Tumor Necrosis Factor and Immune Interferon Act in Concert to Slow the Lateral Diffusion of Proteins and Lipids in Human Endothelial Cell Membranes

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Abstract. Vascular endothelial surface-related activities may depend on the lateral mobility of specific cell surface macromolecules. Previous studies have shown that cytokines induce changes in the morphology and surface antigen composition of vascular endothelial cells in vitro and at sites of immune and inflammatory reactions in vivo. The effects of cytokines on membrane dynamic properties have not been examined. In the present study, we have used fluorescence photobleaching recovery (FPR) to quantify the effects of the cytokines tumor necrosis factor (TNF) and immune interferon (IFN-γ) on the lateral mobilities of class I major histocompatibility complex protein, of an abundant 96,000 M_r mesenchymal cell surface glycoprotein (gp96), and of a phospholipid probe in cultured human endothelial cell (HEC) membranes. Class I protein and gp96 were directly labeled with fluorescein-conjugated monoclonal antibodies; plasma membrane lipid mobility was examined with the phospholipid analogue fluorescein phosphatidylethanolamine (Fl-PE). In untreated, confluent HEC monolayers, diffusion coefficients were 30×10^{-10} cm^2 s^{-1} for class I protein, 14×10^{-10} cm^2 s^{-1} for gp96, and 80×10^{-10} cm^2 s^{-1} for Fl-PE. Fractional mobilities were >80% for each probe. Cultures treated at visual confluence for 3–4 d with either 100 U/ml TNF or 200 U/ml IFN-γ did not exhibit significant changes in protein or lipid mobilities despite significant changes in cell morphology and membrane antigen composition. In HEC cultures treated concomitantly with TNF and IFN-γ, however, diffusion coefficients decreased by 71–79% for class I protein, 29–55% for gp96, and 23–38% for Fl-PE. Fractional mobilities were unchanged. By immunoperoxidase transmission electron microscopy, plasma membranes of untreated and cytokine-treated HEC were flat and stained uniformly for class I antigen. "Line" FPR measurements on doubly treated HEC demonstrated isotropic diffusion of class I protein, gp96, and Fl-PE. Finally, although TNF and IFN-γ retarded the growth of HEC cultures and disrupted the organization of cell monolayers, the slow diffusion rates of gp96 and Fl-PE in confluent doubly treated monolayers were not reproduced in sparse or subconfluent untreated monolayers. We conclude that the slowing of protein and lipid diffusion induced by the combination of TNF and IFN-γ is not due to plasma membrane corrugations, to anisotropic diffusion barriers, or to decreased numbers of cell–cell contacts. We propose that cytokines act directly to regulate the mobility of membrane molecules in confluent HEC monolayers.

THE vascular lining forms the interface between the blood and the underlying vessel wall and tissues. The total luminal surface area presented by human vascular endothelial cell plasma membranes exceeds 6,300 m^2 (Krough, 1929). Endothelial surface-related activities include inducible adherence of blood cells, binding of hormones and nutrients, regulation of the hemostatic/thrombotic balance, attachment to the basement membrane, and facilitation of immune and inflammatory responses. Experimental evidence suggests that each of these activities depends, in part, upon the quantitative level of expression of specific cell surface molecules (reviewed by Gimbrone, 1986). Our working hypothesis is that these activities also depend on the organization and dynamics of specific molecules in the plasma membrane.

Cytokines have been shown to alter the composition of the endothelial cell plasma membrane in vitro. These changes, collectively called "activation," result in altered endothelial cell capacities to function in hemostasis, inflammation, and immunity (reviewed by Cotran and Pober, 1988). For exam-

ple, tumor necrosis factor (TNF)[1] and immune interferon (IFN-γ) have each been shown to augment expression of class I major histocompatibility complex (MHC) antigens on human endothelial cells (HEC) (Pober et al., 1983; Collins et al., 1986), and the combination of these two agents is synergistic for this change (Lapierre et al., 1988). In addition, TNF and IFN-γ each induce reorganization of cultured HEC monolayers. Monolayers treated at visual confluence for 3–4 d with either ≥20 U/ml TNF or ≥16 U/ml IFN-γ assume a fibroblastoid organization, rearrange actin stress fibers from dense peripheral bands to longitudinal bundles, and lose fibronectin from the extracellular matrix (Stolpen et al., 1986). The combination of TNF and IFN-γ acts synergistically at low doses to induce similar morphologic changes. At higher doses these cytokines act in concert to induce unique morphologic changes, such as the development of highly elongated "dendritic" processes (Stolpen et al., 1986). Taken together, these results suggested that the combination of TNF and IFN-γ might have other unique effects on HEC.

In this report we have investigated the effects of TNF and IFN-γ on the lateral mobility of membrane macromolecules in cultured HEC. Fluorescence photobleaching recovery (FPR) was used to quantify the mobility of class I MHC protein and of an abundant 96,000 M_r cell surface glycoprotein (gp96), each labeled with a directly fluoresceinated monoclonal antibody (mAb), and of a fluorescent membrane lipid analogue. Our results show that protein and lipid mobilities are slowed by TNF and IFN-γ in combination, but not by either cytokine alone.

