Endothelial Cell Ca^{2+} Increases upon Tumor Cell Contact and Modulates Cell–Cell Adhesion

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Abstract

The signal transduction mechanisms involved in tumor cell adhesion to endothelial cells are still largely undefined. The effect of metastatic murine melanoma cell and human prostate carcinoma cell contact on cytosolic $[Ca^{2+}]$ of bovine artery endothelial cells was examined in indo-1–loaded endothelial cell monolayers. A rapid increase in endothelial cell $[Ca^{2+}]$ occurred on contact with tumor cells, but not on contact with 8-μm inert beads. A similar increase in endothelial cell $[Ca^{2+}]$ was observed with human neutrophils or monocyte-like lymphoma cells, but not with endothelial cells, red blood cells, and melanoma cell–conditioned medium. The increase in endothelial cell $[Ca^{2+}]$ was not inhibited by extracellular Ca^{2+} removal. In contrast, endothelial cell pretreatment with thapsigargin, which releases endoplasmic reticulum Ca^{2+} into the cytosol and depletes this Ca^{2+} store site, abolished the cytosolic $[Ca^{2+}]$ rise upon melanoma cell contact. Endothelial cell pretreatment with the membrane-permeant form of the Ca^{2+} chelator bis-(*O*-aminophenoxyl)ethane-*N,N,N′,N′*-tetraacetic acid blocked the increase in cytosolic $[Ca^{2+}]$. Under static and dynamic flow conditions (0.46 dyn/cm^2) bis-(*O*-aminophenoxyl)ethane-*N,N,N′,N′*-tetraacetic acid pretreatment of bovine pulmonary artery endothelial cell monolayers inhibited melanoma cell adhesion to the endothelial cells. Thus, tumor cell contact with endothelial cells induces a rapid Ca^{2+} release from endothelial intracellular stores, which has a functional role in enhancing cell-cell adhesion. (*J. Clin. Invest.* 1993. 92:3017–3022.) Key words: melanoma • endothelium • adhesion • calcium

Introduction

The interaction of tumor cells with postcapillary venule endothelium and subendothelial basement membrane is crucial in modulating the frequency of organ-specific metastasis (1). Specific adhesion molecules on endothelial cells (EC)[1] and tumor cells (TC) are involved in this process. Melanoma cell adhesion occurs to unactivated EC in vitro and may be modulated by the interaction between glycosphingolipids (2). EC activation with inflammatory cytokines, such as interleukin 1, tumor necrosis factor-α and interleukin 4, increases TC adhesion through the expression of newly synthesized endothelial adhesion molecules (3, 4), such as the endothelial-leukocyte adhesion molecule-1 and the vascular cell adhesion molecule-1. The same adhesion molecules are also involved in leukocyte adhesion to EC (5, 6). It is noteworthy that these adhesion molecules contain an extracellular Ca^{2+} binding site and require Ca^{2+} to be functionally active (7, 8).

Alterations of cytosolic $[Ca^{2+}]$ (Ca_i), and extracellular $[Ca^{2+}]$ (Ca_o) may influence endothelial cell–leukocyte interaction. Exposure of EC to the calcium ionophore A23187 stimulates secretion of von Willebrand factor and translocation of a selectin adhesion molecule, the intracellular granule membrane protein (GMP-140), to the cell surface (9). GMP-140 is involved in neutrophil early adhesion to EC and can also be recognized by the tumor-associated antigens sialosyl Le^a and sialosyl Le^x (10). Furthermore, the calcium ionophore A23187 acts synergistically with suboptimal concentrations of phorbol ester to augment the expression on EC of the intercellular adhesion molecule-1 (ICAM-1), involved in strengthening neutrophil adhesion (11). Interestingly, in a recent study on heterotypic cell–cell adhesion, neutrophils were found to respond with a transient rise in Ca_i upon binding to interleukin-1–activated EC (12). However, the functional role of Ca^{2+} in the signal transduction mechanisms that modulate cell–cell and, more specifically, TC–EC interactions is poorly understood. In the present study, we examined the effect of tumor cell–EC contact on Ca_i homeostasis of the endothelium and the role of endothelial Ca_i in melanoma cell–EC adhesion.

