TNF-α disruption of lung endothelial integrity: reduced integrin mediated adhesion to fibronectin

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Am J Physiol Lung Cell Mol Physiol 282: L316–L329, 2002; 10.1152/ajplung.00145.2000.—Tumor necrosis factor-α (TNF-α) causes an increase in transendothelial protein permeability of confluent monolayers of calf pulmonary artery endothelial (CPAE) cells, and the addition of plasma fibronectin (pFn) to the culture medium can attenuate this increase in permeability. We determined if reduced integrin function had a role in decreased endothelial cell adhesion to immobilized Fn after exposure of the endothelial monolayers to TNF-α. TNF-α also causes a reorganization of the subendothelial Fn rich matrix and a significant loss in RGD-dependent adhesion of TNF-α treated CPAE cells to pFn coated surfaces. However, flow cytometry revealed no decrease in α5β1 or total β1 integrin expression on the surface of the CPAE cells after TNF-α. Reduced CPAE adhesion to immobilized Fn was, in part, due to a loss of β1-integrin function since the β1-integrin blocking antibody mAb 13 significantly (P < 0.05) prevented the adhesion of normal control CPAE cells but did not further reduce the adhesion of TNF-α-treated cells. In addition, antibodies which activate β1 integrins restored (P < 0.05) adhesion of TNF-α-treated cells to immobilized pFn but did not alter the adhesion of control cells. Despite reduced ability to adhere to immobilized Fn, TNF-α-treated CPAE monolayers demonstrated increased binding and incorporation of fluid-phase pFn into the subendothelial extracellular matrix (ECM) as measured by the analysis of the deoxycholate (DOC) detergent insoluble pool of 125I-Fn in the cell layer. In contrast to the RGD-mediated adhesion of CPAE cells to matrix Fn, the increased binding of soluble pFn after TNF-α was not inhibited by RGD peptides or mAb 13. Thus reduced integrin-dependent adhesion of the CPAE cells to matrix Fn as well as disruption of the Fn matrix may contribute to the increased protein permeability of previously confluent endothelial monolayer after TNF-α. In addition, increased ability for the monolayer to incorporate fluid-phase Fn into the ECM after TNF-α via a non-β1-integrin dependent mechanism may be a compensatory response to stabilize the Fn matrix and the endothelial barrier.

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ADULT RESPIRATORY DISTRESS syndrome as a consequence of increased lung endothelial protein permeability is often observed in critically ill surgical, trauma, or burn patients with gram-negative sepsis (43). Tumor necrosis factor-α (TNF-α), an inflammatory cytokine released from endotoxin-activated monocytes and macrophages sequestered in the lung microcirculation and interstitium during sepsis or lung inflammation, is believed to contribute to disruption of the lung vascular barrier leading to the increase in protein permeability (46, 47). Intravenous injection of recombinant human TNF-α has been shown to elicit cardiovascular and pulmonary disturbances similar to those observed during endotoxemia or gram-negative septic shock (38, 45), whereas the intravenous infusion of neutralizing antibodies to TNF-α reduces lethality in both endotoxemia and bacteremia in subhuman primates (46). Moreover, mice devoid of the 55-kDa TNF-α receptor are more resistant to the development of septic shock after endotoxin or bacterial challenge (34).

Endothelial monolayer barrier integrity is influenced by both cell-cell and cell-matrix interactions (13, 15, 24). The concept that TNF-α can alter vascular integrity is directly supported by various in vitro studies measuring protein permeability of cultured confluent endothelial monolayers after TNF-α exposure (10, 41, 48, 49). Defilipi et al. (12–14) showed that exposure of endothelial cells to a combination of TNF-α and interferon-γ (IFN-γ) reduced endothelial cell adhesion to specific matrix proteins. For example, exposure of human umbilical vein endothelial cells (HUVECs) to TNF-α decreased the expression of α5β3 integrins, resulting in reduced adhesion to substrate laminin, whereas combined treatment with both TNF-α and IFN-γ decreased α5β3-integrin surface expression, leading to reduced adhesion to vitronectin (12–14). Changes in integrin activity have also been implicated in a loss of endothelial cell adhesion, since parallel exposure of human endothelial cells with both TNF-α and IFN-γ reduced the ligand-binding activity of α5β3 integrins, resulting in a loss of adhesion to either denatured collagen, vitronectin, or fibrinogen (42).
Integrin ligation is important in transducing signals from the extracellular matrix (ECM) which in turn can influence cytoskeleton-mediated cell motility and gene expression (1, 15, 23). Endothelial cells express several integrins on their surface, including αβ1- and αβ1-integrins, which interact with a three-amino acid cell attachment sequence, Arg-Gly-Asp (RGD), found in fibronectin (Fn), fibrinogen, and vitronectin (13, 24, 35). Both αβ1- and αβ1-integrins recognize an RGD site accessible in the III-10 module of immobilized or matrix-incorporated Fn (35, 36). However, Dejana et al. (16) demonstrated that αβ3 is the only integrin found clustered in focal contacts when HUVEC cells are plated on Fn. In addition to influencing cell adhesion, several studies have also suggested that αβ1-integrins may facilitate cell-mediated assembly of soluble Fn in the ECM (1, 18, 51), since the binding of the integrins may facilitate cell-mediated assembly sites on adherent cells (22) may require the participation of αβ1-integrins (1, 18, 23, 27, 51).

We previously reported that the addition of TNF-α to confluent calf pulmonary artery endothelial (CPAE) monolayers causes disruption of the underlying fine fibrillar Fn matrix and an increase in the endothelial protein permeability (10, 11, 48, 49). This increase in endothelial protein permeability can be attenuated by the addition of purified human plasma Fn (pFn), presumably through its incorporation in the ECM (10, 48, 49). The current study was designed to determine if exposure of lung pulmonary artery endothelial monolayers to TNF-α would alter their adhesion to immobilized Fn and to determine if such reduced CPAE adhesion to Fn was related to either reduced surface expression of the αβ3-integrins or a change in their activity or functional state.

