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Alteration of Interendothelial Adherens Junctions Following Tumor Cell–Endothelial Cell Interaction *in Vitro*

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The integrity of the vascular endothelium is mainly dependent upon the organization of interendothelial adherens junctions (AJ). These junctions are formed by the homotypic interaction of a transmembrane protein, vascular endothelial cadherin (VE-cadherin), which is complexed to an intracellular protein network including α-, β-, and γ-catenin. Additional proteins such as vinculin and α-actinin have been suggested to link the VE-cadherin/catenin complex to the actin-based cytoskeleton. During the process of hematogenous metastasis, circulating tumor cells must disrupt these intercellular junctions in order to extravasate. In the present study, we have investigated the influence of tumor cell–endothelial cell interaction upon interendothelial AJ. We show that human breast adenocarcinoma cells (MCF-7), but not normal human mammary epithelial cells, induce a rapid endothelial cell (EC) dissociation which correlates with the loss of VE-cadherin expression at the site of tumor cell–EC contact and with profound changes in vinculin distribution and organization. This process could not be inhibited by metalloproteinase nor serine protease inhibitors. Immunoprecipitations and Western blot analysis demonstrate that the overall expression of VE-cadherin and vinculin as well as the composition of the VE-cadherin/catenins complex are not affected by tumor cells while the tyrosine phosphorylation status of proteins within the complex is significantly altered. Our data suggest that tumor cells modulate AJ protein distribution and phosphorylation in EC and may, thereby, facilitate EC dissociation.

INTRODUCTION

Blood and lymphatic circulations are the major ways for metastatic tumor cells to disseminate from the primary tumor to distant sites. In order to establish new tumor foci, circulating tumor cells must escape from the vascular system. The extravasation process can be summarized as a sequence of events which include tumor cell adhesion to the vascular endothelium, tumor cell-mediated opening of endothelial cell-to-cell junctions, adhesion to the underlying basement membrane, and subsequent invasion of the host tissue [1].

The endothelium forms a continuous permselective barrier that regulates the passage of plasma proteins and circulating cells from blood to tissue. The permeability of the endothelium depends upon specialized junctions that are responsible for the lateral plasma membrane cohesion between adjacent endothelial cells (EC). On the basis of morphological and functional characteristics, at least three types of junctions have been identified in EC: tight junctions, gap junctions, and adherens junctions (AJ). The mechanical resistance of interendothelial linkage can be, in a large part, assigned to AJ while tight junctions and gap junctions have been shown to play a role in the control of the permeability to plasma proteins and in the exchange of ions and small size molecules between adjacent EC, respectively [2].

Recently, the transmembrane protein involved in endothelial AJ was identified and characterized [3, 4]. This protein, vascular endothelial cadherin (VE-cadherin) (cadherin 5), belongs to the cadherin family of proteins and is selectively expressed in EC from most types of vessels [3]. As other classical cadherins such as E-, N-, or P-cadherins, VE-cadherin is a transmembrane calcium-dependent adhesion molecules that mediate cell–cell interactions through homophilic binding. The intracellular domain of VE-cadherin interacts with at least three known cytoplasmic proteins: α-catenin, β-catenin, and γ-catenin (plakoglobin) [4]. The association of VE-cadherin with the catenins and the interaction between the VE-cadherin/catenin complex and cytoskeletal proteins govern the strength of interendothelial cohesion and the transendothelial permeability. In fully mature endothelial cell-to-cell junc-

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tions, the intracellular domain of VE-cadherin is bound to α-, β-, and γ-catenins and colocalizes with vinculin which has been suggested to link the VE-cadherin/catenin complex to actin microfilaments [4]. When the binding of VE-cadherin to catenins is inhibited, VE-cadherin association with cytoskeletal proteins is impaired and the formation of a cohesive cell monolayer is prevented [5]. Furthermore, recent studies indicate that the phosphorylation status of the cadherin/catenin complex is determinant for cadherin-mediated adhesiveness. Experiments using v-src-transformed epithelial cells or fibroblasts (expressing high level of $p60^{src}$) have demonstrated that tyrosine phosphorylation of the intracellular domain of E-, P-, or N-cadherin as well as α- and/or β-catenins correlates with the loss of homotypic cell aggregation and ability to form cohesive monolayer [6–9]. Growth factors (EGF and scatter factor) have also been shown to increase cadherin/catenins complex phosphorylation with subsequent loss of cell–cell adhesion [10]. In EC, angiogenic factors as vascular endothelial cell growth factor (VEGF) induce tyrosine phosphorylation of adherens junctions allowing EC motility [11].

Several laboratories have shown that tumor cells from different origin can adhere to EC monolayers and induce the formation of gaps between EC [1, 12–15]. These gaps facilitate the access to the subendothelial basement membrane which in turn stimulates their invasive capacity [16, 17]. In previous works, we have investigated the interactions between human breast adenocarcinoma cells (MCF-7) and the vascular endithelium. We have shown that: (a) MCF-7 cells adhere to the luminal endothelial cell plasma membrane [18], (b) the contact between MCF-7 cells and endothelial cells induce a transient increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in endothelial cells. Our results suggest that these intracellular Ca^{2+} movements are part of a signal transduction pathway initiated by the contact between the two cell types and which govern the opening of endothelial intercellular junctions and tumor cell transendothelial migration [19].

The present study was designed to investigate the influence of tumor cell–EC contact upon endothelial AJ. We show that human breast adenocarcinoma cells (MCF-7), but not normal mammary epithelial cells, induce EC dissociation which correlates with the loss of VE-cadherin expression at the site of tumor cell–EC contact as well as with changes in vinculin distribution. Modulation in the composition and phosphorylation status of the VE-cadherin/catenins complex following tumor cell–EC contact are also investigated.

METHODS

Cell culture. Human EC were isolated from umbilical vein by treatment with collagenase (0.1% from *Clostridium histoliyticum*, Sigma, St. Louis, MO) for 20 min at 37°C [20]. Islets of isolated EC were plated on gelatin-coated dishes and cultured in RPMI 1640 supplemented with 5% fetal calf serum (FCS), 5% pooled human serum, 20 μg/ml of EC growth factor [(ECGF) from bovine brain, Boehringer Mannheim, Mannheim, Germany], 50 μg/ml of heparin (from porcine intestinal mucosa, Sigma), 10 mM Hepes, 50 U/ml penicillin, 50 U/ml streptomycin. Cells were maintained at 37°C in a 95% air, 5% CO_2 incubator and were always used at the first passage. They were positive for von Willebrand factor antigen and showed the typical cobblestone morphology when confluent.