Methods

Cell cultures. Murine melanoma cells B16/G3.26 (a generous gift from Dr. Christopher Stackpole), which are highly metastatic to the lung (13), metastatic human prostate carcinoma cells (TSU-pr1 and LNCap) (American Type Culture Collection, Rockville, MD), bovine pulmonary artery endothelial cells (BPAEC) (American Type Culture Collection), and bovine aorta endothelial cells (BAEC) (National Institute on Aging Cell Repository) were cultured in Dulbecco's modified Eagle's medium (National Institutes of Health Medium Center, Be-

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1. *Abbreviations used in this paper:* BAEC, bovine aorta endothelial cells; BAPTA-AM, bis-(*O*-aminophenoxyl)ethane-*N,N,N′,N′*-tetraacetic acid; BPAEC, bovine pulmonary artery endothelial cells; Ca_i, cytosolic $[Ca^{2+}]$; Ca_o, extracellular $[Ca^{2+}]$; EC, endothelial cells; ER, endoplasmic reticulum; GMP-140, intercellular granule membrane protein; ICAM, intercellular adhesion molecule; $InsP_3$, inositol-1,4,5-triphosphate PAF, platelet-activating factor; RBC, red blood cells; TC, tumor cells.

thesda, MD) with 10% fetal bovine serum (Paragon, Baltimore, MD), 50 μg/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml neomycin (Gibco Laboratories, Grand Island, NY).

Ca_i measurements. EC (passages 16–20 and 5–7 for BPAEC and BAEC, respectively) were harvested with 0.125% trypsin (National Institutes of Health Media Unit) and 2 mM ethylene glycol-bis(beta-amino-ethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (Sigma Immunochemicals, St. Louis, MO) and plated on glass coverslips (Corning Inc., Corning, NY) in culture medium. After 24–36 h, EC reached confluence and were subsequently incubated with culture medium containing 12 μM of the ester derivative (AM form) of the fluorescent Ca^{2+} probe indo-1 (Molecular Probes, Eugene, OR) in a 95% air/5% CO_2 incubator at room temperature for 60 min. Coverslips were then gently washed and kept in the incubator for an additional 60 min to allow for deesterification of the indicator before the beginning of the experiment. Subsequently, EC on glass coverslips were transferred to a Lucite chamber with a glass coverslip bottom on the stage of a modified inverted microscope, on which it was possible to visualize simultaneously the cells and to collect indo-1 fluorescence as previously described (14). The chamber volume was 0.5 ml, and when using a bicarbonate-buffered solution, the chamber gas environment was equilibrated with 5% CO_2 to maintain buffer pH at 7.38. Indo-1 fluorescence was excited at 350±5 nm using a xenon strobe lamp (model 236 stroboscope and model 35S lamp; Chadwick-Helmuth Electronics, Inc., El Monte, CA). Band-pass interference filters (Andover, Lawrence, MA) were used to select wavelength bands of emitted fluorescence at 391–434 nm ("410-nm channel") and 457–507 nm ("490-nm channel"), corresponding to the Ca^{2+}-bound and Ca^{2+}-free forms of the indicator, respectively. The 410:490 nm ratio was used as an index of Ca_i. For these studies, we used a ×63 objective and emitted indo-1 fluorescence was collected from an optical field of view with ~ 10–15 confluent endothelial cells. Autofluorescence of a comparable field of unloaded cells was < 5% of indo-1–loaded endothelial cells. EC were bathed in a bicarbonate buffer of the following composition (in mM): 116.4 NaCl, 5.4 KCl, 1.6 $MgSO_4$, 26.2 $NaHCO_3$, 1.0 NaH_2PO_4, 5.6 D-glucose, and 1.5 $CaCl_2$, continuously gassed with 5% CO_2 (pH = 7.38±0.01; 23°C). For some experiments, we used a bicarbonate-free solution containing (in mM): 137.0 NaCl, 4.9 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 20 Hepes, 15 D-glucose, and 1.5 $CaCl_2$ (pH 7.38±0.01; 23°C). No difference in EC Ca_i response was detected between bicarbonate and bicarbonate-free solutions. The same bicarbonate-free solution without added Ca^{2+} and with 1 mM EGTA was used to achieve Ca_o-free conditions, while in other experiments, extracellular Na^+ was completely removed by substituting NaCl with LiCl and NaH_2PO_4 with KH_2PO_4. We have previously shown that in indo-1–loaded rat aorta endothelial cells there is no significant mitochondrial compartmentalization of the Ca^{2+} indicator (15). However, a technically satisfactory intracellular indo-1 calibration using deenergized endothelial cells and the calcium ionophores ionomycin and 4-bromo A23187 could not be obtained. Such a calibration is dependent on the ability to randomly expose a given endothelial monolayer to solutions of varying $[Ca^{2+}]$, making repeated measurements of emitted indo-1 fluorescence ratio at a given $[Ca^{2+}]$. We were unable to reproducibly equilibrate monolayers to changes in Ca^{2+} concentration to permit a full range of measurements. The difficulties stemmed, apparently, from the low efficacy of the calcium ionophores on the plasma membrane of this cell type. In addition, after endothelial cell loading with indo-1, spectrofluorimetric analysis of digitonin-releasable fluorescence demonstrated the presence of Mn^{2+}-insensitive fluorescence, which may represent a partially deesterified form of the indicator. Subtraction of this Mn^{2+}-insensitive component from total indo-1 fluorescence yielded a spectral scan similar to that obtained from indo-1–free acid solution (not shown). The presence of this Mn^{2+}-insensitive fluorescence would also invalidate calibration of the indicator. The results are, therefore, reported as an emitted indo-1 fluorescence ratio and not as $[Ca^{2+}]$. To minimize loss of the indicator from the cells, experiments were performed at 23°C, as a more rapid indo-1 loss from cells has been shown to occur at 37°C (14). The cells were resuspended either in bicarbonate- or Hepes-buffered solution to match the buffer bathing the endothelial cell monolayer and were added to the chamber (50 μl; 1×10^6 cells/ml). Contact between the endothelial cell monolayer and the cells added to the chamber was established when both cell types were in the same plane of focus, and after each addition, 5–15 cells appeared in the optical field. Alternatively, biologically inert beads (Medical/Surgical Division 3M, St. Paul, MN) of comparable diameter (8 μm) to cells in suspension, or tumor cell–conditioned medium were added in place of cells. In some experiments, EC intracellular Ca^{2+} stores were depleted by exposing the cells to 0.5 μM thapsigargin (Calbiochem-Novabiochem Corp., La Jolla, CA). In other studies, EC Ca_i was buffered by preincubating the cells with the Ca^{2+} chelator bis-(*O*-aminophenoxyl)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA-AM) (Molecular Probes). BPAEC monolayers were incubated with 15 μM BAPTA-AM for 45 min or DMSO alone (0.1%). At the end of the incubation period, 30 min were allowed for deesterification of the Ca^{2+} chelator before the beginning of the experiment.