Materials and Methods

Cell Culture and Cytokine Treatment

HEC were cultured from two to six umbilical cord veins, and passaged once in Medium 199 (Gibco, Grand Island, NY) supplemented with 50 μg/ml endothelial cell growth factor (Meloy Laboratories, Springfield, VA), 100 μg/ml porcine heparin (Sigma Chemical Co., St. Louis, MO), 20% (vol/vol) fetal bovine serum (Gibco), glutamine and antibiotics (M. A. Bioproducts, Bethesda, MD) (Gimbrone, 1976; Thornton et al., 1983). For FPR experiments, cells were plated on 25-mm glass coverslips (Bellco Glass Co., Vineland, NJ); for electron microscopy, cells were plated in 24-well plastic dishes (Costar, Cambridge, MA). All culture surfaces were precoated with 5–10 μg/cm^2 fibronectin (Meloy Laboratories). Where indicated, HEC cultures at visual confluence were treated for three to four additional days with cytokine(s). The recombinant human cytokines used in these studies were TNF (expressed in *Escherichia coli* and purified to homogeneity; sp act 2.5 × 10^7 U/mg protein [Marmenout et al., 1985]) and IFN-γ (expressed in CHO cells and partially purified; sp act 3 × 10^6 U/mg [Devos et al., 1984]; both gifts of Prof. Walter Fiers, State University of Ghent, Ghent, Belgium). TNF and IFN-γ were diluted in standard growth medium to final concentrations of 100 U/ml (L929 cytotoxicity units [Marmenout et al., 1985]) and 200 U/ml (antiviral units [Scahill et al., 1983]), respectively. Using these cytokine treatment protocols, maximal changes in HEC morphology and class I antigen expression were induced (Stolpen et al., 1986; Pober et al., 1986*a*).

Purification of Monoclonal Antibodies and Conjugation to Fluorescein

The murine mAb's W6/32 (reactive with class I MHC antigens [Barnstable et al., 1978]) and E1/1.2 (reactive with gp96 [Pober et al., 1986*a*]) were each purified from murine ascitic fluid by 50% saturated ammonium sulfate precipitation, resolubilization in and dialysis against phosphate-buffered saline (PBS) and centrifugation at 200 *g* for 2 min (Mishell and Shiigi, 1980). The supernatant was adjusted to pH 8.0 and loaded onto a protein A-Sepharose affinity column (Pharmacia Fine Chemicals, Piscataway, NJ) preequilibrated with PBS at pH 7.4. W6/32 (subclass IgG_{2a}) was eluted at pH 4.0 in 50 mM sodium acetate/40 mM sodium chloride; E1/1.2 (subclass IgG_{2b}) was eluted at pH 3.0 in 100 mM glycine (Ey et al., 1978). Protein content was determined by optical density at 280 nm. Peak fractions were pooled and dialyzed extensively against 50 mM sodium carbonate/150 mM sodium chloride/pH 8.5. The dialyzed material was concentrated by nitrogen-driven pressure filtration (Amicon Corp., Danvers, MA) and centrifuged at 100,000 *g* for 1 h to remove antibody aggregates. 8–10 ml of the supernatant was mixed with 1/9 volume dimethylformamide (DMF) and stirred at 0°C. A 20-fold molar excess of fluorescein-5-isothiocyanate (FITC; Molecular Probes, Inc., Eugene, OR) was dissolved in 400 μl DMF and then added dropwise over 15 min to the mAb/DMF solution (adapted from Golding [1976]). Reactants were stirred for an additional 2.5 h in the dark at room temperature. Unreacted FITC was removed by gel filtration chromatography on a Biogel P-6 (Bio-Rad Laboratories, Richmond, CA) column using 50 mM potassium phosphate/pH 8.0 as the elution buffer (Golding, 1976). Protein and fluorescein content were determined by optical density at 280 and 493 nm, respectively. Fractions in the first peak were pooled. The FITC:mAb molar ratio was 5.6 for W6/32 and 7.2 for E1/1.2, compared to a ratio of <3 for commercially obtained FITC-conjugated anti-β_2-microglobulin (anti-β_2M; Becton-Dickinson, Mountain View, CA). Fluoresceinated mAbs were sterile filtered (0.22 μm pore size; Corning Glass Works, Corning, NY), aliquoted, and stored at −20°C.

Labeling of HEC with Fluorescent Antibodies and a Lipid Analogue

Cell surface class I MHC proteins were labeled with either FITC-conjugated anti-β_2M (subclass IgG_1), FITC-conjugated W6/32, or FITC-conjugated monovalent F_{ab} fragments of W6/32 (W6/32-F_{ab}; a gift of Dr. Steven Herrmann, Harvard Medical School, Boston, MA). gp96 was labeled with FITC-conjugated E1/1.2 (Pober et al., 1986*a*). HEC monolayers were washed with magnesium- and calcium-containing Hank's balanced salt solution (HBSS) at 37°C, preincubated for 20 min on ice with a nonbinding murine IgG_{2a} mAb, washed with ice cold HBSS, incubated for 60 min on ice with FITC-conjugated mAb diluted 1:15 in Medium 199 (final mAb concentration of 50–100 μg/ml), and washed three times with HBSS at 37°C. Fluorescence intensity of E1/1.2 staining was unaffected by cytokine treatments, whereas that of W6/32 was increased.

