$[CA^{2+}]_I$-INDEPENDENT SIGNALING MECHANISM IN TUMOR CELL-INDUCED ENDOTHELIAL JUNCTION DISASSEMBLY

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INTRODUCTION

Attachment of tumor cells to endothelial cells (EC) is critical for movement of tumor cells out of the vascular system to establish metastases. An increased expression of β1 as well as β3 integrins has been shown in malignant melanoma cells compared to normal melanocytes, suggesting a potential involvement of cell adhesion molecules in the metastatic cascade [1]. However, how these initial cell adhesion events between tumor and endothelial cells signal downstream pathways that directly regulate the integrity of endothelial barrier require further elucidation.

Intracellular calcium ($[Ca^{2+}]_i$) may play an important role in initiating a sequence of signaling events in response to environmental stimuli. Moreover, VE-cadherin is found to actively participate during most of the stages of transmigration of inflammatory cells. In this study, we monitored the $[Ca^{2+}]_i$ in human umbilical vein endothelial cells (HUVEC) following contacts with human melanoma cells. We show that transient rise in endothelial $[Ca^{2+}]_i$ was elicited specifically by tumor cells, and this response recruited the classical $[Ca^{2+}]_i$ release mechanisms in endothelial cells. Most importantly, we show that endothelial cells do not require $[Ca^{2+}]_i$ for the dissociation of VE-cadherin junctions in response to melanoma cell contact. These findings indicate a clear divergence from the classical leukocyte-endothelial signaling mechanism and suggest an important difference in endothelial signaling pathways recruited by tumor cells in breaching the vasculature.

MATERIALS AND METHODS

Cell culture and preparation. HUVEC (passages 5~9) were maintained in Ham's F12-K media supplemented with 10% fetal bovine serum (FBS; Biofluids Inc., Gaithersberg, MD), 10 μg/ml endothelial cell growth supplement, 100 μg/ml heparin (Sigma Chemical Co., St. Louis, MO), 100 units/ml penicillin-streptomycin (Biofluids Inc.) at 37 ^oC under 5 % CO_2. Prior to a Ca^{2+} assay, cells were incubated with 50 μM Fura-2-AM (Molecular Probes, Eugene, OR) for 30 min and then bathed in assay buffer (137 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 20 mM HEPES, 15 mM D-glucose, 1.5 mM $CaCl_2$, 0.1 % w/v fraction V bovine serum albumin, pH 7.4) for additional 30 min prior to $[Ca^{2+}]_i$ measurement. A2058 human melanoma cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS (Biofluids Inc.) in a standard cell culture condition. Melanoma cells were detached by a brief trypsinization and suspended in culture media for 1 hr at 37 ^oC. Cells were then washed twice with assay buffer and added to HUVEC monolayer in 4:1 during $[Ca^{+2}]_i$ measurement. In addition, tumor cell conditioned media (TCM) was obtained from the supernatant of suspended tumor cells which had been gently rocked for 1 hr. Co-culture media was prepared by incubating endothelial cells with melanoma in 1:4 concentration ratio for 12 hr. The procedure for a digital Ca^{2+} ratiometric assay is detailed elsewhere [2].

Pharmacological inhibitors. In some experiments, fura-2-AM-loaded HUVEC were incubated in culture media containing 1.0 μM thapsigargin (TG; Molecular Probes, Inc.) for 5 min or 2 ~ 10 μM U73122 (Sigma Chemical Co.) for 3 min, followed by a 30 min incubation in fresh media prior to an assay. For Western blot preparation, HUVEC were incubated with 2.6 μM Gö6976 (Calbiochem Co., San Diego CA) or 40 μM BAPTA-AM (Molecular Probes, Inc.) for 30 min following the cell contact assay.

Cell contact assay and Western blots. At designated times following the introduction of melanoma cells (4:1), HUVEC were scraped and resuspended into PBS at 4^oC. Whole cell extracts were prepared by resuspending cells in lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA [pH 8.0], 2 mM Na_3VO_3, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1 % NP-40, 1 mM PMSF, 2 ng/ml pepstatin A). Lysates were incubated on ice for 30 min followed by a centrifugation at 16,000 g for 5 min at 4 ^oC. The pellet was discarded and the supernatant was mixed with 2×SDS running buffer (0.2 % bromophenol blue, 4 % SDS, 100 mM Tris [pH 6.8], 200 mM DTT, 20 % glycerol) in 1:1 ratio. 30 μl was loaded onto a 6 % SDS-PAGE gel and the protein was transferred to 0.2 μm nitrocellulose filter (Schleicher and Schuell, Keene, NH) by electroblotting. Primary

antibodies included anti-VE-cadherin (mouse monoclonal IgG1; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-phospho-tyrosine (mouse monoclonal IgG1; Cell Signaling Technology, Beverly, MA). Secondary antibody was peroxidase-conjugated goat anti-mouse IgG (Sigma Chemical Co.).