Static adhesion assay. BPAEC were grown in a 24-well plate to confluence; melanoma cells were harvested with brief trypsinization (0.125% trypsin and 2 mM EGTA), resuspended in adhesion medium (DME with 0.1% bovine serum albumin; Gibco Laboratories) and added (1×10^5 cells/well) to the EC monolayer. At different time points the wells were washed three times with adhesion medium and melanoma cells adherent to the endothelium were counted under the microscope (×100; five fields in triplicates).

Dynamic adhesion assay. EC were grown to confluence on a 75 × 38-mm glass slide (Corning Inc.). Before the beginning of the adhesion assay, the glass slide was assembled in a parallel plate chamber where hydrodynamic shear stress forces could be calculated as previously described (16) and placed on an inverted microscope (Diaphot; Nikon, Japan). Melanoma cells harvested and resuspended in adhesion medium with 25 mM Hepes (5×10^5 cells/ml) were perfused into the chamber for 25 min with continuous laminar flow, with a syringe pump (Harvard Apparatus, Millis, MA) at 0.46 dyn/cm^2. At the end of the assay, the perfusate was changed to a cell-free buffer and flow was increased for 1 min to achieve shear-stress forces of 12 dyn/cm^2 to remove nonadherent cells. Subsequently, flow was lowered again and TC adherent to the endothelium were counted under the microscope (×40; 15 fields/slide).

Statistical analysis. Results are reported as the mean±SD. Differences between means of unpaired comparisons were evaluated by Student's *t* test. $P < 0.05$ was taken to indicate statistical significance.