Alternatively, HEC plasma membranes were labeled with the lipid probe fluorescein phosphatidylethanolamine (Fl-PE; Avanti Polar Lipids, Inc., Birmingham, AL). A 1 mg/ml solution of Fl-PE in chloroform was dried in a rotary evaporator, brought to a final concentration of 5 μg/ml in Medium 199, vortexed, and sonicated until clear. Cells were incubated with Fl-PE solution for 45–50 min at 35°C and then washed twice with HBSS/1% bovine serum albumin (Sigma Chemical Co.) and once with HBSS alone.

Fluorescence Photobleaching Recovery

Our FPR apparatus and analytical methods are described in detail elsewhere (Golan et al., 1986). "Spot" FPR is a technique used to quantify the isotropic lateral mobility of fluorescent membrane probes (Axelrod et al., 1976); "line" FPR is used to detect and quantify anisotropic diffusion (Stolpen et al., 1988*b*). Briefly, a Gaussian laser beam having a circular (spot FPR) or elliptical (line FPR) profile is focused to a waist at the sample plane of a fluorescence microscope. The elliptical beam is oriented on an individual cell either parallel or perpendicular to the long axis of the cell (Fig. 1). After a brief, intense photobleaching pulse (the bleaching beam), recovery of fluorescence is monitored by periodic low intensity pulses (the measuring beam). Recovery results from the lateral diffusion of unbleached fluorophores into the bleached area. In line FPR, recovery is dominated by diffusion perpendicular to the long axis of the elliptical beam (Stolpen et al., 1988*b*). Nonlinear least squares analysis of fluorescence recovery data yields both the diffusion coefficient (D) and the fraction of fluorescently labeled molecules that are free to diffuse on the time scale of the experiment. The Gaussian beam radius at the sample plane was calibrated daily, as described (Stolpen et al., 1988*a*). Circular Gaussian beams had radii of 1.71–2.15 μm with SD < 5%. Elliptical Gaussian beams were characterized by radii along both the major and minor axes (see legend to Table II for details).

1. *Abbreviations used in this paper*: β_2M, β_2-microglobulin; D, diffusion coefficient; DMF, dimethylformamide; Fl-PE, fluorescein phosphatidylethanolamine; FPR, fluorescence photobleaching recovery; G-PBS, phosphate-buffered saline with 0.2% gelatin; gp96, 96,000 M_r glycoprotein; HEC, human endothelial cell; IFN-γ, immune interferon; TNF, tumor necrosis factor.

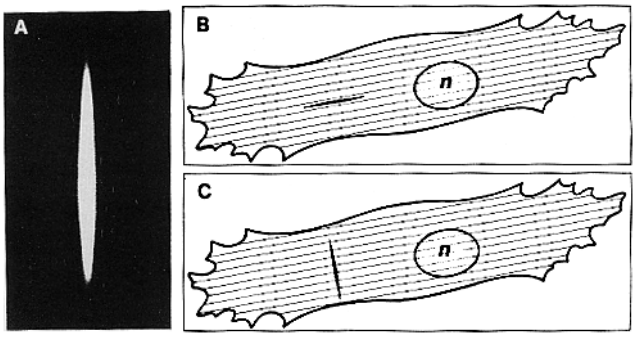


Figure 1. Experimental method for quantifying anisotropic diffusion by the line FPR technique. (*A*) Fluorescence photomicrograph of an elliptical Gaussian laser beam focused on a 1-μm-thick film of aqueous fluorescein-5-thiosemicarbizide (major axis = 8.7 μm; minor axis = 1.2 μm). In line FPR experiments performed on doubly treated HEC, the elliptical beam was focused on a small patch of fluorescently labeled membrane and then oriented either parallel (*B*) or perpendicular (*C*) to the long axis of the cell. The membrane overlying the nucleus (*n*) was not used. In *B*, diffusion perpendicular to the longitudinal actin stress fibers would be measured; in *C*, diffusion parallel to the stress fibers would be measured. A rotatable microscope stage was used to orient the cell with respect to the major axis of the elliptical beam.

Bleaching beam power at the sample was 100–500 μW. The measuring beam power was attenuated 400-fold from this value. Bleaching pulses were 20–30 ms in duration for spot FPR and 90–750 ms for line FPR. Measuring pulses were 25–200 ms in duration for protein diffusion and 5–25 ms for lipid diffusion.

Diffusion was measured on a small patch of uniformly fluorescent membrane. Regions overlying the nucleus or close to the cell periphery were carefully avoided. Cells often overlapped in cytokine-treated cultures, so mobility measurements on cells in these cultures were performed only in regions of single cell thickness. Because the Gaussian laser beams used in these studies diverge insignificantly across the thickness of an endothelial cell, FPR measurements on HEC yielded an average mobility of labeled molecules in apical and basal membranes. In all cases, fluorescence recovery data were well fit by an equation describing the diffusion of a single species, suggesting that there were no major differences between the lateral mobilities of class I protein, gp96, or Fl-PE in apical and basal membranes of either untreated or cytokine-treated HEC.