METHODS

Endothelial cell culture. CPAE cells (CCL-209; American Type Culture Collection, Rockville, MD) were grown in MEM (GIBCO, Grand Island, NY) supplemented with 20% FBS (Hyclone, Logan, UT), nonessential amino acids (10 mM; GIBCO), penicillin (100 U/ml; GIBCO), and streptomycin (100 µg/ml; GIBCO). Experiments were performed between passages 17 and 23. MEM containing 5% FBS was used during the experimental treatments with and without 200 U/ml TNF-α (9 ng/ml) of recombinant human TNF-α (Gibco, Grand Island, NY) supplemented with 20% FBS (Gibco) and penicillin (100 U/ml) and streptomycin (100 µg/ml) in MEM containing 5% FBS was used during the experimental treatments with and without 200 U/ml TNF-α (9 ng/ml) of recombinant human TNF-α (Gibco, Grand Island, NY). CPAE cells (280,000 cells/well) were seeded on glass coverslips in 12-well tissue culture dishes and grown to confluence. After treatment with TNF-α, the cells were fixed in 3% formaldehyde in PBS (GIBCO) for 15 min, permeabilized with 0.5% Triton X-100 at 4°C for 5 min, blocked with 2% BSA + 50 mM glycine + 0.2% Tween 20 in PBS for 1 h, incubated with primary antibody (Ab) for 1 h at room temperature, and washed with PBS before being incubated with secondary Ab for 1 h. Primary Abs included rabbit polyclonal Ab against bovine Fn (1:100 dilution; Calbiochem, La Jolla, CA) and mouse monoclonal antibody (mAb) against the human αβ1-integrin complex (mAb HA5; 1:500 dilution; Chemicon, Temecula, CA). Secondary Abs included rhodamine-isothiocyanate conjugated goat anti-rabbit IgG (Cappel Organon Teknika, Durham, NC) and FITC goat anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN). Coverslips (Fisher Scientific, Pittsburgh, PA) were mounted on slides using AntiFade (Molecular Probes, Eugene, OR) and were viewed using a Nikon Microphot SA fluorescent microscope.

Immunoprecipitation of total cell surface αβ1-integrins. Confluent CPAE cell layers were trypsinized, washed with trypsin inhibitor (1 mg/ml) in PBS, and prelabeled with 0.5 mg/ml Sulfo-NHS-biotin (Pierce, Rockford, IL) for 30 min at 4°C, and 0.05 mM Tris-HCl (pH 7.4), 0.15 mM NaCl, 0.1 mM MgCl2, and 0.1 mM CaCl2 (TBS) was added to stop the reaction. The mAb 9E87 or mAb HA5 against the αβ1 complex (4 µg/ml) was added to 106 suspended cells in 1 ml of TBS. Pellets were treated with extraction buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA (pH 8.0), 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40), and protein-G agarose was added to the supernatants for precipitation. Pelleted samples were washed with extraction buffer, resuspended with gel sample buffer, and boiled for 5 min. Total cell lysates, immunoprecipitated proteins, and IgG controls were separated using 7.5% SDS-PAGE and transferred to nitrocellulose (Schleider and Schuell, Keene, NH). Blots stained with streptavidin-horseradish peroxidase (HRP; Amersham Pharmaic Biochem, Piscataway, NJ) or αβ1- or β1-polycyonal Abs (Chemicon, Temecula, CA) and goat anti-rabbit IgG-HRP were then developed using chemiluminescence (Amersham Pharmacia Biotech).
Biocytometry. Confuent CPAE monolayers treated with or without TNF-α for 18 h were trypsinized, washed, and resuspended in PBS (1 × 10^6 cells/ml). Cells were incubated at 4°C with either mAbs clones 9EG7, HA5, or AIIIB2 (a gift from Caroline Damsky at the University of California-San Francisco) for 30 min, washed with PBS, and labeled for 30 min at 4°C with either FITC-conjugated goat anti-rat IgG (Boehringer Mannheim Biochemical, Indianapolis, IN) or FITC-conjugated goat anti-mouse IgG, respectively. Samples were fixed with 3% paraformaldehyde, and 1× cells were analyzed using a Becton-Dickinson FACScan.

**Binding of soluble pFn to CPAE cells.** Confuent CPAE monolayers grown in 12-well culture plates were treated for 18 h with or without TNF-α. pFn used for binding and incorporation experiments was iodinated using the chloramine-T method, as previously described (37). The cell layers were washed, and 1 ml of MEM containing 1% BSA and 1 μg/ml of human ^125_I-pFn (2.4 × 10^6 counts·min⁻¹ (cpm)·1 μg⁻¹) was added to each well at 4°C. The cell layers were incubated with ^125_I-pFn, washed with cold PBS, dissolved with 1 N NaOH, and counted. Unlabeled pFn (300 μg/ml) was added to control and TNF-α-treated cells with ^125_I-pFn to quantify nonspecific binding, which was subtracted from experimental values. Samples were quantitated using a Wallac 1470 Wizard gamma counter.

**Assay of ^125_I-pFn incorporation in ECM of CPAE monolayers.** CPAE monolayers grown to confluency in 12-well culture plates were exposed for 18 h to TNF-α or diluent medium alone, washed with PBS, and supplemented with 1 ml of MEM containing 5% FBS (Fn depleted) and 1 μg/ml of human ^125_I-pFn (2.4 × 10^6 cpm/μg). The cell layers were incubated at 37°C for 1, 3, 6, or 24 h. To evaluate the incorporation of ^125_I-Fn in the ECM, cells were detergent extracted with deoxycholate (DOC), and the DOC insoluble pool of ^125_I-pFn (covalently incorporated Fn) was quantitated as previously described (37). Fn-depleted FBS was obtained by passing the serum over a gelatin-Sepharose column.