Results

The effect of the interaction between melanoma cells and the endothelium on BPAEC or BAEC Ca_i was examined. The addition of inert beads to the EC monolayer did not elicit any response (Fig. 1). In contrast, in the same monolayer immediately upon contact with melanoma cells there was a rapid and transient increase in endothelial Ca_i, and after the initial peak EC Ca_i remained elevated (Fig. 1). The response appears specific for cell–cell contact and was not caused by mechanical deformation of the EC or to soluble factors, since it was never elicited by inert beads ($n = 5$) or tumor cell–conditioned medium ($n = 4$) (data not shown). In additional experiments, we examined whether the increase in EC Ca_i could be triggered by other cell types or was specific for melanoma cells. We were not able to elicit an increase in EC Ca_i when red blood cells (RBC) (Fig. 2) or endothelial cells (BAEC) in suspension (not shown) came in contact with the EC monolayer. Fig. 2 shows that the addition of RBC to BAEC did not affect EC Ca_i, while the successive TSU-pr1 cell addition to the same monolayer triggered an increase in Ca_i similar to that observed with melanoma cells. Furthermore, the EC Ca_i rise also occurred upon contact with two human prostate carcinoma cell lines, TSU-pr1 (Fig. 2) and LNCap (not shown), human neutrophils (*n*

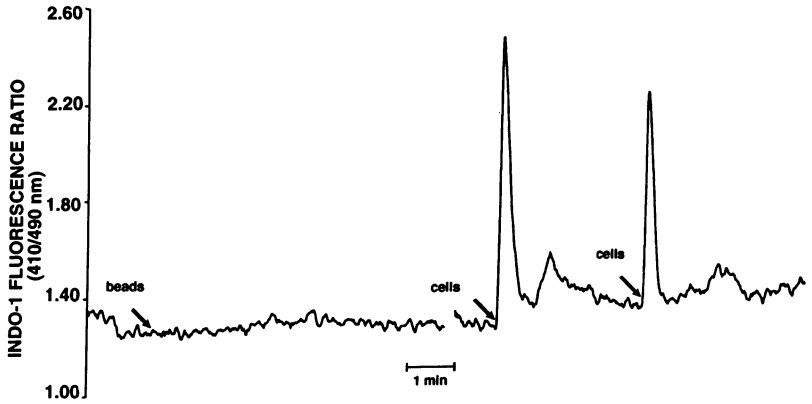

Figure 1. Effect of inert beads and melanoma cells on Ca_i of the same BPAEC monolayer. Inert beads and melanoma cells (50 μl; 1×10^6 melanoma cells or beads/ml; *arrows*) were added to the same EC monolayer during indo-1 fluorescence collection. Similar results with the beads and with the tumor cells were observed in 5 out of 5 and 11 out of 11 experiments, respectively.

= 10; not shown) and U937, a human monocyte-like lymphoma cell line (n = 10; not shown).

To investigate the Ca^{2+} source for the increase in Ca_i, BPAEC in the chamber were perfused with a bicarbonate-free buffer without added Ca^{2+} and with 1 mM EGTA. Under these conditions, Ca^{2+} influx from the extracellular space does not occur. Nevertheless, melanoma cell–EC contact still elicited an increase in Ca_i (Fig. 3 *A*), suggesting that the transient Ca_i increase upon melanoma cell–EC contact is not caused by Ca^{2+} influx from the extracellular space. Furthermore, Δ indo-1 fluorescence ratio from control at 1 min after the initial peak was smaller in the experiments in Ca^{2+}-free buffer than in the studies in 1.5 mM Ca_o (0.03 ± 0.01, $n = 4$ vs 0.18 ± 0.09, $n = 5$; $P < 0.01$). This raises the possibility that Ca^{2+} influx from the extracellular space may keep Ca_i elevated after the initial Ca_i transient. In additional experiments, we examined whether Ca^{2+} release from intracellular Ca^{2+} pools could be the mechanism for the Ca_i transient upon melanoma cell–EC contact. For these experiments, we used thapsigargin, a sesquiserpene lactone that in several cell types (17–19) selectively and irreversibly inhibits the endoplasmic reticulum (ER) Ca^{2+}-ATPase by blocking Ca^{2+} uptake into inositol-1,4,5-trisphosphate ($InsP_3$)-sensitive and $InsP_3$-insensitive intracellular Ca^{2+} pools. The end-result of thapsigargin treatment is intracellular Ca^{2+} stores depletion via an $InsP_3$-independent mechanism. In response to perfusion with 0.5 μM thapsigargin there was a rise in Ca_i that slowly recovered toward control. Melanoma cells were added to the EC monolayer after 25 min exposure to thapsigargin. Under these conditions, the endothelial Ca_i transient was abolished (Fig. 3 *B*). This result shows that upon melanoma cell–BPAEC contact, there was a rapid Ca^{2+} release from the intracellular Ca^{2+} stores, presumably from the ER. However, it cannot be excluded that sustained elevation in Ca_i after exposure to thapsigargin may have inhibited the ER Ca^{2+}-release channel as observed in myocardial cells (20). Similar to other cell types, endothelial cells appear to have a plasmalemmal Na^+/Ca^{2+} exchange (21). Thus, by removing extracellular Na^+, it is possible to enhance Ca_i without depleting ER Ca^{2+}. After BPAEC superfusion with a Na^+-free buffer, Ca_i increased. Nevertheless, the Ca_i transient elicited by melanoma cell addition was also observed under these conditions (Fig. 3 *C*).