Care was taken to maintain the viability of cells during FPR experiments. Coverslips were mounted in a Sykes-Moore chamber (Bellco Glass, Inc., Vineland, NJ) with 1.1 ml of standard HEC growth medium preequilibrated with air/5% CO_2 and warmed to 37°C. The chamber was mounted on a temperature controlled stage (E. Leitz, Inc., Rockleigh, NJ). FPR measurements were performed at 37°C. Over a 90-min period of experimentation, cells maintained under these conditions showed no cytopathic changes and the pH of the culture medium remained at 7.4.

Immunoperoxidase Staining of Cells for Electron Microscopy

HEC monolayers were washed with HBSS at 37°C, fixed for 30 min with 2% paraformaldehyde (Fisher Scientific Co., Pittsburgh, PA)/10 mM sodium periodate/0.75 M lysine/37.5 mM sodium phosphate/pH 6.2, and washed three times with PBS/50 mM ammonium chloride. Fixed cells were washed for 30 min with PBS/0.2% gelatin (G-PBS; Fisher Scientific Co.), incubated for 60 min with W6/32 ascitic fluid diluted 1:100 in G-PBS, washed three times in G-PBS, incubated for 30 min with peroxidase-conjugated rabbit anti-mouse IgG (Accurate Chemical & Scientific Corp., Westbury, NY) and 20% normal human serum in G-PBS, and washed again in G-PBS. Monolayers were postfixed with 2% glutaraldehyde (Electron Microscopy Sciences, Port Washington, PA)/0.1 M sodium cacodylate/pH 7.3 for 15 min, washed with 0.05 M Tris-HCl/pH 7.6, stained with 0.5 mg/ml diaminobenzidine (Sigma Chemical Co.) and 0.01% hydrogen peroxide in 0.05 M Tris-HCl/pH 7.6, and washed with 0.05 M Tris-HCl/pH 7.6 and distilled water. Monolayers were fixed in 2% aqueous osmium tetroxide (Electron Microscopy Sciences), dehydrated through graded ethanols, infiltrated with propylene oxide (Fisher Scientific Co.) and embedded in LX-112 resin (Ladd Research Industries, Inc., Burlington, VT). Sections were examined on a Phillips model 201 transmission electron microscope. A nonbinding isotype-matched murine mAb, UPC-10, was used to demonstrate the specificity of immunoperoxidase staining with W6/32.

Results

Distribution of Fluorescent Probes on HEC

By fluorescence microscopy, E1/1.2, W6/32, W6/32-F_{ab}, anti-β_2M, and Fl-PE were each uniformly distributed on HEC surfaces. There was no evidence of aggregation or domain formation, although the existence of microaggregates or microdomains could not be excluded. Cell surface fluorescence intensity did not vary significantly over the 60–90-min period required for FPR measurements on a single coverslip. Cells labeled with Fl-PE exhibited perinuclear, punctate fluorescence after 1–2 h at 37°C. These areas were not used in FPR experiments.

Effects of TNF and IFN-γ on Protein and Lipid Mobility

Spot FPR measurements were used to quantify the lateral mobility of W6/32-labeled class I MHC proteins, E1/1.2-labeled gp96, and the lipid analogue Fl-PE in plasma membranes of HEC grown to visual confluence. In untreated cells, diffusion coefficients (D) for class I protein and gp96 were 30×10^{-10} cm^2 s^{-1} and 14×10^{-10} cm^2 s^{-1}, respectively (Table I). D for Fl-PE was significantly faster, 80×10^{-10} cm^2 s^{-1} (Table I). Fractional mobilities were consistently >80% for each of the probes. HEC treated at visual confluence for 3–4 d with either 100 U/ml TNF or 200 U/ml IFN-γ manifested changes in cell shape, monolayer organization, and class I antigen expression, as described previously (Collins et al., 1986; Pober et al., 1986*a*; Stolpen et al., 1986). Singly treated HEC cultures did not, however, exhibit significant changes in the mobilities of class I protein, gp96, or Fl-PE (Table I). HEC cultures treated concomitantly for 3–4 d with 100 U/ml TNF and 200 U/ml IFN-γ exhibited a 71–79% slowing of class I protein mobility, a 29–55% slowing of gp96 mobility, and a 23–38% slowing of Fl-PE mobility (Table I). Fractional mobilities remained consistently >80% in all doubly treated cultures. Thus, there was no suggestion that cytokine treatment completely immobilized any fraction of the molecules examined.

Because doubly treated cells manifest higher surface densities of class I proteins than untreated or singly treated cells (Lapierre et al., 1988), increased cross-linking of these proteins by the bivalent W6/32 IgG could have been responsible for the observed slowing of class I protein diffusion in doubly treated cultures. HEC were therefore labeled with FITC-conjugated W6/32-F_{ab}, a monovalent reagent. D for W6/32-F_{ab} on doubly treated cells was found to be 69% less than that on IFN-γ-treated cells (Table I). This degree of slowing is comparable with that measured using intact W6/32 (Table I). FITC-conjugated W6/32-F_{ab} did not provide sufficient fluorescence signal to examine class I protein mobility in untreated or TNF-treated HEC cultures.

The lateral mobility of class I protein on doubly treated HEC was also examined using FITC-conjugated anti-β_2M.