Human MCF-7 cell line was originally established from the pleural effusion of patients with breast carcinoma [21]. They were kindly provided by Dr. G. Leclercq (Institut Bordet, Brussels, Belgium). MCF-7 cells were cultured in DMEM supplemented with 5% FCS, glutamine (292 mg/liter), sodium bicarbonate (2.1 mg/liter), ascorbic acid (50 mg/liter), and penicillin–streptomycin (100 U/ml). Normal human mammary epithelial cells were prepared from mammary gland samples obtained from reduction surgery on healthy women as described by Soule and McGrath [22] and maintained in 50% DMEM (Gibco)–50% Ham's F12 supplemented with 10% decomplemented FCS, hydrocortisone (0.4 μg/ml), cholera toxin (10^{-10} M), insulin (5 μg/ml), penicillin–streptomycin (100 U/ml).

Antibodies and reagents. The following antibodies (Abs) were used: mouse monoclonal antibody (mAb) to VE-cadherin (clone BV9) [4]; rabbit polyclonal antisera to α-catenin (1597) [23] and to β-catenin (1522) [24]; mAbs to γ-catenin and $p120^{cas}$ were obtained from Transduction Laboratories (Lexington, KY); anti-phosphotyrosine mAb (PY20) was obtained from Zymed Laboratories (San Francisco, CA); mAb to vinculin (hVIN1) was obtained from Sigma; second-step horseradish peroxidase-conjugated rabbit anti-mouse and swine anti-rabbit IgG were purchased from Dako (Denmark).

Aprotinin, phosphoramidon, and soybean trypsin inhibitor (SBTI) were purchased from Sigma, Pefabloc was from Boehringer Mannheim and SC-44463 was supplied by Searle (Skokie, IL). Purified tissue inhibitor of metalloprotease-2 (TIMP-2) was prepared as described in [25].

Coculture experiments. For coculture experiments, EC (1.5×10^6 cells/dish) were seeded on gelatin-coated 50-mm-diameter culture dishes and grown to confluence in RPMI 1640 supplemented with 5% FCS and 5% human serum without ECGF and heparin (assay medium). Confluent EC monolayers were then washed once with preheated assay medium and allowed to rest for 1 h at 37°C before any coculture experiment. At that time, no gaps between EC could be observed by phase contrast microscopy under 200× magnification. Cells to be tested were shortly trypsinized, washed once in DMEM containing 5% FCS, and allowed to recover in suspension for 30 min in assay medium at 37°C. Assay medium was then changed and 3×10^6 cells were plated on the top of an endothelial cell monolayer and incubated at 37°C for indicated periods of time. In some experiments, MCF-7 cells were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS: 137 mM NaCl; 2.7 mM KCl; 1.5 mM KH_2PO_4, 6.5 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.4) and washed three times prior to addition to an EC monolayer.

The effect of MCF-7 cell-conditioned medium on endothelial cell cohesion was also tested. MCF-7-conditioned medium was prepared by incubating subconfluent MCF-7 culture in a 75-cm^2 flask for 24 h in 10 ml assay medium. The culture medium was then centrifuged at 200*g* for 5 min and the supernatant was used on EC monolayer (5 ml/50-mm dish).

The following protease inhibitors were tested individually for their effects on tumor cell-induced EC dissociation: TIMP-2 (10 μg/ml), type IV collagenase inhibitor SC-44463 (10 μg/ml), metalloproteinase inhibitor phosphoramidon (100 μg/ml), serine protease inhibitors aprotinin (0.5 μg/ml), Pefabloc (20 μg/ml), and SBTI (10 μg/ml). Such concentrations of proteases inhibitors are able to completely block *in vitro* enzymatic activities and to induce in various culture systems

dramatic biological effects related to proteases inhibition [26–28]. They were added to the tumor cell suspension 5 min before seeding on EC monolayer and were present during the coculture period.

Phase contrast microphotographs were realized on a Nikon Diaphot inverted microscope.

Scanning electron microscopy. For scanning electron microscopy studies, EC were grown on gelatin-coated glass coverslips. Cocultures were realized as described above. Cells were then washed three times in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS^{2+}), fixed with glutaraldehyde (2.5% in PBS^{2+}) for 1 h at room temperature, washed again with PBS, and postfixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.4, for 2 h. Samples were dehydrated in graded ethanol, dried using Peldri II compound (Ted Pella, Inc., Redding, CA), coated with gold-palladium, and examined under a JEOL-JSM840A microscope at an accelerating voltage of 20 kV.

Immunofluorescence. For immunofluorescence studies, EC monolayers and cocultures were fixed for 10 min in 4% paraformaldehyde in PBS^{2+} at room temperature. Cells were permeabilized with 0.05% Triton X-100 in PBS^{2+} for 1 min on ice and exposed for 30 min to the primary antibody at room temperature. FITC-conjugated rabbit anti-mouse IgG was used as secondary antibody (Dako). Labeled cells were then mounted in 50% glycerol. Samples were analyzed on the Insight IQ confocal microscope system (Meridian Instruments, U.S.A.) mounted on an Olympus IMT-2 inverted microscope. Image analysis was performed using the Insight IQ 1.0 software.

