Role of $\alpha_v\beta_3$-integrin in TNF-\(\alpha\)-induced endothelial cell migration

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Gao, Baochong, Thomas M. Saba, and Min-Fu Tsan. Role of $\alpha_v\beta_3$-integrin in TNF-\(\alpha\)-induced endothelial cell migration. Am J Physiol Cell Physiol 283: C1196–C1205, 2002. — Tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), one of the major inflammatory cytokines, is known to influence endothelial cell migration. In this study, we demonstrate that exposure of calf pulmonary artery endothelial cells to TNF-\(\alpha\) caused an increase in the formation of membrane protrusions and cell migration. Fluorescence microscopy revealed an increase in $\alpha_v\beta_3$ focal contacts but a decrease in $\alpha_\alpha\beta_1$ focal contacts in TNF-\(\alpha\)-treated cells. In addition, both cell-surface and total cellular expression of $\alpha_v\beta_3$-integrins increased significantly, whereas the expression of $\alpha_\alpha\beta_1$-integrins was unaltered. Only focal contacts containing $\alpha_v\beta_3$ but not $\alpha_\alpha\beta_1$-integrins were present in cells behind the migration front. A blocking antibody to $\alpha_v$, but not a blocking antibody to $\alpha_\alpha$-integrins, significantly inhibited TNF-\(\alpha\)-induced cell migration. These results indicate that in response to TNF-\(\alpha\), endothelial cells may increase the activation and ligation of $\alpha_v\beta_3$, while decreasing the activation and ligation of $\alpha_\alpha\beta_1$-integrins to facilitate cell migration, a process essential for vascular wound healing and angiogenesis.

ADHESION AND MIGRATION are distinct functions of endothelial cells essential for maintaining the integrity of the endothelium and repairing or forming blood vessels during wound healing or angiogenesis. The balance between adhesion and migration is precisely regulated in response to changing environments in the blood stream. Strong adhesion to the extracellular matrix is required for resting endothelial cells to maintain the integrity of the endothelium (8, 21, 31), whereas modulated adhesion to the matrix is necessary to facilitate cell migration (17, 18, 44). One way cells can modulate the strength of adhesion and facilitate migration is to change the expression and distribution of integrins on the cell surface.

Functional cell-surface integrins are complexes of an \(\alpha\)- and a \(\beta\)-subunit. More than 20 integrin complexes have been identified representing different combinations of at least 16 $\alpha$- and 8 $\beta$-subunits (19, 31). The difference in the subunit composition determines the specificity of the integrin complex for its substrate in the matrix. For example, the $\alpha_\alpha\beta_1$-integrin complex essentially interacts only with fibronectin in the matrix, whereas the $\alpha_v\beta_3$ complex interacts preferentially with laminin (19, 28, 32). Some integrin complexes have multiple preferred substrates in the matrix. One example is $\alpha_v\beta_3$-integrin, which interacts with vitronectin and fibronectin, as well as laminin. Integrins $\alpha_\alpha\beta_1$ and $\alpha_v\beta_3$ are predominant integrin complexes expressed in endothelial cells (8, 37). Both integrin complexes have been implicated in endothelial cell adhesion and migration (17, 18, 33, 42).

The regulation of cell adhesion and migration involves coordinated events including cell signaling, cytoskeleton rearrangement, and surface integrin redistribution. These cellular events are known to be influenced by inflammatory cytokines such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)). TNF-\(\alpha\) is a 17-kDa polypeptide that forms homotrimers on the cell surface. It is synthesized and secreted by many cell types upon stimulation with a variety of toxins and cytokines including TNF-\(\alpha\) itself. Activated macrophages and monocytes are major sources of TNF-\(\alpha\), and a primary target of this specific cytokine is the endothelial cell (23, 25, 36, 38).

