadherin induced by interaction of MDA-MB-231 cells with HUVECs (Fig. 5C). As shown in Fig. 5D treatment of HUVECs with AHCC for 16 h did not change $\beta$-catenin expression. When HUVECs were pretreated with AHCC transendothelial migration of MDA-MB-231 cells was reduced in a dose-dependent manner (Fig. 6A). AHCC attenuated generation of ROS by HUVECs induced by interaction of MDA-MB-231 cells (Fig. 6B). Furthermore, induction of VE-cadherin tyrosine phosphorylation with PMA was diminished when HUVECs were pretreated with 1 mg/l of AHCC for 16 h (Fig. 6C). Treatment of HUVECs with NAC and AHCC reduced basal and PMA-stimulated ROS generation (Fig. 6D). The attenuating effect of NAC and AHCC on TEM of MDA-MB-231 cells was additive (Fig. 6E). Treatment of endothelial cells with 0.5–3 mg/l concentrations of AHCC for 24 h did not show any significant toxicity on endothelial cells, using MTT assay (data not shown).

AHCC attenuates tyrosine phosphorylation of VE-cadherin induced by ovarian and prostatic cancer cells

We previously demonstrated that interaction of invasive ovarian and prostatic cancer cells with HUVECs induces tyrosine phosphorylation of VE-cadherin (Haidari et al., 2012a). To determine whether AHCC attenuates disruption of EAJ after the interaction of other invasive cancer cells, we studied invasive prostatic (PC-3) and ovarian (SKOV3) cancer cells. As shown in Fig. 7A and B, AHCC diminished VE-cadherin tyrosine phosphorylation induced by PC-3 and SKOV3 cells. To determine if the attenuating effect of AHCC on MDA-MB-231 cell-induced tyrosine phosphorylation of VE-cadherin is limited to HUVECs, human aortic endothelial cells (HAEC) were used. As shown in Fig. 7C attachment of MDA-MB-231 cells to HAECs increased VE-cadherin tyrosine phosphorylation of VE-cadherin is limited to HUVECs, human aortic endothelial cells (HAEC) were used. As shown in Fig. 7C attachment of MDA-MB-231 cells to HAECs increased VE-cadherin tyrosine phosphorylation of VE-cadherin induced by ovarian and prostatic cancer cells.

Discussion

The role of ROS in transendothelial migration of breast cancer cells has not been determined. In the present study we demonstrated that
ROS mediate disruption of EAJ and enhance transendothelial migration of invasive breast cancer cells through increasing phosphorylation of VE-cadherin. Treatment of endothelial cells with antioxidant, including AHCC protects endothelial cells against impairment of EAJ induced by interaction of invasive breast cancer cells with endothelial cells. Our studies suggest that AHCC attenuate phosphorylation of VE-cadherin induced after interaction of invasive breast, prostatic and ovarian cancer cells with endothelial cells.

Our group previously showed that interaction of invasive breast cancer cells with endothelial cells results in disruption of integrity of EAJ (Haidari et al., 2012a). Inhibition of HRas abolished VE-cadherin tyrosine phosphorylation induced by interaction of MDA-MB-231 cells with HUVECs. ROS is not only downstream but also upstream signaling molecules of protein kinase C, PKC (Inoguchi et al., 2003; Cosentino-Gomes et al., 2012). Because interaction of MDA-MB-231 cells with HUVECs led to increase in ROS, we tested this hypothesis that PKC contributes to VE-cadherin tyrosine phosphorylation induced by interaction of MDA-MB-231 cells with HUVECs. Inhibition of PKC-α blocked PMA-induced VE-cadherin tyrosine phosphorylation (Fig. 2C). HRas inhibition diminished PMA-induced VE-cadherin tyrosine phosphorylation but not to the extent of PKC-α inhibition. This suggests that HRas partially mediates PMA-induced VE-cadherin tyrosine phosphorylation. This finding is in line with a previous report showing the activation of Ras by ROS in endothelial cells (Wung et al., 1999). As indicated in Fig. 2D, inhibition of HRas abolished MDA-MB-231 cells-induced tyrosine phosphorylation of VE-cadherin while PKC-α suppression partially inhibited the alteration. This result suggests that HRas is the main factor in this alteration and PKC-α is acting as a contributory factor to the HRas effect. Based on this results one can speculate that induction of VE-cadherin tyrosine phosphorylation by HRas is mediated by both PKC-α dependent and independent pathways. Indeed, it has been demonstrated that PKC activates HRas downstream effector Raf-1 (Lorenz et al., 2003). In addition, the possible contribution of other PKC isoforms in VE-cadherin tyrosine phosphorylation induced by interaction of breast cancer cells with HUVECs must be considered for interpretation of our results.

Our studies suggest that AHCC attenuates disruption of EAJ by inhibiting MDA-MB-231 cells-induced tyrosine phosphorylation of VE-cadherin.
VE-cadherin. AHCC is primarily composed of carbohydrates (70%), protein (13%), ash contents (9%), fats (2%), and fiber (2%). A significant portion of the carbohydrates (approximately 20%) is composed of α1,4-glucansanil (Shah et al., 2011). It is manufactured by Amino Up Chemical Co., Ltd., Sapporo, Japan and used most predominantly in Japan as a supplement in cancer chemotherapy. The safety of AHCC has been confirmed in animal experiments and with healthy volunteers (Spierings et al., 2007; Fujii et al., 2011). The biological effects of AHCC have been attributed to its glucan fraction (Matsushita et al., 1998). Previous studies have proven that the oral administration of AHCC has a wide variety of therapeutic effects, including anticancer effects both in animal models and in clinical trials (Matsui et al., 2002a,b; Gao et al., 2005; Ye et al., 2003). Mushroom extracts are known to have immunomodulating and antitumor effects in humans as well as in animals (Shah et al., 2011). Several mushroom products possess potential anticancer activity in vitro or in animal models. AHCC administered to rats reduced the growth and metastasis of mammary adenocarcinoma cells (Fujii et al., 2011). The anticancer activity of AHCC has been ascribed to its immunomodulatory effects on both innate and adaptive immune responses (Gao et al., 2005). In this study we introduce a novel molecular mechanism for anti-cancer effects of AHCC, preserving integrity of endothelial barrier function against disruption by interaction of breast cancer cells. In consistent to our finding, previous studies indicated that AHCC is a potent antioxidant that protects against disorders induced by oxidative stresses (Ye et al., 2003, 2004). The component of AHCC that is responsible for its protective effects against disruption of endothelial barrier was not explored in this study and is currently under investigation.

Conclusions

Our study provides supportive evidence for the role of oxidative stress in disruption of endothelial barrier following interaction of breast cancer cells with endothelial cells and suggests that our findings may be extendable to other invasive cancer cells. In addition, our results suggest that AHCC has protective effects against the breakdown of endothelial cell barrier function induced by interaction of breast cancer cells with endothelial cells.

Conflict of interest statement

This work was funded by a grant from the Amino Up Chemical Company (Sapporo, Japan) and the MacDonald General Research Fund (grant # 09RDM002) to M.H. The authors have no conflict of interest to declare.
References