Immunoprecipitation and Western blot analysis. For immunoprecipitation, EC were ^{35}S labeled as follow. Confluent EC monolayers were washed two times with methionine/cysteine-free RPMI 1640 and incubated overnight in the same medium containing 10% dialyzed FCS and 100 μCi/ml Pro-Mix (70% L-[^{35}S]methionine, 30% L-[^{35}S]cysteine; Amersham, UK). Monolayers were then washed three times with assay medium and incubated alone or in coculture with unlabeled tumor cells as described above. Cell layers or cocultures were then washed three times with ice-cold PBS^{2+} and extracted on ice for 15 min in PBS^{2+} containing 1% Triton X-100, 1% Nonidet P-40, 1 mM PMSF, 40 U/ml aprotinin, 20 μM leupeptine, 1 mM sodium orthovanadate (extraction buffer; 300 μl/dish) and scraped. Extracts were centrifuged at 15,000 rpm for 5 min at 4°C and the pellets were discarded. Immunoprecipitation was performed as described in Lampugnani *et al.* [4]. Briefly, cell extracts (equal amount of TCA-precipitable radioactivity for each sample) were pretreated with protein G–Sepharose (Pharmacia, Upsalla, Sweden; 25 μl/sample) for 1 h at 4°C. The supernatant was incubated with 25 μl protein G–Sepharose pretreated with 400 μl of hybridoma culture supernatant containing 10 μg mouse immunoglobulins directed against VE-cadherin (BV9) at 4°C for 2 h under gentle agitation. Hybridoma culture supernatant containing 10 μg of immunoglobulins directed against an unrelated antigen were used as control. The resin pellet was then washed five times with PBS, 0.5 mM NaCl, 0.05% NP-40, 1 mM PMSF, 40 U/ml aprotinin and boiled in 50 μl sample buffer (0.25 M Tris–HCl, pH 6.8, 10% SDS, 4% sucrose, 0.1% bromophenol blue) containing 5% β-mercaptoethanol as reducing agent. Proteins were then separated by 7.5% polyacrylamide gel electrophoresis, stained with Coomassie blue, destained, and treated with EN^{3}HANCE for fluorography. Gels were dried and exposed to X-ray film.

For Western blotting, the gels were soaked for 20 min in transfer buffer (50 mM Tris–HCl, 95 mM glycine, 1 mM $CaCl_2$) after electrophoresis and the proteins were electrotransferred on nitrocellulose filter (Hybond C, Amersham, UK). The quality of the transfer was assessed by staining the filters with Ponceau S (Chroma, Köngen, Germany). The filters were then blocked in PBS^{2+} with 1% BSA for 2 h (Janssen Pharma, Beerse, Belgium) and incubated overnight with the appropriate antibody (5 μg/ml in PBS^{2+} with 0.1% BSA). Immunoreactive bands were detected using peroxidase-conjugated rabbit anti-mouse immunoglobulins followed by ECL (Amersham, UK) and autoradiography. In some experiments, filters were stripped according to the manufacturer, blocked, and rehybridized as described above.

Western blot analyses were also performed on total cell extracts. In these experiments, EC monolayer were labeled with [^{35}S]-methionine and cocultures were performed as described above. MCF-7 cells (3×10^6) seeded on gelatin-coated dishes were used for comparison. EC monolayers, MCF-7 cells, and cocultures were washed three times with ice-cold PBS^{2+} and immediately treated with boiling sample buffer (200 μl/dish) containing 5% β-mercaptoethanol. An equal amount of radioactivity for control EC monolayer and coculture samples were loaded on the gels. Electrophoresis, transfer, and detection were performed as described above.

All experiments have been performed at least three times with independent EC primary cultures.

RESULTS

Rupture of EC Monolayer Integrity by Tumor Cells: Morphological Events

The interactions between MCF-7 cells and EC monolayer were studied at different times of coculture by light and scanning electron microscopy. Confluent EC monolayer forms a continuous lining on the bottom of the culture dish and displays a classical cobblestone morphology (Fig. 1A). Fifteen minutes after seeding on the monolayer, MCF-7 cells adhered to EC luminal surface and were preferentially localized on interendothelial junctions. At that time, no sign of EC disjunction could be detected. The formation of gaps between EC was evidenced after 30 min of coculture and was restricted to the site of tumor cell adhesion (Fig. 1B). Pseudopodia emerging from tumor cells were in contact with both EC and subendothelial matrix. Longer incubation time resulted in the formation of "holes" in the EC monolayer around the tumor cells (Fig. 1C, 90 min after plating). EC in contact with tumor cells progressively lost their junctions with the neighboring cells (Figs. 1B and 1C), while EC distant from tumor cells remained flattened with apparently intact intercellular junctions. As the EC monolayer integrity was impaired, tumor cells spread on the exposed subendothelial matrix and migrated toward EC initiating new contacts and thereby inducing an extensive loss of endothelial cell-to-cell cohesion.

The interactions of normal human mammary epithelial cells with EC monolayer was also studied. Few cells adhered to the EC monolayer, remained rounded and did not induce EC dissociation even after 24 h of coculture (not shown).

The putative implication of a proteolytic cleavage of endothelial cell-to-cell junctions induced by tumor cells was investigated using different metalloproteinase (TIMP-2; SC44463; phosphoramidon) and serine protease (aprotinin, Pefabloc, and SBTI) inhibitors at concentration known to block enzymatic activities and to be effective on relevant cellular models. No effect of

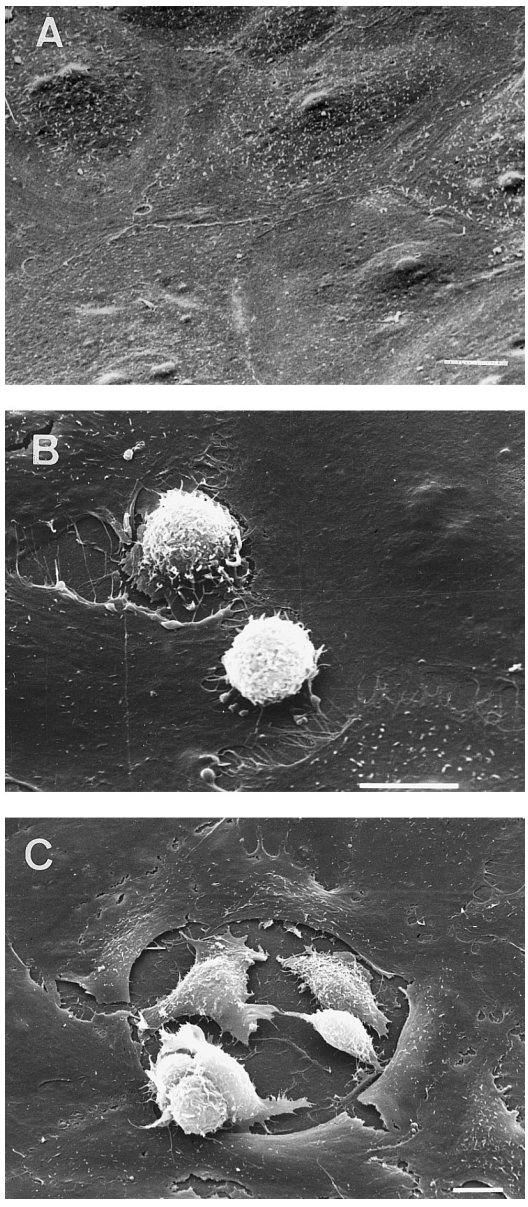

FIG. 1. Scanning electron micrographs of EC monolayer (A) and EC–MCF-7 cells cocultures after 30 min (B) and 90 min (C) of incubation. Bars, 10 μm.