**Microscopic analysis of cell adhesion to substrate Fn.** Fn (0.5–2 μg/ml) diluted in PBS was adsorbed on 24-well cell culture plates and blocked with 1% BSA at 4°C. Confuent CPAE cell layers treated with TNF-α for 18 h were trypsinized, and 5 × 10^4 CPAE cells suspended in 0.5 ml of MEM were added to each well for 30 min at 37°C. Thereafter, wells were washed three times with PBS, and the number of adherent cells per 1 mm² was counted using an inverted microscope. Depending on the protocol, cell layers were preincubated with either a RGD-containing peptide (GRGDSP; GIBCO), a control RGE-containing peptide (GGRGSP; GIBCO), mAb 13 (Becton-Dickinson, Franklin Lakes, NJ), mAb 12G10 (Chemicon), or mAb 9EG7 for 30 min at 4°C before analysis in the adhesion assay.

**Electrical cell impedance sensor analysis of cell adhesion to substrate Fn.** Cell adhesion was also studied with an electrical cell impedance sensor (ECIS) from Applied Biophysics (Troy, NY). This system can analyze the dynamic behavior of cells in culture by continuously recording changes in electrical resistance resulting from altered cell attachment and/or spreading on gold-plated sensors fixed within the ECIS culture wells (19). Confluent CPAE monolayers were pretreated with TNF-α for 18 h, lifted by trypsinization, suspended (5 × 10^4 cells) in 400 μl of MEM supplemented with 1% BSA, and added to eight-well ECIS plates precoated with human pFn (25 μg/ml) or 1% BSA. The electrical resistance in ohms was recorded over a 60-min interval. To verify RGD-dependent adhesion to the Fn-coated surface, 250 μg/ml of the RGD peptide or the control RGE peptide was added.

**Statistical methods.** Data are presented as means ± SE. Data were analyzed by a Student’s t-test or a two-way ANOVA, with significance from controls determined by a Tukey test. A confidence level of 95% (P < 0.05) was used to establish statistical significance.

**RESULTS**

**Protein permeability increase, Fn rearrangement, and intercellular gap formation after TNF-α exposure to CPAE monolayers.** Figure 1 presents the endothelial protein permeability of the CPAE monolayers after TNF-α treatment. Protein clearance across control lung endothelial monolayers was 0.031 μl/min. This was not significantly elevated during the initial 6 h but did increase significantly (P < 0.05) by 18 h post-TNF-α. We used immunofluorescence microscopy to study the endogenous bovine Fn-rich subendothelial matrix (Fig. 2, left) and differential interference contrast (DIC) microscopy to visualize intercellular gap formation (Fig. 2, right). Rearrangement or disruption of the Fn matrix was readily apparent at 18 h, with a loss of the fibrillar Fn network from the ECM. Parallel DIC analysis demonstrated a tight and uniform monolayer with slight changes in morphology by 6 h. Gap formation was evident at 18 h after exposure to TNF-α, consistent with the significant increase in protein permeability. These results suggest a temporal relationship between the structural rearrangement of the Fn matrix, increased protein permeability, and gap formation of the endothelial monolayer, all which seemed to occur in parallel after TNF-α treatment. Based on these data, we used an 18-h TNF-α treatment period for the remainder of this study.

Fig. 1. Temporal effect of tumor necrosis factor-α (TNF-α) on protein permeability of calf pulmonary artery endothelial (CPAE) monolayers. Confluent monolayers were exposed to 200 U/ml of purified human recombinant TNF-α for 2, 4, 6, or 18 h. Control wells were incubated with medium alone for 18 h. Integrity of the endothelial cell barrier was determined using transendothelial protein clearance (μl/min). Values for protein clearance represent means ± SE with 4–10 wells/group. *Significantly greater than control (P < 0.05).
We next examined the effect of TNF-α/H9251 presence of the Fn/H9251 both the seen to align (Fig. 3, layers were treated with 200 U/ml TNF-α/H9251 Fig. 2. Temporal effect of TNF-α/H9251

...demonstrated by immuno...endothelial cells (black arrows), as demonstrated by immunofluorescent examination of endogenous bovine Fn (left) and differential interference contrast (DIC) microscopic analysis (right), respectively.

**Loss of αβ1-integrin association with fine Fn fibers of the ECM after TNF-α exposure to CPAE monolayers.** We next examined the effect of TNF-α exposure on the presence of the Fn αβ1-integrins in relation to the fine Fn fibers from the ECM by costaining for both the αβ1-integrins and endogenous Fn fibers and visualizing them by fluorescence microscopy. In confluent control monolayers (Fig. 3, top left), αβ1-integrins are seen to align (Fig. 3, top right) with fine Fn fibers at the periphery of the cells. With higher magnification, cells could be seen within the center of these dense bundles (data not shown). In contrast, after treatment with TNF-α the presence of both fine Fn fibers (Fig. 3, bottom left) and the αβ1-integrins (Fig. 3, bottom right) within the bundles was lost although cells were still present. Another integrin found on the surface of endothelial cells that is capable of interacting with the RGD site of Fn is the αβ3-integrin (13). Costaining for both the αβ3-integrins with mAb LM609 and the bovine Fn fibers did not show colocalization within circular Fn bundles, but rather diffuse staining of the αβ3-integrins around the periphery of cells (data not shown). The reduced colocalization of the fine Fn fibers with αβ1-integrins after TNF-α suggested that the increase in protein permeability may reflect decreased αβ1-mediated cell adhesion to Fn in the matrix.

**Effect of TNF-α on adhesion of endothelial cells to immobilized Fn.** We confirmed a decrease in adhesion of TNF-α-treated CPAE cells to pFn-coated wells by direct cell counting via microscopic analysis. No significant differences occurred between control and TNF-α-treated cells plated on wells coated with 0.5 μg/ml pFn or BSA alone (Fig. 4A). However, a significant (P < 0.05) decrease of 35 and 50% in adhesion of TNF-α-treated CPAE cells to wells coated with 1.0 and 2.0 μg/ml pFn, respectively, was observed compared with control cells. The assay was repeated with the addition of RGD- and RGE-containing peptides to evaluate the role of integrins in the observed adhesion to the immobilized Fn surface. Control and TNF-α-treated CPAE cells were plated on wells coated with pFn (2 μg/ml) after preincubation with RGD or RGE peptide (500 μg/ml). RGD peptide caused a significant (P < 0.05) loss of adhesion to Fn in both the control and TNF-α-treated groups (Fig. 4B).