Table I. Effect of TNF and IFN-γ on the Lateral Diffusion of Class I MHC Protein, gp96, and Fl-PE in HEC Membranes

Surface component	Fluorescent probe	D × 10^{10} cm^2 s^{-1}			
		Untreated	TNF	IFN-γ	TNF and IFN-γ
Class I MHC protein	FITC-W6/32	30 ± 8* (10)‡	35 ± 9 (10)	27 ± 6 (11)	6.3 ± 3.6§ (14)
Class I MHC protein	FITC-W6/32	29 ± 4 (10)	30 ± 11 (11)	23 ± 6 (12)	8.5 ± 3.2§ (12)
Class I MHC protein	FITC-W/32-F_{ab}	ND	ND	22 ± 6 (14)	6.9 ± 1.7§ (15)
gp96	FITC-E1/1.2	13 ± 4 (11)	12 ± 5 (12)	13 ± 4 (12)	5.9 ± 1.4§ (12)
gp96	FITC-E1/1.2	14 ± 6 (11)	18 ± 7 (12)	13 ± 5 (12)	10 ± 4‖ (11)
Lipid	Fl-PE	82 ± 26 (26)	70 ± 17 (11)	71 ± 22 (15)	51 ± 15¶ (14)
Lipid	Fl-PE	74 ± 13 (11)	72 ± 19 (15)	81 ± 8 (10)	57 ± 15** (11)

First passage HEC were plated on fibronectin-coated glass coverslips and treated at visual confluence for three to four additional days with 100 U/ml TNF and/or 200 U/ml IFN-γ, as indicated. Data in each row of the table were obtained on the same day from a single HEC culture which had been replicate plated. D, diffusion coefficient; ND, not determined. Mean fractional mobilities were >80% in all experiments.

* Mean ± SD.

‡ Number of measurements.

§‖¶** Significance level for difference in D values (in the same row) between IFN-γ-treated (FITC-W6/32-F_{ab}-labeled) or untreated (all other fluorescent labels) and doubly treated HEC, as determined by two-tailed Student's *t* test. § $P < 0.001$; ‖ $P < 0.10$; ¶ $P < 0.002$; ** $P < 0.02$.

D for this sample was 6.4 ± 2.3 × 10^{-10} cm^2 s^{-1} (mean ± SD for 10 measurements), which was not significantly different from that obtained using FITC-conjugated W6/32 (Table I). FITC-conjugated anti-β_2M, obtained commercially, also did not provide sufficient fluorescence signal to examine untreated HEC cultures.

Electron Microscopy

Aizenbud and Gershon (1982, 1985) have shown that membrane corrugations can cause an apparent slowing of lateral diffusion. This effect occurs because FPR methods measure only those motions orthogonal to the bleaching laser beam. To determine whether membrane nonplanarity was responsible for the cytokine-induced decreases in lateral diffusion, we used transmission electron microscopy to examine the topography of HEC plasma membranes and the distribution of immunoperoxidase-labeled class I antigens. Plasma membranes of both untreated and cytokine-treated HEC were found to be flat and smooth (Fig. 2). Class I antigens were distributed uniformly on both the apical and basal membranes. There was no "ruffling" or "pitting" of the plasma membrane in cultures treated concomitantly with TNF and IFN-γ (Fig. 2 *d*).

Cytokine-treated cultures exhibited characteristic features of activated endothelium. Cells were tall and had cytoplasms packed with biosynthetic organelles. TNF and IFN-γ also caused marked disruption of monolayer organization. Whole cells and cell processes were seen to overlap one another (Fig. 2, *c* and *d*). Vascular endothelial cells at sites of delayed hypersensitivity reactions in vivo have been shown to exhibit a similar morphology (Willms-Kretschmer et al., 1967; Graham and Shannon, 1972; Dvorak et al., 1976; Polverini et al., 1977; Freemont and Ford, 1985).

Line FPR Measurements on Doubly Treated HEC

We have previously shown that actin stress fibers in doubly treated HEC are arranged in longitudinal, parallel bundles (Stolpen et al., 1986). Line FPR measurements of the diffusion of anti-β_2M-labeled class I protein, E1/1.2-labeled gp96, and Fl-PE were performed on doubly treated HEC, to determine whether actin stress fibers act as a one-dimensional barrier to lateral diffusion. Such a barrier could explain the cytokine-mediated decrease in protein mobility. Diffusion was measured along two orthogonal axes oriented parallel and perpendicular, respectively, to the long axis of the cell (see Fig. 1). Neither class I protein, gp96, nor Fl-PE was found to diffuse anisotropically in doubly treated cells (Table II).

Effects of Cell Density on gp96 and Lipid Diffusion

TNF and IFN-γ in combination cause marked disruption of HEC monolayer organization (Stolpen et al., 1986). Doubly treated cultures lose the tightly packed, epithelioid organization of confluent, untreated monolayers. Rather, the cells in such cultures overlap extensively as if they had lost contact inhibition of migration. Spaces appear between neighboring cells, creating multiple gaps in the monolayer. To test the hypothesis that TNF and IFN-γ decrease lateral mobility indirectly, by decreasing the number of cell–cell contacts and thereby releasing cells from contact inhibition, we examined the effects of cell density on the mobility of gp96 and Fl-PE in untreated HEC cultures. Table III shows that changes in monolayer density had no effect on the mobility of either gp96 or lipid in untreated cultures. In particular, cells in sparse or subconfluent HEC cultures did not exhibit the slowing of gp96 or lipid diffusion which was seen in confluent, doubly treated cultures.