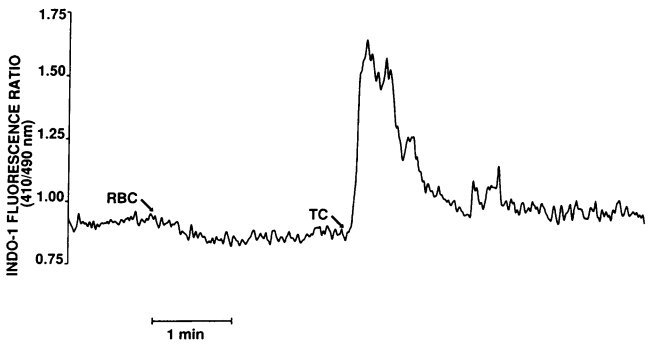

Figure 2. Effect of red blood cells and prostate carcinoma cells on Ca_i of the same BAEC monolayer. RBC and TSU-pr1 cells (*TC*) (50 μl, 1×10^6 cells/ml; *arrows*) were added to the same EC monolayer. Similar results were observed in five out of five experiments.

The functional role of the increase in endothelial Ca_i on melanoma–BPAEC interaction was examined in BPAEC treated with BAPTA-AM, an intracellular Ca^{2+} chelator. After incubation with 15 μM BAPTA-AM for 45 min, BPAEC did not exhibit an increase in Ca_i upon contact with melanoma cells (Fig. 4). Thus, in additional experiments, we examined melanoma cell adhesion to BPAEC monolayers under control conditions and to BPAEC monolayers pretreated with BAPTA-AM for 45 min. Both static and dynamic adhesion assays were used to examine the effect of BAPTA-AM pretreatment on melanoma cell adhesion to BPAEC monolayer. Under static conditions, EC pretreatment with BAPTA-AM inhibited TC adhesion only at 3 and 5 min ($47.3 \pm 15.4\%$ and $49.4 \pm 9.7\%$ inhibition respectively), while it was ineffective at 15 min (Fig. 5 *A*). In contrast, under dynamic conditions, which more closely mimic in vivo adhesion, there was a significant inhibition of adhesion ($70.3 \pm 18.5\%$ inhibition) even after 25 min at 0.46 dyn/cm^2 (Fig. 5 *B*). Thus, EC treatment with BAPTA-AM abolished the increase in Ca_i upon melanoma cell contact and significantly reduced subsequent melanoma cell adhesion to BPAEC monolayer. Inhibition of TC adhesion to BAPTA-AM–treated EC appeared more effective in a dynamic flow system, possibly because of additional adhesion enhancement occurring after the initial contact under dynamic conditions. In addition, it is noteworthy that in the dynamic adhesion assay we observed a rolling phenomenon similar to that previously described by Lawrence and Springer during neutrophil addition to artificial bilayers containing GMP-140 (22). Melanoma cell rolling was confirmed by observing ($\times 200$) rotation of TC; it was characterized by transient melanoma cell attachment to the EC monolayer with a decrease in cell velocity followed by an increase in velocity during which the cells appeared to detach from the EC and then to reattach downstream.