Over the past decade, considerable effort has been focused on TNF-\(\alpha\)-induced apoptosis, whereas the mechanism of TNF-\(\alpha\)-induced endothelial cell migration is relatively unknown. Studies show that TNF-\(\alpha\) can display either proangiogenic or antiangiogenic effect depending on experimental conditions (12, 22, 26). One of these conditions appears to be the dosage or concentration of TNF-\(\alpha\) used in vivo or in vitro. It promotes the formation of tubular structure at relatively low dosages but becomes inhibitory to angiogenesis and induces apoptosis at relatively high dosages (12, 22, 29). In vitro, TNF-\(\alpha\) concentrations between 100 and 250 units/ml induced the highest levels of tubule formation, whereas tubule formation was sig-
significantly reduced at TNF-α concentrations higher than 500 units/ml (43). TNF-α concentrations around 250 units/ml were also observed in the blood of patients with serious inflammation and sepsis or in healthy human subjects challenged with endotoxin (36, 39). Accordingly, we used TNF-α at a concentration known to induce cell migration to identify the role of cell-surface integrins in TNF-α-induced endothelial cell migration.

MATERIALS AND METHODS

Materials. Bovine pulmonary artery endothelial (CPAE) cells were obtained from American Type Culture Collection (Manassas, VA). Recombinant human TNF-α (20 units/ng) was obtained from Cellular Products (Buffalo, NY). Monoclonal antibodies to α5β3 (clone LM609), α5β1 (clone HA5), and actin (MAB1501) and polyclonal antibodies to α5- (AB1928) and β3- (AB1932) integrins were obtained from Chemicon International, (Temecula, CA). The blocking antibody to α5 (clone BI1G2) was developed by C. H. Damsky and obtained from the Developmental Studies Hybridoma Bank established under the auspices of the National Institute of Child Health and Human Development (NICHD) and maintained by the Department of Biological Sciences, The University of Iowa, Iowa City, IA. All integrin antibodies used in this study recognize both activated and nonactivated form of integrins. Protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and N-p-tosyl-l-lysine chloromethyl ketone (TLCK) were purchased from Sigma (St. Louis, MO).

Endothelial cell culture. CPAE cells at passage 16 were cultured as described previously (14). The cells were cultured in minimum essential medium (MEM; GIBCO Invitrogen, Carlsbad, CA) containing 20% fetal bovine serum (FBS; GIBCO Invitrogen). TNF-α exposure was carried out in MEM containing 5% FBS. All cells used in this study were cultured to confluence and treated with or without TNF-α at 200 units/ml for 18 h before analysis (migration assay, adhesion assay, immunofluorescence microscopy, or immunoprecipitation).

Determination of membrane protrusion formation and cell migration with an in vitro wound-healing assay. Confluent endothelial cells on glass coverslips were treated with or without TNF-α, and wounds were created on cell monolayers by using the “scratch wound” protocol (10, 15, 34) with a razor blade. The debris was removed by washing the cells with serum-free MEM, and the cells were incubated in a 37°C incubator for 5 h in serum-free MEM. The cells were photographed and the number of migrating cells and the percentage of cells with membrane protrusions were determined under an inverted microscope. A total of nine areas were selected randomly on each coverslip under a 40× objective. Cells on three to six coverslips of either control or TNF-α-treated sample were quantified in each experiment. To detect integrins in focal contacts, the cells were fixed, permeabilized, and incubated with antibodies to α5β3 (LM609) or α5β1 (HA5) and fluorescence-labeled secondary antibodies (Molecular Probes, Eugene, OR).

To determine the effects of blocking antibodies on cell migration, confluent endothelial cells on glass coverslips were treated with or without TNF-α and scrapped with a razor blade. The debris was removed by washing the cells with serum-free MEM. The cells were then incubated in a 37°C incubator for 5 h in the presence or absence of blocking antibodies to either α5- (BI1G2) or α5β3 (LM609)-integrin complexes. The number of cells migrated into the wound area was determined as described above.