**Temporal effect of TNF-α on adhesion of endothelial cells to immobilized Fn as measured by ECIS.** ECIS (19) was used to examine the temporal aspects of cell adhesion to wells coated with 25 μg/ml pFn over 60 min after addition of the cells to the culture medium. Resistance began to increase in both control and TNF-α-treated groups by 20–25 min after plating (Fig. 5), with significance (P < 0.05) observed by 30 min. Thereafter, the TNF-α-treated group remained significantly lower for the remainder of the 1-h incubation. The presence of cells on the unwashed electrodes from all groups was confirmed by visualization using an inverted microscope after the ECIS recordings were completed. As measured by ECIS (Fig. 5), the addition of RGD peptides inhibited both control and TNF-α-treated CPAE cell attachment and spreading on the Fn surface, whereas RGE peptides had no effect (data not shown). Using an inverted microscope, we observed that control cells were spread on the electrode, whereas those exposed to the RGD peptide were found to be rounded in appearance (data not shown). Thus exposure of CPAE cells to TNF-α caused a reduction in their RGD-dependent adhesion to Fn.

**Influence of integrin-blocking Abs on the adhesion of TNF-α-treated CPAE cells to immobilized pFn.** We examined the role of integrins in adhesion of CPAE cells to immobilized pFn using integrin-blocking Abs. Control and TNF-α-treated cells were preincubated with 10 μg/ml mAb 13 (β1-integrin blocking), 10 μg/ml mAb LM609 (αβ3-integrin blocking), or no mAb at all and were applied to wells coated with 2.0 μg/ml pFn. Significant (P < 0.05) losses in adhesion to Fn were observed with control cells preincubated with mAb 13 and mAb LM609 (Fig. 6). Each Ab alone reduced cell
Fig. 3. TNF-α treatment disrupts localization of αβ1-integrins to the fine Fn fibers of the subendothelial matrix of CPAE monolayers. Confluent endothelial cells were treated with either medium alone (Control) or medium containing 200 U/ml of TNF-α for 18 h at 37°C. Cell layers were fixed, permeabilized, and stained for dual-labeling fluorescence microscopy to detect the localization of endogenous Fn (top) and αβ1-integrins (middle) using a polyclonal antibody (Ab) to bovine Fn and HA5, a monoclonal antibody (mAb) to αβ1-integrins, respectively. Overlay of Fn and αβ1-integrin is shown at bottom; arrows indicate examples of colocalization of αβ1-integrins and fine Fn fibers, and dotted ovals demonstrate areas with a loss of such localization as seen throughout TNF-α-treated monolayers.

Fig. 4. Effect of TNF-α exposure on CPAE cell adhesion to Fn-coated surfaces. Confluent monolayers were pretreated with medium alone (Control) or medium containing 200 U/ml of TNF-α for 18 h, trypsinsized, added to culture wells coated with 0, 0.5, 1.0, or 2.0 μg/ml plasma fibronectin (pFn), incubated for 30 min at 37°C, and washed with PBS. Adherent cells were counted using an inverted microscope. A: pretreatment of CPAE monolayers with purified TNF-α decreased the ability of CPAE cells to adhere to immobilized pFn substrate. Data are represented as the average of adherent cells in 3 x 1 mm² areas ± SE (3 experiments performed in triplicate). *Significantly less than controls within same Fn concentration group (P < 0.05). B: suspended cells were pretreated with 500 μg/ml RGD or control RGE peptide for 30 min at 4°C before addition to culture wells coated with 2.0 μg/ml of pFn. The decrease in the number of adherent cells with RGD peptide demonstrates that binding of immobilized Fn by CPAE cells is RGD dependent. Data are presented as the average of adherent cells in 3 x 1 mm² areas ± SE (2 experiments performed in triplicate). **Significantly greater than RGE control; ***significantly less than TNF-treated RGE control (P < 0.05).
consistent with the molecular masses of the blots with streptavidin-HRP revealed two bands consistent with proteins immunoprecipitated with mAb HA5. Staining mediated by Western blot analysis of biotinylated surface proteins indicated by arrow. RGD peptide (250 ng/ml) was added to control (control + RGD) and TNF-treated (TNF + RGD) wells, demonstrating an RGD dependence of adhesion of CPAE cells to immobilized pFn as the increase in electrical resistance is inhibited. Control RGE peptide (250 µg/ml) did not block adhesion to Fn-coated wells (data not shown). Data are presented as means ± SE of 21–22 wells for control or TNF-α and 3 wells for samples with RGD or RGE peptides.

adhesion by ~40%, down to the level of TNF-α treated cells. In contrast, adhesion of TNF-α-treated cells to immobilized pFn was not inhibited (P > 0.05) by mAb 13 or mAb LM609 (Fig. 6). Interestingly, preincubation with both antibodies together did not further reduce adhesion in either group (data not shown). These data further suggested that a loss of integrin-mediated adhesion to surface Fn occurs in CPAE cells treated with TNF-α.

Surface expression of α5β1-integrin by flow cytometry. We then speculated that the loss of integrin-mediated adhesion to Fn by CPAE cells treated with TNF-α may be the result of a loss of α5β1-surface expression. To measure surface expression on CPAE cells, we performed flow cytometry using mAb HA5 to detect the α5β1-complex. First, the specificity of mAb HA5 and cross-reactivity for the bovine α5β1-complex was confirmed by Western blot analysis of biotinylated surface proteins immunoprecipitated with mAb HA5. Staining blots with streptavidin-HRP revealed two bands consistent with the molecular masses of the α5- and β1-integrin subunits (Fig. 7A), and reprobing with a polyclonal Ab against α5-integrin confirmed the upper band was α5.