these inhibitors was found on tumor cell-induced EC dissociation as assessed by phase contrast microscopy. The time course of EC dissociation was similar to what was found in the absence of inhibitors (Fig. 1).

Immunolocalization of VE-Cadherin and Vinculin in EC Monolayer and MCF-7–EC Coculture

Immunofluorescence with mAbs directed to VE-cadherin (BV9) and to vinculin (hVIN-1) was performed on EC monolayer and EC–MCF-7 cell coculture and were analyzed using laser confocal microscopy. VE-cadherin labeling on confluent contact-inhibited EC monolayer revealed a complex interdigitating structure localized at intercellular junctions and distributed along several focal planes (Fig. 2A). Following tumor cell–EC contact, VE-cadherin organization remained unchanged during the first 15 min. Within 30 min, however, the continuous staining of the EC junctions was disrupted at the site of tumor cell impact (Fig. 2B). Longer incubation times were accompanied by a complete loss of VE-cadherin labeling from the edges of the cells (Fig. 2C) and by the emergence of an increased number of brightly labeled vesicules inside EC cytosol (Figs. 2B–2D). Confocal microscopy analysis of 30-min cocultures suggests that these vesicules derive from VE-cadherin-positive intercellular structures (Fig. 2D, arrows).

In confluent EC culture, vinculin labeling was mainly localized in interendothelial junctions (Fig. 3A). The staining pattern was limited to a thin line edging EC and did not show the complex structure observed with VE-cadherin mAbs. Only rare focal adhesion plaques, close to the cell periphery, were labeled. In coculture, EC vinculin rapidly translocated from cell border to focal adhesion plaques. Well-differentiated focal adhesion plaques could be evidenced on the basal side of EC within 30 min and were clearly expressed within 90 min (Fig. 3B). Changes in the distribution of both VE-cadherin and vinculin described above only occurred in EC having a physical contact with live tumor cells. There were not observed with tumor cells conditioned medium, fixed tumor cells, nor normal human mammary epithelial cells (data not shown).

Western Blot Analysis of VE-Cadherin and Vinculin

The influence of tumor cells on the expression of VE-cadherin and vinculin in EC was investigated by Western blot analysis. As shown in Fig. 4A, immunoreactive VE-cadherin was clearly detectable in both control EC monolayer and EC–MCF-7 cell cocultures. As expected, no signal was detected in MCF-7 cell extracts attesting for the absence of crossreactivity of VE-cadherin mAbs with E-cadherin expressed by MCF7 cells. No important modifications in VE-cadherin expression were detected following EC cocultivation with MCF-7 cells (Fig. 4A).

Vinculin was present in both EC and MCF-7 cell extracts (Fig. 4B). The increase in the amount of vinculin detectable in coculture experiment can thus be ascribed to the sum of the vinculin content from both cell types (Fig. 4B).

Immunoprecipitation of the VE-Cadherin/Catenins Complex

In order to investigate the effects of tumor cell–EC interactions on the composition of the VE-cadherin/