Determination of membrane protrusion formation with cell adhesion assay. Human fibronectin was purified from cryoprecipitate (American Red Cross) by using gelatin-Sepharose affinity chromatography according to the procedure of Engel and Ruoslahti (11). Human cryoprecipitate (15 ml) was diluted 1:1 with the column equilibration buffer and loaded onto a 10-ml gelatin-Sepharose column (Pharmacia Biotech, Piscataway, NJ) at a flow rate of 0.5 ml/min. The column was washed with 1 M NaCl in phosphate-buffered saline (PBS) and eluted with 4 M urea in the washing buffer. The eluted fraction was dialyzed overnight in 0.2 M phosphate buffer, pH 7.4, and the fibronectin concentration was determined by using the extinction coefficient ε280 = 12.8.

Glass coverslips in 12-well plates were incubated overnight with purified fibronectin at 2 μg/ml in coating buffer (50 mM NaHCO3, pH 9.6) at 4°C. Endothelial cells treated with or without TNF-α were lifted into suspension with trypsin-EDTA buffer (GIBCO Invitrogen) and seeded onto fibronectin-coated or noncoated coverslips at 105 cells/well. The cells were incubated in either serum-free medium on fibronectin-coated surfaces or MEM containing 20% FBS on noncoated surfaces at 37°C for 30 min. Nonadhered cells were removed by washing with PBS, and adhered cells were examined and photographed under an inverted microscope. Cells with membrane protrusions were quantified as described above.

Determination of the effect of blocking antibodies on endothelial cell adhesion on fibronectin-coated surfaces. Endothelial cells in suspension were preincubated with blocking antibodies to either α5β3 (LM609)- or α5β1 (BI1G2)-integrins on ice for 30 min before being seeded onto glass coverslips coated with 2 μg/ml fibronectin. Coverslips coated with 10 μg/ml bovine serum albumin (BSA) were used as controls for nonspecific adhesion. Cells were incubated in serum-free medium at 37°C for 30 min. Nonadhered cells were removed by washing with PBS. The number of adhered cells was determined by counting under an inverted microscope as described above.

Determination of cell-surface integrin expression by surface biotinylation, immunoprecipitation, and Western blotting. Confluent CPAE cell monolayers treated with or without TNF-α were labeled with Biotin (Pierce, Rockford, IL) at 0.5 mg/ml in PBS for 60 min at 4°C. Cells were then lysed in the lysis buffer (150 mM NaCl, 5 mM EDTA, 1% sodium deoxycholate, 1% Triton X-100, and 20 mM Tris at pH 7.4) containing protease inhibitors (0.3 mM PMSF and 0.1 mM TLCK). The cell lysate was clarified by centrifugation in a Microfuge and preclarified by incubation with protein G agarose (GIBCO Invitrogen). Integrins α5β3 or α5β1 were immunoprecipitated with antibodies LM609 and HA5, respectively, followed by incubation with protein G agarose. The agarose-bound integrins were solubilized in boiled SDS-gel sample buffer under nonreducing conditions and clarified by spinning in a Microfuge. Precipitated integrins were separated on two identical 7.5% SDS gels and transferred onto nitrocellulose membranes. One membrane was used to determine cell-surface integrins with streptavidin conjugated to horseradish peroxidase and enhanced chemiluminescence (ECL) Western blotting detection solutions (both from Amersham, Piscataway, NJ). The other membrane was used to determine total cellular integrins in biotinylated cells with antibodies to either α5 (AB1928) or β3 (AB1932) and ECL Western blotting detection solutions. The bands on films were quantified by densitometric scanning using a BioRad imaging densitometer (Bio-Rad, Hercules, CA).
Determination of total cellular integrin expression by immunoprecipitation and Western blotting. Confluent CPAE cells treated with or without TNF-α were lysed in the lysis buffer, and α5β1- or α6β1-integrins were immunoprecipitated from the cell lysate by using monoclonal antibodies to the integrins as described above. Precipitated integrins were separated on SDS gels and transferred onto nitrocellulose membranes. The nitrocellulose membranes were probed for separation on SDS gels and transferred onto nitrocellulose from the cell lysate by using monoclonal antibodies to the integrins. The nitrocellulose membranes were probed for using antiactin antibody MAB1501.