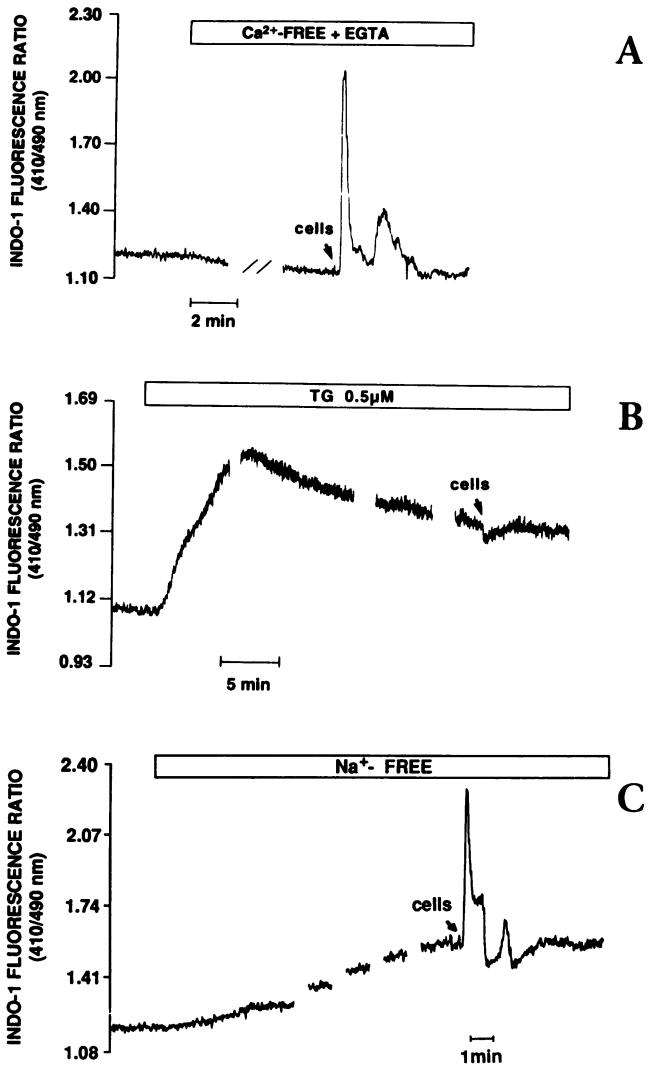

Figure 3. Effect of extracellular and intracellular Ca^{2+} on melanoma cell-induced Ca_i rise in BPAEC. (*A*) To prevent Ca^{2+} influx from the extracellular space, BPAEC were perfused with a Hepes-buffered solution without added Ca^{2+} and with 1 mM EGTA. After a slight initial decrease in indo-1 fluorescence ratio because of Ca^{2+} efflux a stable baseline was obtained. Under these conditions, melanoma cells were added to the endothelium and triggered a rapid increase in Ca_i. The same result was observed in four out of four experiments. (*B*) BPAEC were perfused with 0.5 μM thapsigargin. There was an initial effect of thapsigargin to increase indo-1 fluorescence ratio followed by a slow return toward control. Under these conditions, melanoma cells added to the endothelium did not enhance Ca_i; a similar result was observed in five out of five experiments. (*C*) Exposure to Na^+-free buffer increased Ca_i in BPAEC. Melanoma cells added under conditions of elevated Ca_i (*arrow*) still caused a rapid and transient increase in EC Ca_i. A similar result was observed in three out of three experiments.