Flow cytometry was performed using mAb HA5 on cells harvested from control and TNF-α-treated monolayers. A graph of a representative analysis is shown in Fig. 7B. The averages of mean fluorescence intensity from combined experiments showed no significant (P > 0.05) difference between the control and TNF-α-treated groups (Fig. 7C). In addition, no loss of α5β1-integrin expression was seen when flow cytometry was performed on cells lifted with trypsin compared with cells lifted with 5 mM EDTA (B. Gao, K. Powell, T. M. Saba, unpublished results), which confirmed that the trypsinization procedure used to lift CPAE cells did not change α5β1-integrin surface expression. In addition, recent flow cytometric analysis using mAb LM609 actually showed an approximate twofold increase in the expression of α5β1-integrin on the surface of TNF-α-treated CPAE cells compared with controls (B. Gao, K. Powell, T. M. Saba, unpublished results). Thus the reduced adhesion of TNF-α-treated CPAE cells to immobilized Fn was not the result of reduced α5β1- or α5β3-surface expression.

Surface expression of 9EG7-detectable epitope by flow cytometry. The β1-integrin subunit can play a major role in the activation of the integrin complex and exists in distinct conformations or activity states that influence their interactions with ligand (2, 32). Moreover, expression of the mAb 9EG7-dependent epitope on the β1-integrin reflects a high affinity or ligand-bound state that can be influenced by cations, chelating agents, antibodies, inside-out signaling, or ligand occupation (2, 26, 32). Mastrandrello et al. (26) demonstrated a decrease in mAb 9EG7-dependent expression associated with a loss of Fn-dependent adhesion in MG-63 cells transfected with chimeric receptors containing the β1-cytoplasmic domain. Since we hypothesized that reduced adhesion of TNF-α-treated cells to immobilized pFn might correlate with reduced surface expression of the mAb 9EG7-dependent epitope, we next investigated the possibility that TNF-α treatment
had altered the activity state of α5β1-integrins. First, cross-reactivity to bovine β1-subunits was confirmed by probing Western blots of immunoprecipitated α5β1-integrin with mAb 9EG7 (data not shown). Flow cytometry performed using mAb 9EG7 and mAb AIIB2, an Ab against β1-integrins, revealed a slight but non-significant (P > 0.05) increase in both fluorescence intensity for the mAb 9EG7-dependent epitope and the β1-integrin on TNF-α-treated CPAE cells (Fig. 8). Thus the decreased adhesion of TNF-α-treated cells to Fn was not caused by a significant decrease in β1-integrin surface expression.

Effect of Mn2+ on the mAb 9EG7-detectable epitope in bovine endothelial cells. Bazzoni et al. (3) demonstrated that phorbol myristate acetate (PMA) increased the adhesion of K562 cells to Fn without an increase in mAb 9EG7-dependent epitope expression. The same study showed that mAb 9EG7 stimulated the adhesion to Fn via α5β1-integrins in the presence of Mn2+, a divalent cation that increases mAb 9EG7-dependent epitope expression (2, 3, 25). To verify the existence of a functional mAb 9EG7-dependent epitope in bovine endothelial cells, biotinylated CPAE cells were incubated with mAb 9EG7 in the presence or absence of 5 mM Mn2+. The results of immunoprecipitation with this Ab showed two major bands after staining with streptavidin (Fig. 9A, lanes 2 and 3) that aligned with bands of α5- and β1-subunits immunoprecipitated with mAb HA5 (lane 1). These two bands were the only major bands present above 67.5 kDa on the blot, suggesting that α5β1-integrin was a major complex immunoprecipitated by mAb 9EG7. The increased band intensity with Mn2+ treatment (lane 3) confirmed that the mAb 9EG7-dependent epitope exists on α5β1-integrins expressed on the surface of CPAE cells. Reprobing with a polyclonal Ab against the α5-subunit confirmed the upper band (Fig. 9B). Accordingly, Mn2+ induced the surface expression of the mAb 9EG7-dependent β1-epitope in bovine endothelial cells, thus verifying the existence of a functional mAb 9EG7 epitope in our bovine cells.

Effect of β1-activating antibodies on cell adhesion. Addition of mAb 9EG7 can activate the α5β1-integrin without Mn2+, presumably by shifting the β1-subunit from an inactive to an active state (33). If true, then the TNF-α treatment of CPAE cells may have caused inactivation of α5β1-integrins, resulting in reduced adhesion to Fn, which should be recoverable by β1-activating Abs. To test this concept, suspended control and TNF-α-treated CPAE cells were preincubated with 10 μg/ml of the β1-activating antibodies mAb 12G10 and mAb 9EG7 before being plated on Fn-coated wells. Neither activating Ab significantly (P < 0.05) increased adhesion of control cells to immobilized Fn,

Fig. 7. Effect of TNF-α exposure on the surface expression of α5β1-integrins on CPAE cells. A: trypsinized CPAE cells were surface biotinylated and incubated with mAb HA5 before extraction. Samples were separated by 7.5% SDS-PAGE, blotted, and stained with streptavidin to detect the labeled surface proteins precipitated by mAb HA5. The blot was reprobed with polyclonal anti-α5 Ab to confirm the indicated band (data not shown). mAb HA5 precipitates bovine α5- and β1-integrin subunits. IP, immunoprecipitation. B: after 3 days of culture, CPAE monolayers treated with medium alone (Control) or medium containing 200 U/ml TNF-α for 18 h were trypsinized, washed, and counted. Samples containing 1 × 10⁶ cells were analyzed by flow cytometry after labeling with 4 μg of the mouse mAb clone HA5 and an FITC-conjugated secondary Ab (2°Ab). Data are representative of 7 experiments. C: mean fluorescence intensity. Exposure to TNF-α for 18 h did not significantly decrease (P > 0.05) the surface expression of α5β1-integrins in confluent CPAE cells.
yet both Abs significantly (P < 0.05) increased adhesion of TNF-α-treated cells by 30–40%, thus reversing the adhesion deficit (Fig. 10). The restoration of TNF-treated CPAE cells by the β1-activating antibodies was, in turn, prevented by the β1-blocking mAb AIIB2 (data not shown). These β1-activating antibodies rescue adhesion of TNF-α-treated but not control cells, supporting the concept that TNF-α treatment causes a significant population of β1-integrins to become inactive.