Immunofluorescence microscopy. CPAE cells cultured on coverslips were fixed with 3% formaldehyde, permeabilized in 0.5% Triton, and stained with either an antibody against human α5β1-integrin (clone HA5) or an antibody to human α5β1-integrin (clone LM609) at 2 μg/ml. This was followed by incubations with secondary antibodies conjugated to Alexa-488 (Molecular Probes, Eugene, OR). The coverslips were mounted with ProLong Anti-Fade (Molecular Probes) and examined using a BX60 fluorescence microscope (Olympus, Melville, NY) and photographed using a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Statistical analysis. All measurements were performed at least three times with duplicate samples. Results are presented as means ± SD. Levels of significance are determined by a two-tailed Student’s t-test (13), and a confidence level of >95% (P < 0.05) was used to establish statistical significance.

RESULTS

Effect of TNF-α on endothelial cell migration and the formation of membrane protrusions. We examined the migration of endothelial cells treated with TNF-α at 200 units/ml for 18 h, because previous studies indicate that functional changes in endothelial monolayers occur between 12 and 24 h of TNF-α exposure at this dosage. These functional changes include dissociation of α5β1-integrins from focal contacts (14, 30), increased recycling of integrins (14), reduced cell adhesion to fibronectin (30), cell-cell gap formation (7, 14, 30), and increase in protein permeability (6, 7, 40).

The migration of endothelial cells was evaluated by using a well-established in vitro wound-healing assay (10, 15, 34). In these experiments, endothelial cell monolayers treated with or without TNF-α were wounded with a razor blade. After a 5-h incubation, the number of cells migrated into the wound area was determined under an inverted microscope. Results showed that TNF-α-treated cells displayed a significant increase in cell migration (Fig. 1, A–C). In addition, increased formation of membrane protrusions was observed in TNF-α-treated cells at the migration front (Fig. 1, A, B, and D), suggesting a possible role of the membrane protrusions in the increased cell migration.

We next asked the question whether the increased formation of membrane protrusion was a characteristic of all TNF-α-treated cells, not only cells at the migration front. One way to answer this question is to determine the formation of membrane protrusions in a cell adhesion assay under subconfluent conditions. To determine the effect of matrix proteins, we determined the formation of membrane protrusions on surfaces coated with fibronectin, a common substrate for both α5β1- and α6β1-integrins. In this experiment, cells in monolayers treated with or without TNF-α were lifted into suspension and seeded onto glass coverslips coated with or without fibronectin. Cells were then incubated briefly at 37°C either in a serum-free medium on fibronectin-covered surfaces or in the presence of 20%...
serum on noncoated surfaces. Nonadhered cells were removed by a washing with PBS. Adhered cells were photographed under an inverted microscope. Results in Fig. 2 show clearly that TNF-α-treated cells display more membrane protrusions than control cells in this assay on both fibronectin-coated and noncoated surfaces. These observations under subconfluent conditions suggest that increased formation of membrane protrusions may be a characteristic of all TNF-α-treated cells, not only cells at the migration front in the wound-healing assay.

Effect of TNF-α on the localization of αβ3- and αβ1-integrins in focal contacts. αβ3 and αβ1 are predominant integrin complexes expressed in endothelial cells. These integrin complexes have been shown to play important roles in cell migration (31, 32, 37). It is possible that the increased formation of membrane protrusions after TNF-α exposure were mediated by an increase in the ligation of these integrins. If this were the case, one would expect to see integrin-containing focal contacts in membrane protrusions, especially in cells at the migration front.

To test this possibility, endothelial cells treated with or without TNF-α were assayed for cell migration as in Fig. 1, and cells were fixed and stained with antibodies recognizing either αβ3- or αβ1-integrin complexes. As shown in Fig. 3, only αβ3-containing focal contacts were detected in membrane protrusions of cells at the migration front (Fig. 3, B and D). In contrast, αβ1-integrins in cells at the migration front were observed only in structures resembling endocytic vesicles (Fig. 3, A and C), not in focal contacts. However, focal contacts containing αβ1-integrins were readily identified in cells immediately behind the migration front (Fig. 3, A and C). In comparison, control cells not treated with TNF-α had much fewer αβ3 focal contacts and significantly lower levels of membrane protrusion formation (Fig. 3, compare B and D). These data support the notion that TNF-α-induced formation of membrane protrusion and cell migration may rely on the increase in the ligation of αβ3-integrins.