Discussion

In this study, we have shown that the initial contact of metastatic tumor cells with unactivated endothelial cells induces a rapid and transient EC Ca_i rise that is caused by Ca^{2+} release from an intracellular store rather than by Ca^{2+} influx from the extracellular space. An increase in EC Ca_i was also observed with leukocytes which, similarly to tumor cells, can bind and cross the endothelium. In contrast, no increase in Ca_i of EC monolayer was observed upon contact either with red blood cells or with endothelial cells in suspension. Thus, the Ca_i rise may be triggered by specific adhesion molecules present on tumor cell surface, since endothelial cells and red blood cells were not able to enhance Ca_i. Furthermore, we excluded the possibility that soluble factors may be involved in the phenomenon, since melanoma cell conditioned medium was not able to increase EC Ca_i. It is also noteworthy that Δ indo-1 fluorescence ratio from control at 1 min after the initial peak was greater in the experiments implemented in 1.5 mM Ca_o than in those in Ca^{2+}-free buffer. This suggests that Ca^{2+} influx into endothelial cells may also be triggered by contact with the TC and may be responsible for the sustained increase in indo-1 fluorescence that occurs after the initial peak. However, this cannot be conclusively established with the experimental protocol used in this study, because the sustained increase in indo-1 fluorescence may also be caused by TC reaching the endothelium at different times after their addition to the chamber. It is also of interest that the first tumor cell that reaches the endothelium triggers the most rapid and marked increase in Ca_i; subsequent TC–EC interactions, within the ensuing 2–3 min, cause markedly smaller increases in Ca_i (Fig. 1). This observation suggests that initial TC–EC contact may "prime" the surrounding endothelial cells and modulate their Ca_i response to the contact with cancer cells that may occur shortly thereafter. This preliminary observation will require additional studies to establish whether the rapid Ca_i rise upon initial TC–EC contact may be a mechanism for cell-to-cell signaling as described for other nonendothelial cells (23, 24). The signal transduction mechanism for the increase in Ca_i reported in the present study remains to be elucidated. It is noteworthy that EC respond to interventions that cause phosphatidylinositol turnover and enhance $InsP_3$ and Inositol-1,3,4,5-tetrakisphosphate ($InsP_4$) production with a biphasic Ca_i response. An initial rapid and transient increase in Ca_i caused by $InsP_3$-mediated ER Ca^{2+} release is followed by a lower and more sustained increase in Ca_i caused by Ca^{2+} influx from the extracellular space that may result from $InsP_4$ (25). In the absence of biochemical measurements, it cannot be determined whether the observations reported in this study are associated with an enhanced phosphatidylinositol turnover; nevertheless, such possibility may be considered. The results of the present study differ from those of a previous report where flow cytometry analysis (FACS®; Becton Dickinson Immunocytometry Systems, Mountain View, CA) and a suspension of leukocytes and EC were used to examine the effect of the interaction between those two cell types on EC Ca_i, and it was found that no increase in Ca_i occurred in EC (12). However, different experimental conditions may account for the discrepancy between the results of that study and those reported in this work. We have also shown that the increase in endothelial cell Ca_i upon contact with melanoma cells has a functional significance because it modulates melanoma cell adhesion.

Current knowledge on TC adhesion to endothelia is deficient as compared to leukocyte adhesion. However, findings on leukocyte adhesion may also be relevant to tumor cells, since, for example, the interaction of both melanoma cells and lymphocytes with EC appears to be mediated by the same adhesion molecule, vascular cellular adhesion molecule-1 (3). Moreover, metastatic cells, like leukocytes, adhere to and cross the

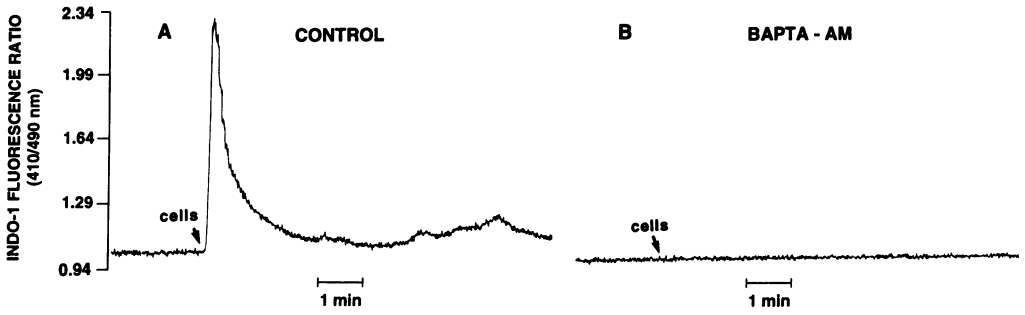

Figure 4. Effect of BAPTA-AM pre-treatment on BPAEC Ca_i. Melanoma cells were added (*arrows*) to EC and indo-1 fluorescence was collected in control (*A*) and in a BPAEC monolayer pre-treated with 15 μm BAPTA-AM for 45 min (*B*); a similar result was observed in three out of three experiments.