Table II. Line FPR Measurements of Class I MHC Protein, gp96, and FL-PE Diffusion in Doubly Treated HEC Membranes

Surface component	Fluorescent probe	D × 10^{10} cm^2 s^{-1} ‖*	D × 10^{10} cm^2 s^{-1} ⊥
Class I MHC protein	FITC-anti-β_2M	7.9 ± 4.3‡ (11)§	8.6 ± 3.5 (12)
gp96	FITC-E1/1.2	7.0 ± 1.8 (11)	7.5 ± 2.4 (13)
Lipid	Fl-PE	70 ± 25 (6)	78 ± 25 (7)

First passage HEC were plated on fibronectin-coated glass coverslips and treated at visual confluence for three to four additional days with both 100 U/ml TNF and 200 U/ml IFN-γ. Data in each row of the table were obtained on the same day from a single HEC culture. D, diffusion coefficient. Mean fractional mobilities were >83% in all experiments. The $1/e^2$ radii (in μm) of the elliptical Gaussian laser beams were (minor axis/major axis): for class I protein diffusion measurements, 1.3/10.6; for gp96 measurements, 1.2/10.5; for Fl-PE measurements, 2.4/13.0.

* ‖, parallel, and ⊥, perpendicular, indicate the orientations of the elliptical beam with respect to the axis of HEC actin stress fibers (see Fig. 1).

‡ Mean ± SD.

§ Number of measurements.

Discussion

In this report, we have shown that two endothelial cell surface proteins, class I MHC protein and gp96, diffuse rapidly ($D \cong 30 \times 10^{-10}$ cm^2 s^{-1} and $D \cong 14 \times 10^{-10}$ cm^2 s^{-1}, respectively) in untreated, TNF-treated , and IFN-γ-treated HEC. Similar values for class I protein diffusion have previously been shown for W6/32-labeled IFN-γ-treated human dermal fibroblasts ($D = 12 \times 10^{-10}$ cm^2 s^{-1}; Stolpen et al., 1988*b*) and W6/32-F_{ab}-labeled B lymphoblastoid cells ($D = 21 \times 10^{-10}$ cm^2 s^{-1}; Bierer et al., 1987). The lipid analogue Fl-PE diffuses three to five times faster than protein in untreated or singly treated HEC ($D \cong 70$–80×10^{-10} cm^2 s^{-1}). TNF and IFN-γ in combination cause a three- to fivefold decrease in the diffusion rate of W6/32- or W6/32-F_{ab}-labeled class I protein and a one and one half- to twofold decrease in the diffusion rates of gp96 and Fl-PE. Thus, Fl-PE diffuses 5–10 times faster than protein in doubly treated cells. These data suggest that the mobility of proteins in untreated and singly treated HEC is partially restricted relative to that of lipid, and that TNF and IFN-γ in combination induce further restriction of protein mobility. Further, these data suggest that the cytokine-mediated slowing of class I protein diffusion does not result from the cross-linking of surface proteins by the bivalent W6/32 IgG.

TNF and IFN-γ could act to restrict protein and lipid mobility through a number of different mechanisms. Cytokine-induced changes in membrane geometry could produce an apparent slowing of lateral diffusion (Aizenbud and Gershon, 1982, 1985). Transmission electron microscopy shows, however, that cytokine treatment does not increase corrugation or pitting of HEC plasma membranes. Furthermore, membrane nonplanarity should produce proportionate changes in the mobility of all diffusible membrane constituents, whereas the mobilities of class I protein and Fl-PE decrease disproportionately after treatment with TNF and IFN-γ. These data argue against cytokine-mediated changes in HEC membrane geometry.

Cytoskeletal interactions slow transmembrane protein diffusion in both erythrocyte (Golan and Veatch, 1980; Sheetz et al., 1980) and lymphocyte (Henis and Elson, 1981; Wu et al., 1982) membranes. It is hypothesized that steric interactions and/or reversible, low affinity binding of transmembrane proteins to specific sites on the cytoskeletal lattice restrict membrane protein mobility (Nicolson, 1976; Ash et al., 1977; Koppel et al., 1981; Jacobson et al., 1984, 1987; Bourguignon et al., 1985, 1986). TNF and IFN-γ could restrict the mobility of class I protein and gp96 by increasing steric "hindrance," by increasing the affinities of cytoskeletal-membrane protein interactions, or by decreasing the mean separation between cytoskeletal binding sites. Significant interactions between an anisotropic actin cytoskeleton and a mobile membrane protein should lead to anisotropic diffusion of that protein, i.e., the cytoskeleton should act as a one-dimensional barrier to lateral diffusion. We have previously shown that rhodamine-phalloidin-stained actin stress fibers in TNF- and IFN-γ-treated HEC are arranged in longitudinal bundles which are parallel to the long axis of the cell (Stolpen et al., 1986). Using the line FPR technique, we show