To obtain a closer look at the formation of αβ3 focal contacts on migrating cell, we carried out a time course of cell migration into the wound area and compared the rate of migration of control and TNF-α-treated endothelial cells. Results (Fig. 4) showed that cell migration could be detected in TNF-α-treated cell monolayers 1 h after wounding (Fig. 4E), whereas similar levels of cell migration were not observed until 4 h after wounding.
in control monolayers (Fig. 4C). In addition, focal contacts containing \(\alpha_v\beta_3\)-integrins formed in all migrating cells, especially on membrane protrusions (Fig. 4, C–H).

An important question was whether the increased \(\alpha_v\beta_3\) and decreased \(\alpha_5\beta_1\) focal contacts occurred not only in cells at the migration front but also in cells in confluent monolayers. To answer this question, endothelial monolayers treated with or without TNF-\(\alpha\) were fixed and stained with antibodies recognizing both activated and nonactivated form of either \(\alpha_v\beta_3\)- or \(\alpha_5\beta_1\)-integrins. Results show (Fig. 5) that the expression of \(\alpha_v\beta_3\)-integrins in control cells in monolayers is only detectable at cell-cell junctions (Fig. 5B), whereas \(\alpha_5\beta_3\)-containing focal contacts can be readily identified around the cell periphery in TNF-\(\alpha\)-treated cells (Fig. 5D). In addition, TNF-\(\alpha\)-treated cells also display increased gap formation, suggesting a loss of cell-cell interactions after cell monolayers were exposed to TNF-\(\alpha\). This is consistent with earlier observations under similar conditions (6, 14, 30). Increased \(\alpha_v\beta_3\) focal contacts around the periphery of the TNF-\(\alpha\)-treated cells may be a cellular response to increase cell adhesion in compensating the loss of cell-cell interactions. In contrast to \(\alpha_v\beta_3\) focal contacts, robust \(\alpha_5\beta_1\)-containing focal contacts were detected in control cells (Fig. 5A), and an apparent decrease in \(\alpha_5\beta_1\) focal contacts was observed in TNF-\(\alpha\)-treated cells (Fig. 5C). These results indicate that TNF-\(\alpha\) caused an increase in the activation/ligation of \(\alpha_v\beta_3\) and a decrease in the activation/ligation of \(\alpha_5\beta_1\)-integrins in all TNF-\(\alpha\)-treated endothelial cells, not only in cells at the migration front.

**Effect of TNF-\(\alpha\) on the expression of \(\alpha_v\beta_3\) and \(\alpha_5\beta_1\)-integrins.** Changes in focal contacts observed in Figs. 3 and 4 could have been caused by changes in cell-surface expression and/or total cellular expression of the integrins. However, individual integrins cannot be detected by microscopy unless they have been recruited into focal contacts. We therefore investigated TNF-\(\alpha\)-induced changes in the expression of \(\alpha_v\beta_3\) and \(\alpha_5\beta_1\)-integrins in endothelial cells using biochemical approaches.

Integrins can display different activation states, and the state of integrin activation is influenced by their
interactions with ligands, antibodies, and cations such as Mn²⁺ (1, 24, 28, 41). Binding of a ligand or Mn²⁺ can switch an integrin complex from a “low-affinity state” (nonactivated form) to a “high-affinity state” (activated form). The transition of the affinity states involves conformational changes of the integrins, which can be detected by specific antibodies recognizing motifs exposed only when integrins are activated. To quantify the expression of all forms of integrins, we used antibodies to recognize both activated and nonactivated forms of integrins for immunoprecipitation.