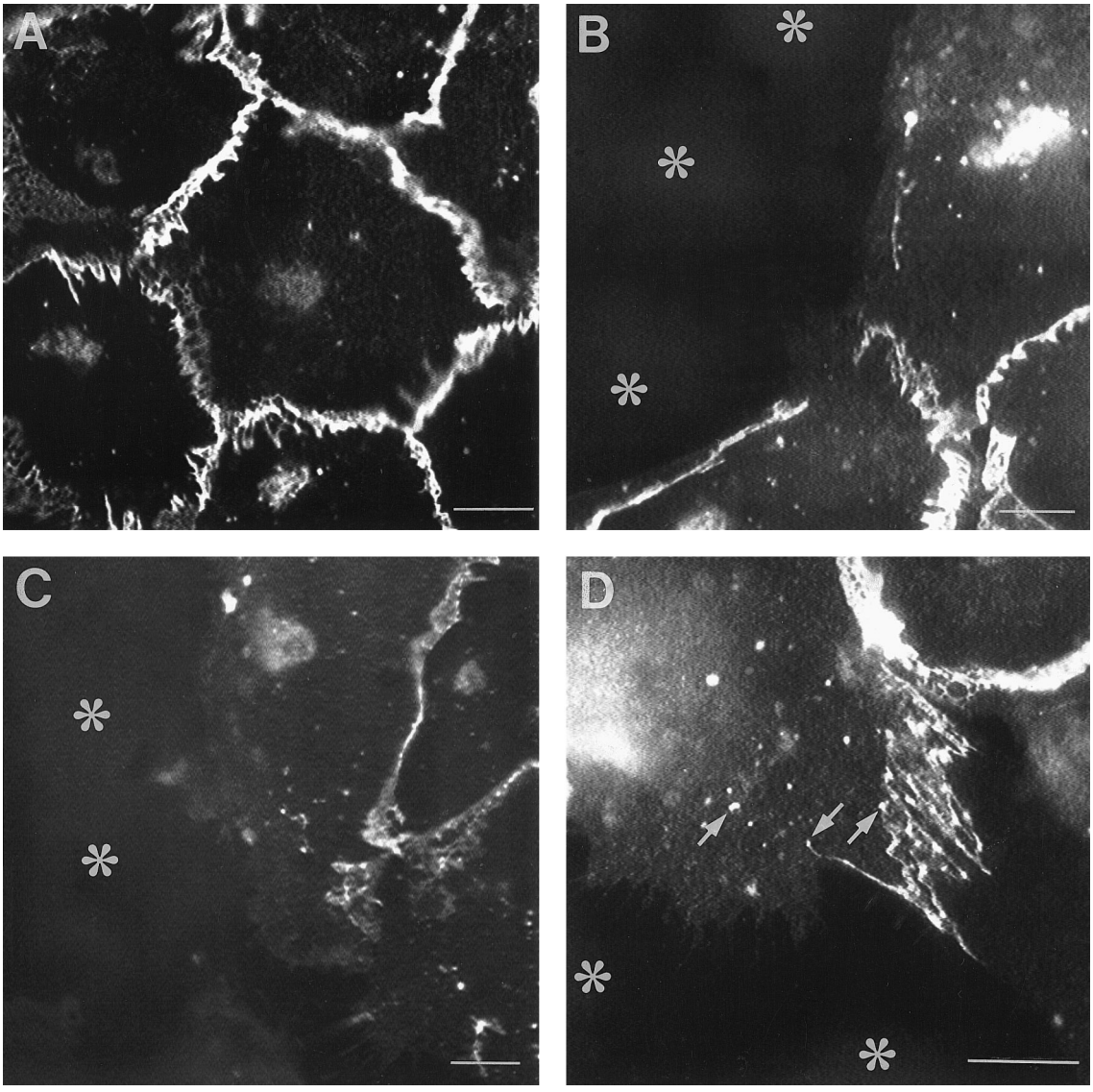

FIG. 2. Immunolocalization of VE-cadherin in EC monolayer and EC–MCF-7 cell cocultures. EC monolayer alone (A) or in coculture with MCF-7 cells for 30 min (B) and 90 min (C) were fixed, permeabilized, and stained for VE-cadherin. Higher magnification of reorganizing VE-cadherin in 30-min coculture is shown in D. Arrows indicate brightly labeled vesicules accompanying AJ disruption. The locations of tumor cells are indicated (*). Bars, 10 μm.

catenins complex, we have performed immunoprecipitations using VE-cadherin mAbs on cell extracts prepared from ^{35}S-labeled EC monolayers cultured alone or cocultured with unlabeled MCF-7 cells. As expected from previous studies by Lampugnani *et al.* [4], the immunoprecipitates contained four major bands with apparent M_r characteristic for VE-cadherin (130 kDa), α-catenin (102 kDa), β-catenin (93 kDa), and γ-catenin (85 kDa) (Fig. 5). Each protein of the complex could be separately identified in the immunoprecipitates by Western blotting using specific Abs directed against VE-cadherin (BV9), α-catenin (1597), β-catenin (1522), and γ-catenin (clone 15) (Fig. 5A). An additional labeled protein with a M_r of 66 kDa was also detected on the autoradiogram. However, it was present in the control immunoprecipitate suggesting that it does not specifically take part to the VE-cadherin/catenins complex (Fig. 5B, CTR). The association between VE-cadherin, α-catenin, β-catenin, and γ-catenin remained stable during the time course of tumor cell–EC interaction (Fig. 5B). The four bands were detectable in the cell extracts from control EC monolayer as well as from 30- and 90-min cocultures.

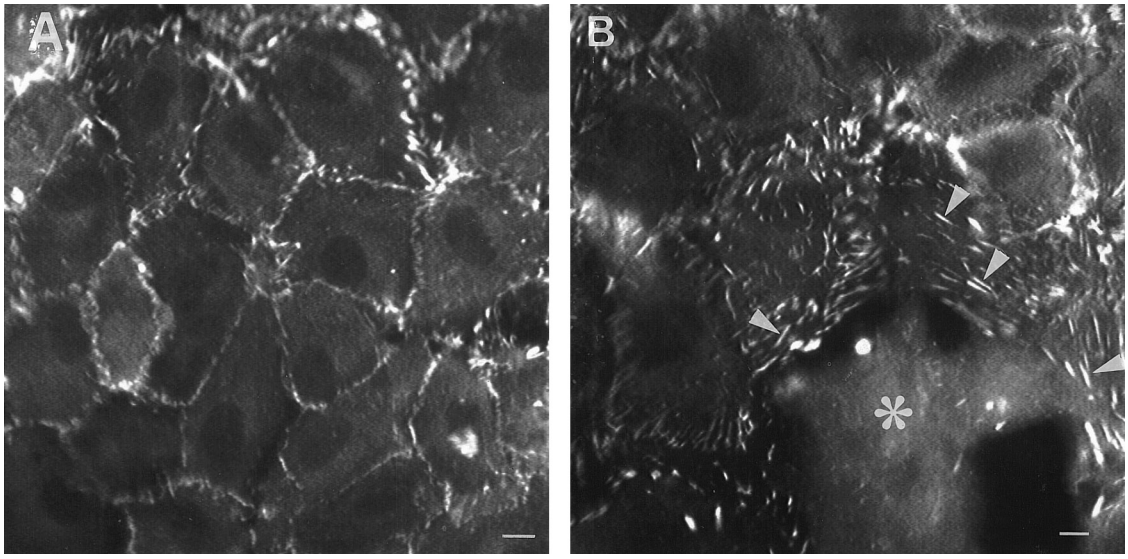

FIG. 3. Immunolocalization of vinculin in EC monolayer and EC–MCF-7 cell cocultures. EC monolayer alone (A) or in coculture with MCF7 cells for 90 min (B) were fixed, permeabilized, and stained for vinculin. Arrowheads indicate newly formed vinculin-positive focal adhesion plaques. The location of tumor cells is indicated (*). Bars, 10 μm.

Tyrosine Phosphorylation Status of the VE-Cadherin/Catenins Complex in EC Following Contact with Tumor Cells

The level of tyrosine phosphorylation of the VE-cadherin/catenins complex was evaluated in VE-cadherin-specific immunoprecipitates from EC monolayer and tumor cell–EC coculture by Western blotting using anti-phosphotyrosine mAbs (PY20). The presence of tyrosine-phosphorylated proteins in EC monolayer immunoprecipitates was barely detectable (Fig. 6A, EC). Following tumor cell contact however, three major tyrosine-phosphorylated bands were evidenced. Two proteins with apparent M_r of 100–105 and 110 kDa appeared first in 30-min coculture immunoprecipitates (Fig. 6A, EC + MCF-7 30 min). Within 90 min of coculture, a significant increase in the phosphorylation status of these two bands was observed as well as the emergence of a third tyrosine-phosphorylated protein species migrating at 130 kDa (Fig. 6A, EC + MCF-7 90 min). A marked reduction of the intensity of these three bands was then observed in longer time coculture (not shown).