endothelium (1). Thus, it was not surprising that under our experimental conditions, neutrophils and monocytes elicited a Ca_i response similar to that of tumor cells. A general model of leukocyte-EC recognition (26) suggests an active process requiring three sequential events: the first step, described as leukocyte "rolling," is mediated by lectin-carbohydrate interaction (GMP-140/oligosaccharides). This initial adhesion is transient and reversible unless followed by leukocyte activation caused by secretion of platelet-activating factor (PAF) or other cell contact-mediated signals (second step). PAF is not constitutively present in resting EC, it is synthesized rapidly upon agonist exposure and it requires elevation of Ca_i for its synthesis (27). PAF associated with EC is involved in the recruitment of leukocyte CD11-CD18 integrins and in enhancing the tightness of GMP-140-mediated adhesion (28). Interaction of the activation-dependent leukocyte integrin with its EC counterreceptor ICAM-1/ICAM-2 (third step) results in strong and sustained attachment. EC Ca_i is also required for cell surface expression of both GMP-140 and ICAM-1, and modulates cell-cell adhesion via this mechanism (9, 11). Furthermore, neutrophils respond with a transient Ca_i rise upon contact with EC (12) and the increase in neutrophils Ca_i enhances the binding affinity of the leukocyte integrin CD18/11a (29, 30). In a recent study, it was shown that in a dynamic flow system comparable to ours, initial adhesion of B16 melanoma cells to unactivated mouse and human endothelial cells is mediated by the interaction between glycosphingolipid GM3(NeuAca2-3Galb1-4Glcb1-Cer), and lactosylceramide (LacCer,Galb1-4Glcb1-Cer), expressed on TC and EC, respectively (2). Thus, the latter and the present study suggest that EC activation is not necessary for specific melanoma cell adhesion. In addition, under static conditions, adhesion to BAPTA-AM-treated endothelial cells was reduced only at 3 and 5 min, while in the dynamic flow system, adhesion was inhibited also at 25 min (Fig. 5). This result may be caused by an effect of the rapid increase in Ca_i on mechanisms of cell-cell adhesion that lead to EC activation and expression of adhesion-enhancing molecules such as ICAMs and selectins.

Furthermore, in our dynamic flow system, TC attachment to EC resembled leukocyte adhesion, since TC "rolling" occurred. This "rolling" phenomenon may result from interaction between glycosphingolipids present either on TC or EC, as recently described (2). Under low shear stress melanoma cells were able to "roll," but not to attach firmly to BAPTA-AM-treated EC monolayers. Thus, by blocking EC Ca_i rise, we were able to inhibit further strengthening of adhesion.

In conclusion, unactivated EC Ca_i may play a crucial role in tumor cell adhesion by reinforcing the initial weak interaction and mechanisms of "juxtacrine activation" similar to those described in leukocyte-EC adhesion may also mediate TC-EC adhesion.

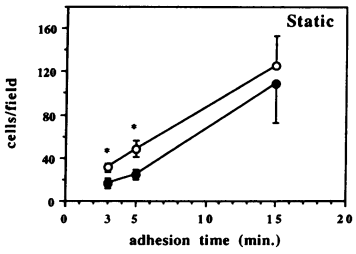

Figure 5. Inhibition of TC adhesion to BPAEC by BAPTA-AM pre-treatment under static and dynamic flow conditions. (*A*) Static adhesion assay. BPAEC were grown until confluence in 16-mm diameter plates. Melanoma cells (1×10^5/well) were added to EC, and the wells were washed at different times after addition. Tumor cell adhesion to control (*open circles*) or to BAPTA-AM-treated EC (*filled circles*) is expressed as number of cells per microscopic field (×100). Data are the mean±SD of three different experiments. $*P < 0.005$ at 3 and 5 min. (*B*) Dynamic adhesion assay. Confluent BPAEC monolayer on glass slide assembled in the parallel plate chamber. TC (5×10^5/ml) were perfused through the chamber at 0.46 dyn/cm^2 (see Methods). TC adhesion 25 min after addition to control (*solid bar*) or to BAPTA-AM-treated (*hatched bar*) EC is expressed as number of adherent cells per microscopic field (×40). Data are the mean±SD of three different experiments. $*P < 0.0005$ for BAPTA-AM-treated EC.

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