To determine the surface expression of the integrins, the cell surface was first biotinylated and then αvβ5- or α5β1-integrins were immunoprecipitated from the cell lysate. The immunoprecipitated integrins were then quantified by Western blotting using streptavidin conjugated to horseradish peroxidase (Fig. 6). To determine the total cellular expression of the integrins, αvβ3 or α5β1 was immunoprecipitated from the whole cell lysate and the integrins were quantified by Western blotting using antibodies to either α5- or β3-integrin subunit (Fig. 7). These antibodies were used to quantify α5β1- and αvβ3-integrin complexes, because α5-subunit has been found only in αvβ1 complexes and β3-subunit forms complexes only with αv in endothelial cells (9, 28, 31).

Results (Figs. 6 and 7) indicated that TNF-α caused a significant increase in both the cell-surface and total cellular expression of αβ3-integrins. In contrast, the expression of αvβ3-integrins did not change significantly despite the clear decrease in α5β1-containing focal contacts observed (Fig. 5). Thus the increase in αvβ3-containing focal contacts observed in TNF-α-treated endothelial cells (Figs. 3–5) was at least partially due to the increased surface expression of the integrins. On the other hand, the data were consistent with the concept that an inactivation, rather than a decrease in surface expression of α5β1-integrins, was the basis for the reduction of focal contacts containing α5β1-integrins observed in TNF-α-treated endothelial cells (Fig. 5).

Effect of blocking antibodies to αvβ3- and α5β1-integrins on TNF-α-induced cell migration. The above observations suggest that increased αvβ3-containing focal contacts may have served as anchors for membrane protrusions, without which membrane protrusions may retract and cell migration may be abolished. If this were true, one would expect to see an attenuation of cell migration when the αvβ3-ligand interactions are blocked.
To test this hypothesis, the cell migration assay was performed in the presence of blocking antibodies to either α5β3- or α5β1-integrins. A blocking antibody to α5-subunit was used to block the function of α5β1-integrin complexes, because α5-subunit has only been found in α5β1-integrin complexes (28, 31). We first examined the effect of the antibodies on cell adhesion to determine the concentration at which the antibodies can act effectively. Because fibronectin is a substrate for both α5β3- and α5β1-integrins in the matrix, we determined whether the antibodies could block cell adhesion on fibronectin-coated surfaces. We observed that both blocking antibodies inhibited the adhesion of endothelial cells with significant blocking effects observed at 5 μg/ml for anti-α5β3 and a fivefold dilution for anti-α5 antibodies (Fig. 8).

We next determined the effect of the blocking antibodies on cell migration. Results showed that cell migration was inhibited in TNF-α-treated cells by the blocking antibody to α5β3-integrins in a concentration-dependent manner (Fig. 9). In contrast, the blocking antibody to α5-integrins had little effect on TNF-α-induced cell migration (Fig. 10), even at concentrations that significantly inhibited cell adhesion (Fig. 8). These observations suggest that α5β3-integrins play an important role in TNF-α-induced cell migration.

**DISCUSSION**

The results presented in the current study demonstrated that TNF-α at 200 units/ml, a concentration commonly found in severely septic patients, could cause endothelial cells to increase the formation of membrane protrusions and cell migration. These changes were accompanied by an increase in both cell-surface and total cellular expression of α5β3-integrins. In contrast, the expression of α5β1-integrins remained unchanged. The increased formation of membrane protrusions and cell migration in TNF-α-treated cells was facilitated by the increased expression of α5β3 on the cell surface and increased recruitment of α5β3-integrin into focal contacts. Several lines of evidence presented in this study support these conclusions. First, a significant increase in α5β3-integrin expression was detected on the surface of TNF-α-treated endothelial cells. Second, a marked increase in α5β3-containing focal contacts was observed after cells were exposed to TNF-α. Third, only α5β3-containing focal contacts, but not α5β1-containing focal contacts, were detected in membrane protrusions of cells at the migration front. Fourth, a blocking antibody to α5β3-integrins, but not a...
blocking antibody to α5-integrins, significantly inhibited TNF-α-induced cell migration.