Soluble 125I-pFn binding and ECM incorporation to CPAE monolayers after TNF-α treatment. Because the α5β1-integrin has been shown to mediate soluble Fn binding to human fibroblasts (18), we then explored the possibility that the binding of soluble pFn to the cell layers may also be reduced in TNF-α-treated monolayers in which α5β1-integrin activity was reduced. 125I-pFn binding at 4°C was measured after 5, 10, 20, 30, or 60 min of incubation with TNF-α. Contrary to our expectations, the binding of soluble Fn was significantly (P < 0.05) increased five- to sixfold after TNF-α treatment at 60 min (Fig. 11A).

Kowalczyk et al. (21, 22) demonstrated that the restrictive barrier formed by endothelial cell-cell interactions can limit the accessibility of soluble pFn to matrix assembly sites. To determine if the unexpected increase in soluble 125I-pFn binding to the CPAE cell layer after TNF-α exposure could be explained by increased accessibility to the basolateral region resulting from loss of barrier function after TNF-α, binding ex-
Experiments were repeated using preconfluent CPAE monolayers studied at 24 and 48 h postseeding to remove the restrictive nature of the confluent endothelial barrier. Binding of fluid-phase $^{125}$I-pFn to both control and TNF-$\alpha$-treated monolayers decreased as the monolayer reached confluence. However, at all time points (24, 48, and 72 h), the TNF-$\alpha$-treated endothelial cells bound significantly ($P < 0.05$) more $^{125}$I-pFn than untreated controls, even at 24 h postseeding (~2.5-fold increase). These observations suggest that, while accessibility can affect binding of $^{125}$I-pFn to endothelial cell layers, it cannot fully explain the increased binding of soluble pFn observed after TNF-$\alpha$ treatment.

Because TNF-$\alpha$-treated cells bound more soluble fluid-phase pFn, we predicted increased incorporation of soluble pFn in the matrix by TNF-$\alpha$-treated monolayers. To test this prediction, we measured the DOC-insoluble pool of $^{125}$I-pFn in the monolayers as an index of Fn matrix assembly, as previously described (10, 11, 28, 29, 37). Significantly ($P < 0.05$) more $^{125}$I-pFn was detected in the DOC-insoluble fraction of TNF-treated CPAE cell layers than for controls by 3 h, with an approximate threefold increase in incorporation at 24 h (Fig. 12). Thus both an increase in binding and incorporation of soluble pFn exists after exposure of endothelial cells to TNF-$\alpha$.

**Effect of RGD peptides and mAb 13 on fluid-phase pFn binding to TNF-$\alpha$-treated CPAE monolayers.** It has been suggested that $\alpha_5\beta_1$-integrins can facilitate pFn incorporation in the ECM (1, 18, 51). However, despite the reduction of $\beta_1$-integrin function, we observed an increase in soluble pFn binding and ECM incorporation after TNF-$\alpha$ treatment. Because it is possible that $\beta_1$-integrins, although losing adhesive activity for immobilized Fn, may still play a role in soluble ligand binding, we repeated the binding studies using control and TNF-$\alpha$-treated CPAE monolayers exposed to RGD peptides and mAb 13 to investigate a role for $\beta_1$-integrins in soluble pFn binding. TNF-$\alpha$-treated monolayers again showed increased binding of $^{125}$I-pFn compared with controls, but only the control cells were inhibited ($P < 0.05$) when incubated with the RGD peptide compared with RGE peptide (Fig. 13A).

Using mAb 13 to specifically block $\beta_1$-integrins, Fogerty et al. (18) observed an inhibition of soluble Fn binding to human fibroblast monolayers. Surprisingly, soluble pFn binding to control and TNF-$\alpha$-treated

![Figure 9](image-url)

**Fig. 9.** Analysis of the $\beta_1$-integrin epitope detectable by mAb 9EG7 on CPAE cells. A: suspended cells were biotinylated, labeled with mAb 9EG7 or mAb HA5 in Tris-buffered saline (TBS) ± 5 mM Mn$^{2+}$, extracted, and immunoprecipitated. Samples were separated by 7.5% SDS-PAGE, Western blotted, and stained with streptavidin-horseradish peroxidase. Major bands from the 9EG7 immunoprecipitate (lanes 2 and 3) aligned with $\alpha_5$- and $\beta_1$-integrin bands from HA5-immunoprecipitated samples (lane 1). B: blot in A was stripped and reprobed with a polyclonal Ab to $\alpha_5$, confirming that the addition of 5 mM Mn$^{2+}$ increases the coprecipitation of $\alpha_5$ by mAb 9EG7 (lanes 2 and 3).

![Figure 10](image-url)

**Fig. 10.** Effect of activating Abs against $\beta$-integrin on the adhesion of TNF-$\alpha$-treated CPAE cells to surface-immobilized pFn. Confluent CPAE monolayers were treated with either medium alone (Control) or medium containing 200 U/ml of TNF-$\alpha$ for 18 h and trypsinized. Suspended cells were pretreated for 30 min at 4°C with 10 μg/ml of the $\beta_1$-integrin-activating Abs mAb 12G10, mAb 9EG7, or no Ab before cells were added to culture wells coated with 2 μg/ml of pFn. Activation of the $\beta_1$-integrin did not significantly increase ($P > 0.05$) adhesion of control cells but did significantly increase ($P < 0.05$) that of TNF-treated cells (*) compared with cells treated with TNF-$\alpha$ but preincubated with no Ab. The restoration of TNF-treated cells activated by mAb 9EG7 was blocked with 10 μg/ml of the $\beta_1$-blocking mAb AlIB2 but not with a nonblocking control mAb HA5. Data are expressed as the average of adherent cells in $3 \times 1$-mm$^2$ areas ± SE of 3 experiments performed in quadruplicate.
monolayers was not blocked at 4°C by the addition of 10 μg/ml mAb 13 (Fig. 13B, left). Because we were unsure whether inhibition at 4°C influenced the possible allosteric-based inhibition of mAb 13 (13), we repeated the binding study at 37°C (Fig. 13B, right). In this case, the control group showed a decrease (P < 0.05) in pFn binding after incubation with mAb 13 while the TNF-α-treated group did not display such a decrease with mAb 13. Blocking the α,β3-integrin with mAb LM609 did not inhibit pFn binding to control or TNF-α-treated groups at either 4°C or 37°C (data not shown). These data further support the conclusion that TNF-α treatment of CPAE monolayers leaves β1-integrins in an inactive state, resulting in reduced cell adhesion to pFn-coated surfaces. In response, there appears to be an increase in integrin-independent binding and ECM incorporation of soluble Fn, perhaps as a compensatory mechanism.