In order to gain insight into the nature of the phosphorylated proteins, the nitrocellulose membrane used for phosphotyrosine detection was stripped and the first lane was rehybridized with Abs to VE-cadherin, α-catenin, and β-catenin. The bands evidenced at 100–105 and 130 kDa using anti-phosphotyrosine antibodies was superimposable to α-catenin and VE-cadherin specific band, respectively (Fig. 6B).

Immunofluorescence study using anti-phosphotyrosine mAbs on permeabilized cells revealed a very discrete staining of EC margins in confluent EC monolayer (Fig. 7A). In coculture experiment, an increased staining was evidenced on the free edge of early-dissociated EC (Figs. 7B and 7C, arrowheads). This staining was discontinuous and closely resembled the pattern shown by reorganizing VE-cadherin 30 min after tumor cell contact (Fig. 2B). On the contrary, endothelial intercellular junctions were not labelled (Fig. 7B and 7C). Simultaneously, focal adhesion-like structures were evidenced in cocultures suggesting an increased in protein kinase activity associated with focal adhesion plaques neoformation (Fig. 7B, arrows).

DISCUSSION

The migration of leukocytes and metastasizing tumor cells across the endothelium requires the opening of endothelial cell-to-cell junctions by a still unknown mechanism. Assembly and deassembly of interendothelial junctions is a complex process in which VE-cadherin and AJ-associated proteins have been suggested to act as major regulators [4, 11, 29]. In the present study, we have investigated the influence of tumor cell–EC contact on AJ proteins expression, association, and phosphorylation status.

As previously described with many tumor cell types [1, 12–15], breast adenocarcinoma cells (MCF-7) dramatically impaired interendothelial cohesion (Fig. 1). The opening of the interendothelial junctions induced by tumor cells, in our system, was strictly limited to

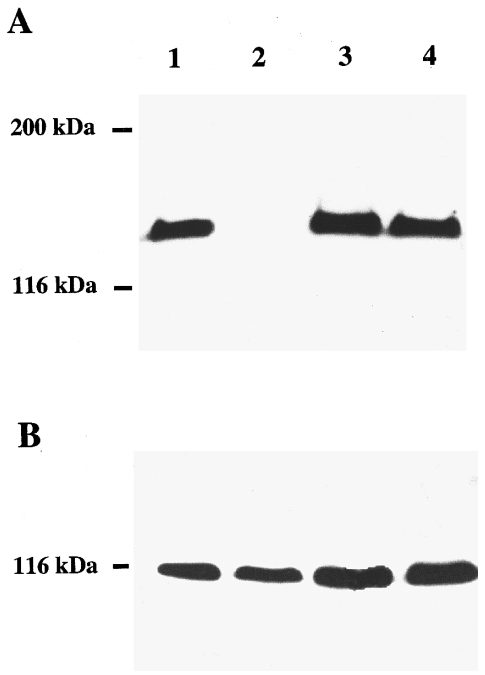

FIG. 4. Western blot analysis of VE-cadherin and vinculin in EC monolayer, MCF-7 cells, and cocultures. Total cell extracts from EC (lane 1), MCF-7 cells (lane 2), or 30 min (lane 3) and 90 min (lane 4) cocultures were electrophoresed, transferred to nitrocellulose filters, and probed using VE-cadherin (A) or vinculin (B) mAbs. All samples were prepared in parallel from the same EC primary culture. Equal amounts of radiolabeled material were loaded for each condition.

the site of tumor cell contact with the endothelium and could not be reproduced when using tumor cell culture supernatant or fixed tumor cells. Furthermore, EC dissociation could be induced by a single tumor cell within a time period consistent with tumor cell extravasation observed *in vivo* by videomicroscopy [15]. These observations suggest that the initiation of EC dissociation is spatially limited to the physical contact site between the two cell types and is not induced by soluble factor(s) freely available to EC. The specificity of the mechanisms involved in these interactions are further supported by the facts that neither normal human mammary epithelial cells nor fixed tumor cells interfered with endothelial monolayer integrity.

We show that the loss of interendothelial contact induced by tumor cells is concomitant with a intense remodeling of endothelial AJ. In confluent EC monolayers, VE-cadherin has the same distribution as vinculin along the cell margin (Figs. 2 and 3). In the presence of tumor cells, however, the staining pattern of both molecules was strongly modified. VE-cadherin-specific staining progressively disappeared from cell-to-cell boundaries and remained as brightly labeled vesicles in the cytoplasm (Fig. 2). Optical sectioning of VE-cadherin-labeled EC using laser confocal microscopy shows that: (i) these vesicules are located into the cytosol rather than on the basal or luminal plasma membrane of EC and (ii) they result from the disruption of intercellular structures (Fig. 2D, arrows). These observations suggest that VE-cadherin is internalized following interaction with tumor cells. The presence of few labeled vesicules in quiescent contact-inhibited EC may account for the natural turnover of the protein (Fig. 2A).

Simultaneously, vinculin was translocated from the cell border to well-differentiated focal adhesion plaques located on the basal EC plasma membrane in contact with the extracellular matrix (Fig. 3). Independent studies have shown that vinculin colocalizes with VE-cadherin in confluent EC cultures [30] and is able to associate with actin microfilaments directly [31] or via the binding to α-actinin [32]. Furthermore, a high homology was found between the C-terminal domain of