The development of inflammation is mediated by cytokines released upon bacterial infection. Proinflammatory cytokines such as TNF-α mediate vascular inflammation by inducing cell-cell and cell-matrix dissociation of endothelial cells (7, 14, 23, 30). In vitro, the dissociation of either cell-cell or cell-matrix interactions can cause increased protein permeability across the endothelial monolayer (4, 7, 30, 40). This may be the basis for the increased endothelial protein permeability across the endothelium observed in vivo with inflammation and sepsis. A similar process occurs in the formation of new blood vessels. Angiogenic factors such as VEGF cause cell-cell and cell-matrix dissociation followed by migration and proliferation of endothelial cells (5). On the other hand, many angiogenic factors have also been shown to cause increased permeability across the endothelial monolayer and inflammatory response (5, 9, 43). It is therefore likely that both processes share a part of the same cell-signaling pathway.

TNF-α has been shown to induce the release of metalloproteinases (35), vascular endothelial growth factor A (VEGF-A), and interleukin-8 (43), all of which are potent angiogenic factors. TNF-α has also been shown to modulate the expression of VEGF receptors (16, 26). The current study has demonstrated a possible involvement of integrin signaling in TNF-α-induced cell migration via a coordinated regulation between α5β1-integrins. On the other hand, it is well known that the angiogenic effect of TNF-α varies with cell lines and experimental conditions (12, 22, 26). Therefore it remains to be determined whether the TNF-α-induced coordinated regulation of α5β1-integrins observed in CPAE cells also occurs in other endothelial cell lines or under in vivo conditions.

The integrin complex α5β3 interacts with a wide range of matrix proteins. It is, however, not expressed at high levels compared with α5β1 on resting endothelial cells (37). A likely reason for its increased expression on TNF-α-treated cells is to allow cells to survive on a changing matrix. Resting endothelial cells produce a fibronectin-rich matrix both in vivo and in vitro, and their interactions with the matrix are mediated predominately by α5β1-integrins (8, 9). TNF-α has been shown to cause the release of proteinases that can modify the matrix of endothelial cells (35). This matrix modification may be one reason for the observed decreased localization of α5β1-integrins and the increased localization of α5β3-integrins in focal contacts.

The current study demonstrated changes in α5β3 surface expression and focal contacts in endothelial cells after TNF-α exposure. It also suggested a possible coordinated regulation on the expression and ligation of two different integrins. This is evident not only in protein expression but also in the localization of these integrins in focal contacts. Integrin α5β3 was detected only at cell-cell junctions in untreated cells, whereas focal contacts containing α5β3-integrins were readily identified in cells after TNF-α exposure. In contrast, α5β1-integrins were present in robust focal contacts in treated cells, whereas focal contacts containing α5β3-integrins were observed in membrane protrusions of cells at the migration front. These coordinated changes in α5β3- and α5β1-integrins induced by TNF-α may mediate the observed membrane protrusion formation and cell migration.

Considerable evidence suggests that signaling among integrins is modulated by “cross talk” mediators. Integrin α5β3-mediated vitronectin internalization appeared to require the ligation of α5β1-integrins (27). Ligation of α5β3-integrins was found to suppress α5β1-mediated activation of calcium/calmodulin-dependent protein kinase II (CamKII) (2), which appeared to be required for integrin-mediated phagocytosis and cell migration. CamKII at high levels, however, may in-
hibit the interaction of \(\alpha_\beta_1\)-integrin with fibronectin (3). Kim et al. (20) demonstrated that the ligation of \(\alpha_\beta_1\)-integrins could potentiate \(\alpha_\beta_2\)-mediated endothelial cell migration on vitronectin by suppressing the activity of protein kinase A. It is possible that the differential regulation on the expression of \(\alpha_\beta_1\)- and \(\alpha_\beta_2\)-integrins induced by TNF-\(\alpha\) is mediated by a cross-talk mediator. Future studies to identify such a mediator may provide a better understanding of the mechanism by which TNF-\(\alpha\) induces the increase in \(\alpha_\beta_2\)-integrin expression and endothelial cell migration, processes that may be essential for vascular wound healing and angiogenesis.

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