DISCUSSION

We have previously shown that the addition of either recombinant TNF-α, soluble RGD-containing peptides, or polyclonal antibodies against α5β1-integrins to the culture medium of previously confluent CPAE monolayers increased their protein permeability in association with disruption of the fine fibrillar Fn-rich matrix (10, 48, 49). The addition of soluble human pFn can prevent and reverse the increase in monolayer protein permeability caused by TNF-α, and specificity was apparent, since other RGD-containing proteins such as fibronectin and vitronectin will not work (10, 48, 49). In the current study, we observed reduced colocalization of α5β1-integrins with fine Fn fibers in the ECM after TNF-α exposure, which correlated with reduced adhesion of the CPAE cells to immobilized Fn. Curtis et al. (11) demonstrated that the protein permeability increase and Fn matrix rearrangement is not the result of proteolysis of Fn within the subendothelial ECM after TNF-α treatment, since no Fn degradation could be detected in the medium or cell layer/matrix. In addition, protease inhibitors did not prevent the increase in protein permeability after TNF-α (11). Thus we hypothesized that this reduced ability of CPAE cells to adhere to matrix Fn may be because of changes in

![Graph A](http://ajplung.physiology.org/)

**Fig. 11.** TNF-α treatment increases binding of soluble 125I-pFn to CPAE monolayers. CPAE monolayers were treated with medium alone (Control) or medium containing 200 U/ml TNF-α for 18 h. A: confluent cells were washed, and 1 ml of MEM containing 1% BSA and 1 μg/ml of human 125I-pFn (2.4 × 10^6 counts·min⁻¹·cpm·μg⁻¹) was added to each well and incubated for 5, 10, 20, 30, or 60 min at 4°C. Monolayers were washed with cold PBS, dissolved with 1 N NaOH, and quantitated. Each point represents 2 experiments, performed in triplicate wells. Data are expressed as nonspecific binding. TNF-α exposure resulted in increased binding of soluble pFn to both preconfluent and confluent CPAE monolayers. Each point represents 3 experiments performed in triplicate (mean ± SE). *Significantly greater (P < 0.05) than controls within same incubation interval groups (A) or postseeding interval groups (B).

![Graph B](http://ajplung.physiology.org/)

**Fig. 12.** Increased incorporation of 125I-pFn in the ECM of CPAE monolayers exposed to TNF-α. Confluent CPAE monolayers were treated with medium alone (Control) or medium containing 200 U/ml TNF-α for 18 h at 37°C and washed, and 1 ml of MEM containing 5% FBS (Fn-depleted) and 1 μg/ml of human 125I-pFn (2.4 × 10^6 cpm/μg) was added to each well. Samples were incubated at 37°C for 1, 3, 6, or 24 h. To evaluate the incorporation of the 125I-pFn in the ECM, cells were detergent-extracted with deoxycholate (DOC), and the DOC-insoluble 125I-pFn was quantitated. Each point represents 2 experiments, performed in triplicate wells. Data are expressed as means ± SE in units of ng/10^6 cells. *Significantly greater (P < 0.05) than controls within same incubation interval group.
either the surface expression of $\alpha_5\beta_1$-integrins or the activation state of the $\beta_1$-subunit. Cytokines such as TNF-\(\alpha\), interleukin-1$\beta$, and IFN-\(\gamma\) have been shown to modulate the surface expression of integrins on a variety of cells (12, 14, 17, 31). Although exposure of HUVECs (40) to 100 ng/ml TNF-\(\alpha\) can decrease the expression of the $\alpha_5\beta_1$-integrin, we were unable to detect any significant reduction in the expression of $\alpha_5\beta_1$-integrins after exposure of CAPE monolayers to ~9 ng/ml TNF-\(\alpha\), as used in our experimental protocol.

Although mAb 13, a blocking Ab against the $\beta_1$-subunit, significantly inhibited adhesion of control cells to pFn, only partial adhesion blocking occurred. Other integrins on the cell surface may also be involved in the adhesion of CAPE cells to immobilized Fn such as $\alpha_\beta_3$, $\alpha_\beta_5$, and $\alpha_1\beta_3$. We observed that blocking the $\alpha_\beta_3$-integrin with mAb LM609 also inhibited adhesion to immobilized Fn. Defilippi et al. (13, 14) showed that cotreatment of HUVECs with TNF-\(\alpha\) and IFN-\(\gamma\) decreased the surface expression of $\alpha_\beta_3$-integrin, whereas B. Gao, K. Powell, and T. M. Saba (unpublished results) demonstrated that surface expression of $\alpha_\beta_3$-integrins on CAPE cells doubled after exposure to TNF-\(\alpha\) for 18 h, as used in the current study. There was also no additive blocking effect against adhesion when cells were coincubated with mAb 13 and mAb LM609 together, raising the possibility that the $\alpha_\beta_1$- and $\alpha_\beta_3$-integrins work in concert in mediating the adhesion of CAPE cells to immobilized Fn. In support of this concept is the finding that M21 melanoma cells require surface expression of both $\alpha_\beta_1$- and $\alpha_\beta_3$-integrins to attach and spread on Fn-coated surfaces (7). Although $\alpha_\beta_1$- and $\alpha_\beta_3$-integrins may both play a role in maintaining pulmonary endothelial monolayer barrier function, the increased protein permeability caused by TNF-\(\alpha\) appears not to be the result of decreased surface expression of these receptors. Furthermore, unlike $\alpha_\beta_1$-integrins, $\alpha_\beta_3$-integrins were not colocalized with Fn in the matrix, suggesting a specific role for $\alpha_\beta_1$-integrins in the adhesion of CAPE monolayers to Fn-rich matrix. Other RGD-dependent integrins, such as $\alpha_\beta_5$ and $\alpha_\beta_\beta_3$, may also play a role in CAPE cell adhesion to immobilized Fn, and their role may be addressed in future studies.