RESULTS

Endothelial $[Ca^{2+}]_i$ activation in response to tumor cell contact occurs through PLC-IP_3 pathway. Endothelial cells responded to melanoma cell contact with a single sharp $[Ca^{2+}]_i$ peak, followed by a plateau declining to approach baseline over time (Figure 1A). Melanoma cell contact induced an increase of 120.7 ± 21.6% in peak $[Ca^{2+}]_i$ (n=11) magnitudes over resting baseline $[Ca^{2+}]_i$. Polystyrene beads did not induce significant $[Ca^{2+}]_i$ response. Neither TCM nor the supernatant obtained from a co-culture induced $[Ca^{2+}]_i$. Taken together, these data indicate that a specific cell-cell contact was required to trigger $[Ca^{2+}]_i$; mechanical effects and soluble factors did not contribute to eliciting $[Ca^{2+}]_i$ response. To ascertain the initial signaling events following tumor cell contact with the endothelium, U73122, an inhibitor of phospholipase C (PLC), was used. A dose-dependent attenuation of $[Ca^{2+}]_i$ was observed in endothelial cells following the tumor cell contact. A complete abrogation of $[Ca^{2+}]_i$ response was observed at 10 μM (n=4). Moreover, TG, an irreversible inhibitor of endoplasmic reticulum (ER) Ca^{2+}-ATPase, blocks Ca^{2+} uptake into intracellular Ca^{2+} stores, was applied to EC at 1.0 μM for 5 min. Perfusing tumor cells did not further elicit endothelial $[Ca^{2+}]_i$ activity over the elevated baseline $[Ca^{2+}]_i$. These results indicate that that $[Ca^{2+}]_i$ response utilizes PLC / IP3 pathways that release Ca^{2+} from the ER stores following tumor-endothelium contacts.

Tyrosine phosphorylation of VE-cadherin does not require $[Ca^{2+}]_i$. Melanoma contact with EC monolayer induced a time-dependent increase in tyrosine phosphorylation, as shown in Figure 1B. Tyrosine phosphorylation reached a maximum at 45 min following the introduction of tumor cells. Furthermore, as shown in Figure 2, normalized levels of tyrosine-phosphorylation of VE-cadherin following tumor contact with endothelium (45 min) was not significantly different compared to the positive control (lane 2; Figure 2) when intracellular Ca^{2+} chelating agent (BAPTA-AM) was used at 40 μM (lane 4, p=0.17; Figure 2). PLC inhibitor U73122 used at 10 μM did not affect phosphotyrosine levels (lane 5; Figure 2). TG-treatment, which was shown to eliminate further endothelial $[Ca^{2+}]_i$ activity following tumor contact, also had no effect (lane 6; Figure 2). As a potential candidate for the downstream effector of $[Ca^{2+}]_i$ pathway, protein kinase C (PKC) was targeted using a specific inhibitor Gö6976. However, the levels of the phosphorylated tyrosine following the tumor-endothelial contact in presence of this PKC inhibitor (lane 7; Figure 2) was not significantly different from the positive control to indicate that PKC pathways may not be important in regulation of VE-cadherin activity.

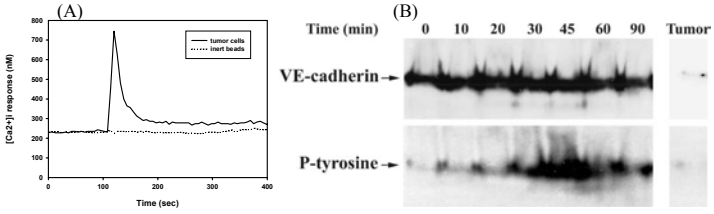

Figure 1. Tumor cell contact with endothelium induces an increase in (A) EC $[Ca^{2+}]_i$ & (B) tyrosine phosphorylation

DISCUSSION

Melanoma cells resemble leukocytes in inducing endothelial $[Ca^{2+}]_i$, which was specifically generated by cell-cell interaction and not by mechanical force or soluble factors. We suggest that a specific pool of intracellular Ca^{2+} sensitive to PLC/IP_3-dependent pathways, is induced in response to melanoma-endothelial contacts. In addition, VE-cadherin has been suggested to play a major role in regulating tumor cell transendothelial migration [3]. However, lack dependence of VE-cadherin regulation on endothelial $[Ca^{2+}]_i$ has been shown by using the PLC inhibitor U73122. Furthermore, draining the intracellular Ca^{2+} stores by application of TG had no impact on VE-cadherin phosphorylation following melanoma cell contact. These results suggest that VE-cadherin junctional disassembly following melanoma cell contact with the endothelium does not require endothelial $[Ca^{2+}]_i$. As a comparison, endothelial Ca^{2+} is considered to be a critical upstream second messenger during leukocytic diapedesis, especially in transvascular migration of monocytes through microvascular endothelial cells [4]. In their study, absence of Ca^{2+} significantly reduced the number of transmigrating monocytes while not affecting the cell adhesion as well as granulocyte extravasation [4]. Therefore, these studies suggest that the involvement of $[Ca^{2+}]_i$ may be dependent on the type of transmigrating cells. PKCα have been shown to play a direct role in VE-cadherin dissociation in response to thrombin stimulation [5]. However, our data indicated that PKC was not important in mediating the melanoma-initiated disassembly of VE-cadherin junctions. Taken together, these observations suggest a novel, $[Ca^{2+}]_i$-independent mechanism by which melanoma cells initiate breakdown of endothelial adherens junctional barrier and invade at the sites of secondary metastasis.

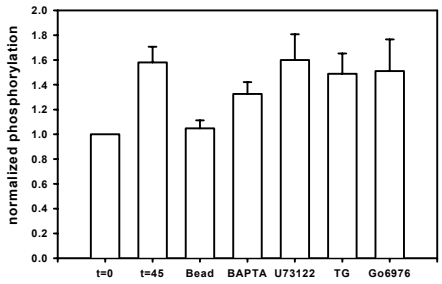

Figure 2. Inhibitors had no effect on phosphotyrosine levels

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