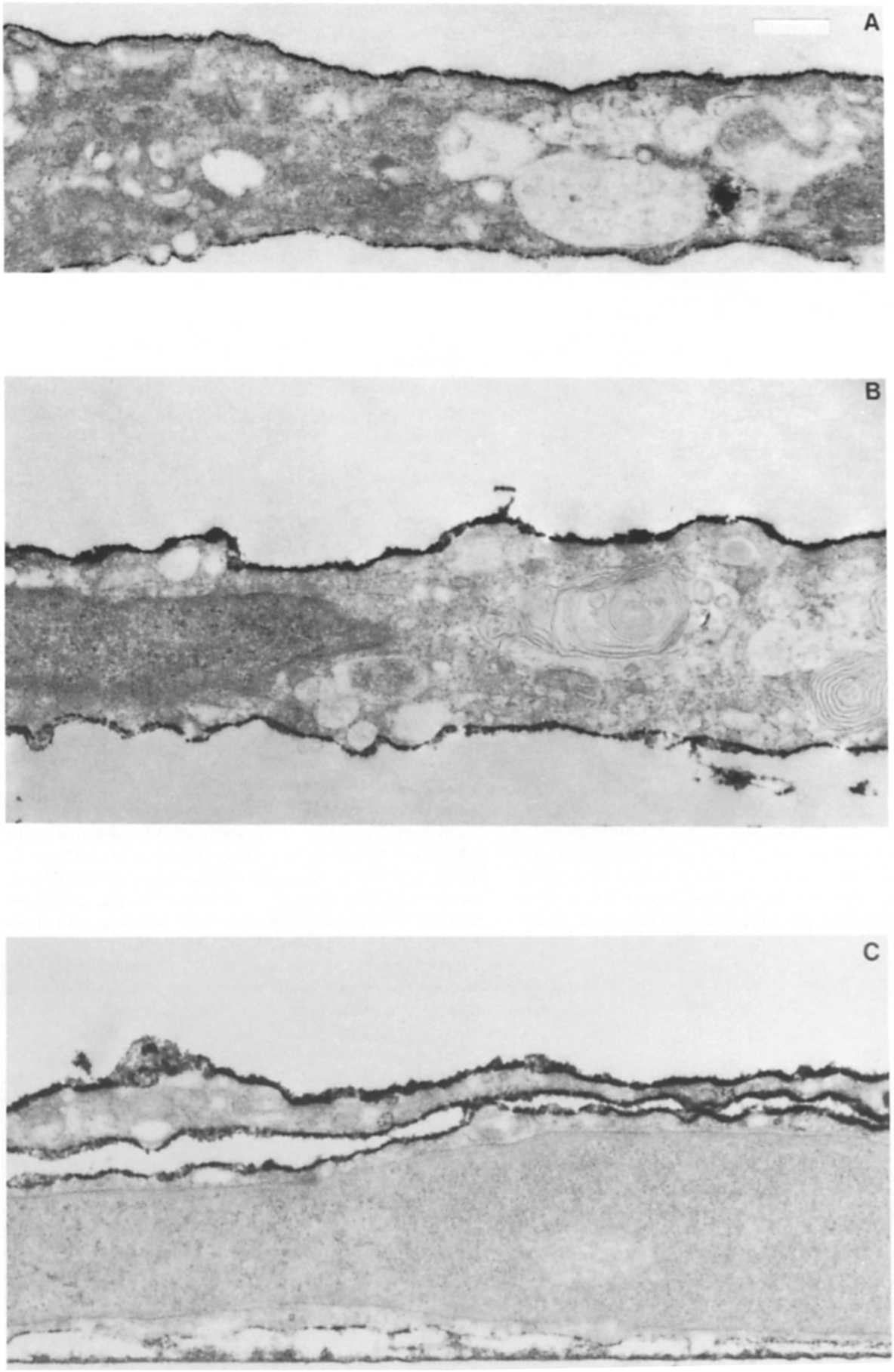
Table III. Effect of Monolayer Density on gp96 and Lipid Diffusion in Untreated HEC Cultures: Comparison with Doubly Treated Cultures

Surface component	Fluorescent probe	Untreated Isolated	Untreated Subconfluent	Untreated Confluent	TNF + IFN-γ Confluent
gp96	FITC-E1/1.2	10.4 ± 3.4* (10)‡	10.0 ± 2.9 (10)	10.8 ± 2.0 (10)	6.7 ± 2.9 (11)
gp96	FITC-E1/1.2	11.1 ± 3.4 (5)	10.2 ± 3.1 (11)	10.9 ± 1.9 (10)	7.2 ± 3.8 (12)
Lipid	Fl-PE	140 ± 14 (10)	125 ± 19 (11)	139 ± 14 (10)	98 ± 22 (12)
Lipid	Fl-PE	89 ± 18 (12)	ND	74 ± 13 (11)	57 ± 15 (11)

First passage HEC were plated on day 0 at the following densities: isolated, 1×10^4 cells/cm^2; subconfluent, 2×10^4 cells/cm^2; and confluent, 5×10^4 cells/cm^2. Cultures were treated continuously from day 1 as indicated: doubly treated cultures received 100 U/ml TNF and 200 U/ml IFN-γ. Spot FPR measurements were performed on day 4. Data in each row of the table were obtained on the same day from a single HEC culture. Data for lipid mobility in untreated and doubly treated confluent cultures (data set No. 2) are reproduced from Table I. ND, not determined. Mean fractional mobilities were >94% in all experiments.