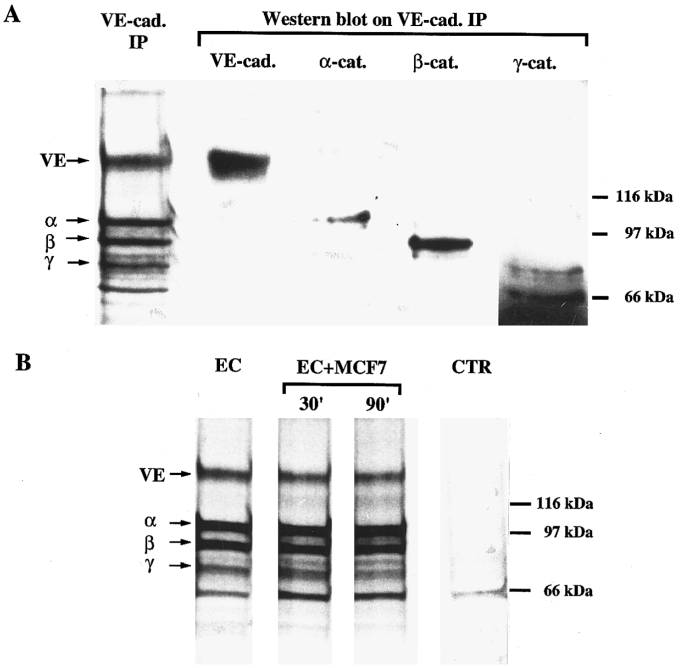

FIG. 5. VE-cadherin/catenin complex immunoprecipitation from EC monolayer and EC–MCF-7 cell cocultures. Confluent EC monolayers were [^{35}S]methionine labeled and incubated for 30 and 90 min with unlabeled MCF-7 cells. Cell extracts were immunoprecipitated using mAbs against VE-cadherin. VE-cadherin immunoprecipitates from EC monolayer alone were electrophoresed and either autoradiographied (VE-cad.IP) or transferred to nitrocellulose filter for further identification of the immunoprecipitated bands by Western blot using Abs against VE-cadherin (VE-cad), α-catenin (α-cat.), β-catenin (β-cat.), and γ-catenin (γ-cat.) (A). VE-cadherin immunoprecipitates from EC monolayer (EC) and coculture (EC + MCF-7) at different times were migrated on the same gel and autoradiographied (B). In the control condition, extracts were immunoprecipitated using unrelated mAbs (B, CTR).

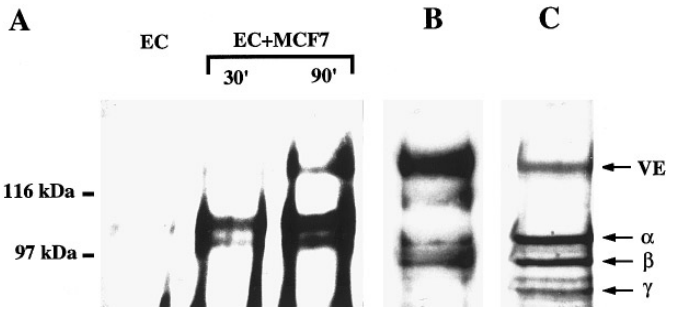

FIG. 6. Anti-phosphotyrosine Western blot analysis of VE-cadherin immunoprecipitates. Extracts from EC monolayer (EC) and EC–MCF-7 cell cocultures at different times (EC + MCF-7) were immunoprecipitated using mAbs against VE-cadherin. The immunoprecipitates were electrophoresed, transferred to nitrocellulose filter, and probed with anti-phosphotyrosine mAbs (PY20) (A). In some experiments, the filters were stripped and the first lane was reprobed with Abs against VE-cadherin, α-catenin, and β-catenin as described under Methods (B). The pattern obtained in (B) was compared to the anti-phosphotyrosine Western blot (A) and to the radiolabeled VE-cadherin immunoprecipitate from EC monolayer (C) showing the position of VE-cadherin (VE), α-catenin (α), β-catenin (β), and γ-catenin (γ).

α-catenin and the self-association domain of vinculin suggesting a possible association between these two proteins. Vinculin was thus proposed as a potential link between cadherin/catenin complexes and the actin cytoskeleton through its interaction with α-catenin [33]. Here, we show that the contact between tumor cell and EC induces the loss of VE-cadherin and vinculin colocalization.

The loss of AJ correlates with a differential redistribution of VE-cadherin and vinculin rather than an inhibition of their expression. Indeed, evaluation of the VE-cadherin and vinculin content in EC on Western blots did not show any important modifications in response to tumor cell contact (Fig. 4). Furthermore, the hypothesis of a proteolytic breakdown of AJ proteins induced by tumor cells was disproved first by the lack of effect of metalloprotease and serine protease inhibitors on EC dissociation (not shown) and second by the fact that all coculture experiments were performed in the presence of 10% serum which contains high level of protease inhibitors.

The importance of the interactions between the intracellular domain of VE-cadherin and the catenins in maintaining EC cohesion have been recently documented by Navarro *et al.* [5] using truncated VE-cadherin lacking the binding regions for catenins. They show that VE-cadherin extracellular domain is sufficient for early steps of cell adhesion and recognition but does not provide strength and selective barrier property to EC monolayers. Furthermore, a recent study on the kinetics of AJ formation have shown that