The ability of $\beta_1$-integrin-activating Abs to restore the adhesion of TNF-\(\alpha\)-treated CAPE cells to Fn surfaces, without affecting the adhesion activity of control cells, suggests that a loss of $\alpha_\beta_1$-integrin activity may be responsible for the observed increased protein permeability. The role for $\alpha_\beta_1$ is further supported by the ability of mAb 9EG7 to immunoprecipitate $\alpha_\beta_1$-integrin subunits. One must also consider the possibility that TNF-\(\alpha\) treatment may have caused a shift of a population of $\alpha_\beta_1$-integrins from an active to an inactive state, incapable of binding immobilized pFn. Mould et al. (33) have shown that an inactive pool of $\alpha_\beta_1$-integrins incapable of binding ligand can indeed exist and that mAb 9EG7 can rescue this inactive pool with respect to ligand binding. An association between decreased surface expression of the mAb 9EG7-dependent epitope and decreased cell adhesion to Fn has also been previously suggested (26). We did not observe decreased expression of this epitope after TNF-\(\alpha\) in the current study. Despite a lack of changes in surface expression in our study, this epitope appeared to be functionally responsive to Mn$^{2+}$, a well-known characteristic of this epitope (2, 3). However, a direct relationship between mAb 9EG7-dependent epitope expression and cell adhesion to substrate Fn does not always hold true. For example, PMA treatment of K562 cells increased the ability to adhere to Fn without increased
surface expression of the mAb 9EG7-dependent epitope (3). The possibility that TNF-α-induced loss of adhesion may be mediated by the inactivation of integrins is also supported by several other studies. Ruegg et al. (42) cotreated human endothelial cells with 200 ng/ml TNF-α and 330 ng/ml IFN-γ and observed a loss of adhesion to immobilized vitronectin, denatured collagen, and fibrinogen, but not to Fn. This loss of adhesion was the result of inactivation of α5β1-integrins, even in the presence of their increased surface expression. The mechanism by which β3 deactivation can be caused by TNF-α, as suggested by our current data, can only be speculated. Blystone et al. (4) demonstrated that α5β1-integrins can negatively influence α5β1-integrin activity with respect to Fn-mediated phagocytosis by suppressing calcium/calmodulin-dependent protein kinase II (CaMKII) activity. Furthermore, Bouvard et al. (5) have shown that CaMKII mediates CHO cell adhesion to Fn by regulating the affinity state of the α5β1-integrin. Based on these findings, one could speculate that the twofold increase in surface expression of α5β1-integrin on CPAE cells after TNF-α exposure may have caused a reduction of CaMKII activity, thereby reducing α5β1-integrin activity and contributing to the decrease in adhesion of CPAE cells to the Fn matrix. In addition to their role in cell adhesion to ECM, α5β1-integrins may facilitate the binding of soluble Fn to the cell layer and its matrix assembly (1, 18, 23, 27, 51). Our findings of decreased β1-integrin-mediated binding of soluble pFn after TNF-α are consistent with the belief that a population of α5β1-integrins expressed on the surface of TNF-α-treated CPAE cells are in a low-affinity or inactive state. However, the reduction we observed in β1-mediated pFn binding was greatly offset by a dramatic increase in pFn binding to the cell layer, which could not be blocked by mAb 13 or RGD peptide. The α5β1-integrins have the ability to assist in the assembly of soluble Fn in ECM when transfected in CHO cells (50), but mAb LM609, which blocks such matrix assembly of Fn, did not inhibit binding of soluble pFn to the cell layer in our current study. Thus the increased binding of soluble pFn does not appear to be integrin mediated; however, it may be mediated by tissue transglutaminase, since its extracellular activity is increased in CPAE cells after TNF-α, causing nonreducible Fn multimer formation in the matrix (8).

In response to binding an immobilized ligand, such as Fn, major integrins will cluster within focal adhesion complexes, resulting in interactions with actin-associated cytoskeletal proteins (6). The structural link between the ECM, integrins, and the actin cytoskeleton stabilizes cell adhesion and contributes to the mechanical basis by which integrins can influence endothelial cell shape. Changes in the ability of the cells to bind to the ECM results in cytoskeletal reorganization and global changes in cell shape (30, 44). Sims et al. (44) showed that changes in cell shape can result from cytoskeletal tension, generated by an actomyosin filament sliding mechanism, and that this tension can be physically resisted by cell surface integrins bound to immobilized adhesion sites within the ECM, including the RGD or cell attachment site in Fn. Thus the ability of integrins to bind to matrix proteins may exert a unique control on cell shape. Loss of this binding could result in a loss of monolayer confluence and the development of intercellular gaps, with an increase in endothelial protein permeability like we observed.

In summary, our findings demonstrate that TNF-α, which increases protein permeability and reorganizes the Fn matrix, also reduces the ability for CPAE cells to adhere to immobilized Fn. This loss of adhesion to an Fn substrate appears to be related to inactivation or a reduced function of α5β1-integrins. However, in parallel, TNF-α treatment caused both an increase in β1-integrin-independent binding of soluble pFn to the CPAE cell layer and its ECM incorporation. This suggests that TNF-α released in the plasma during postoperative bacterial sepsis or lung inflammatory injury may have a dual role. It may initially increase the permeability of the lung vascular barrier to facilitate the transvascular flux of plasma proteins essential to pulmonary host defense but, thereafter, lead to enhanced incorporation of pFn in the subendothelial matrix to rapidly stabilize the endothelial barrier.

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