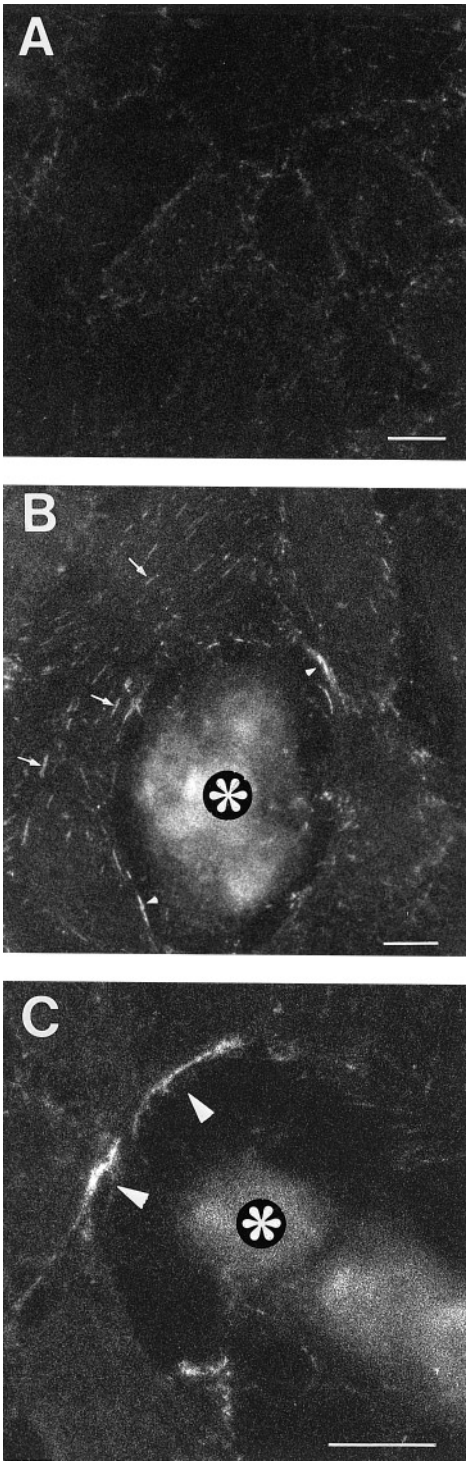

FIG. 7. Immunolocalization of tyrosine-phosphorylated proteins in EC monolayer (A) and 30-min EC–MCF-7 cell coculture (B and C). Cells were fixed, permeabilized, and stained with PY20 mAbs. Arrows indicate labeled focal adhesion-like structures. Strongly positive structures bordering EC-free edge are pointed out by arrowheads in B and C. The location of a tumor cell aggregate is indicated (*). Bars, 10 μm.

γ-catenin associates with VE-cadherin/α-catenin/β-catenin complex only at late stage of AJ assembly and is determinant for the establishment of stable intercellular junctions [4]. In the present study, we have analyzed the composition of the VE-cadherin/catenin complex in EC monolayer and tumor cell–EC coculture. In quiescent contact-inhibited EC, at least four specific bands were detected in VE-cadherin immunoprecipitate and were identified as VE-cadherin, α-catenin, β-catenin, and γ-catenin by Western blot analysis (Fig. 5A). The presence of all three catenins in the immunoprecipitate is consistent with the expression of fully differentiated AJ linking EC together. Upon contact with tumor cells, the composition of the complex is unchanged suggesting that the association between VE-cadherin and α-, β-, and γ-catenins remains stable as EC disjunction progresses (Fig. 5B). In agreement with our observation are the data from Lampugnani *et al.* [4] showing that the induction of EC dissociation by reducing the extracellular Ca^{2+} concentration was accompanied by a dramatic reduction of the VE-cadherin/catenins complex expression at cell junctions but does not impair the composition of the complex. Altogether, these results suggest that the adhesive function of VE-cadherin can be modulated by mechanisms that do not affect its association with intracellular catenins. In contrast with our observations, however, are the recent results reported by Del Maschio *et al.* [34]. Although these authors have shown that polymorphonuclear neutrophils do induce the disappearance of VE-cadherin from endothelial cell-to-cell junctions, their results suggest that this process correlates with the dissociation of β-catenin and plakoglobin (γ-catenin) from the complex. Similarly, thrombin-induced EC disjunction was shown to correlate with the disassembly of the VE-cadherin/catenin complex [35]. Furthermore, VE cadherin reorganization observed in these studies occurred within 5 min after treatment while it appears only 30 min after treatment in our experiments. These apparent discrepancies support the idea that different molecular mechanisms may control the adhesive properties of VE-cadherin.

Previous studies have also stressed the major role of tyrosine phosphorylation/dephosphorylation mechanisms in the assembly/disassembly of intercellular junctions in epithelial cells and embryonic fibroblasts [6–10]. As demonstrated by these authors, an increase in tyrosine phosphorylation of cadherin, α-catenin, and/or β-catenin correlated with a decrease of cadherin-dependent intercellular adhesion. Presently, we show that three major bands are heavily phosphorylated on tyrosine residues in the VE-cadherin immunoprecipitate as a result of cocultivation with tumor cells (Fig. 6). The crossreactivity of two of these proteins with specific Abs directed against VE-cadherin and α-catenin suggest that these later are phosphorylated in the early time of tumor cell–EC interaction. Earlier studies on E- or P-cadherin complex phosphorylation using $p60^{src}$-transfected cell lines [8, 9, 36] have described β-catenin as a major substrate for protein tyrosine kinase. However, in embryonic fibroblasts expressing N-cadherin, Hamagushi *et al.* [7] have reported an important increase in α-catenin phosphorylation following $p60^{src}$ transformation which correlated with the suppression of cell adhesiveness. These results suggest that the pattern of protein phosphorylation within the cadherin/catenins complex may be different according to the cell type and the nature of the cadherin expressed. As for N-cadherin, α-catenin phosphorylation may be determinant for the adhesive properties of VE-cadherin.

The third phosphorylated band (110–115 kDa) evidenced in the VE-cadherin immunoprecipitate could not be identified in the present study. A $p120^{cas}$ isoform migrating at an intermediate molecular weight between α-catenin and VE-cadherin has been described previously [35]. $p120^{cas}$ is a substrate for $p60^{src}$ and may be implicated in signal transduction mechanisms through AJ [37]. However, $p120^{cas}$ could not be identified in VE-cadherin immunoprecipitates by Western blots although it was present in human fibroblast lysate used as a positive control (data not shown).

The increase in the phosphorylation of the VE-cadherin/catenin complex during tumor cell–EC interaction was further supported by immunofluorescence studies using anti-phosphotyrosine mAbs (Fig. 7). The low level of tyrosine phosphorylation of AJ proteins (Fig. 6A) correlated with the very faint signal observed at EC margin in quiescent monolayer (Fig. 7A). In early coculture (30 min), an increased signal was detected along EC free edge as well as in elongated rod-like structure on the basal EC surface (Figs. 7B and 7C). This staining pattern was very similar to that observed using separately VE-cadherin mAbs (Fig. 2B) and vinculin mAbs (Fig. 3B) and suggest that both reorganizing VE-cadherin and newly formed focal adhesion plaques are phosphorylated. This occurred only in EC directly interacting with tumor cells.

Altogether the data presented here demonstrated that tumor cell-induced EC disjunction occurs simultaneously with VE-cadherin/catenins complex phosphorylation, VE-cadherin reorganization, and focal adhesion plaques differentiation. Our observation suggests that the contact between tumor cell and EC triggers a series of molecular events which locally impair endothelial cell-to-cell cohesion and therefore would facilitate the access to the subendothelial basement membrane and invasion into the perivascular area.

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