* Diffusion coefficient (mean ± SD) × 10^{10} cm^2 s^{-1}.

‡ Number of measurements.



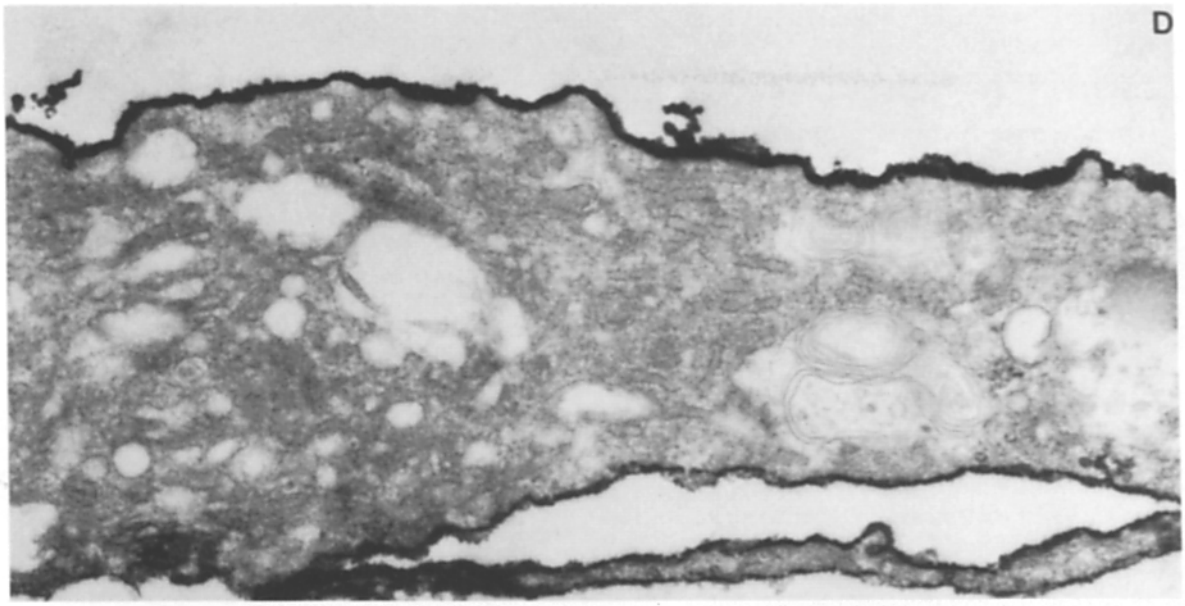


Figure 2. Transmission electron micrographs of immunoperoxidase-stained cultured HEC. HEC were grown to visual confluence and treated for four additional days with no additives (*A*), 100 U/ml TNF (*B*), 200 U/ml IFN-γ (*C*), or 100 U/ml TNF and 200 U/ml IFN-γ (*D*). Class I MHC antigens were labeled with W6/32 and immunoperoxidase-conjugated rabbit anti-mouse immunoglobulin. HEC plasma membrane contours are smooth and relatively flat, regardless of cytokine treatment. Class I MHC protein is detected on both apical and basal membranes. Bar, 0.5 μm.

here that class I protein, gp96, and Fl-PE do not diffuse anisotropically in doubly treated HEC. These observations extend our previous line FPR studies, in which class I protein and Fl-PE were found to diffuse isotropically in HEC treated with IFN-γ alone (Stolpen et al., 1988*b*). (Note that line FPR may fail to detect diffusion anisotropy on a much larger or smaller scale than the characteristic dimensions of the elliptical Gaussian laser beam.) Apparently, the mobilities of class I protein and of gp96 are not restricted by cytoskeletal elements containing filamentous actin. This conclusion is further supported by the observation that changes in protein mobility are observed only in doubly treated cultures, despite the similar arrangement of actin stress fibers in singly and doubly treated HEC. It is possible that an isotropic, nonphalloidin-staining "membrane skeleton" (Marchesi, 1985) restricts the mobilities of class I protein and of gp96. Alternatively, these two HEC membrane proteins may not interact significantly with any cytoskeletal elements, consistent with the observations of Edidin and Zuniga (1984) (see also Livneh et al., 1986).

The combination of IFN-γ and TNF induces unique changes in the organization and morphology of HEC cultures (Stolpen et al., 1986). Doubly treated cells exhibit an unusual "dendritic" shape. Cell–cell contacts are markedly disrupted; contiguous cells no longer abut one another uniformly, but are instead interconnected by numerous thin, actin-containing dendritic processes. In addition, TNF and IFN-γ have been shown to retard HEC growth in culture (Stolpen et al., 1986). These observations raised the possibility that the combined actions of TNF and IFN-γ on HEC protein and lipid diffusion could be mediated indirectly through the disruption of intercellular communication. Here, we have found that untreated cultures at low cell density do not reproduce the slowing of gp96 or Fl-PE diffusion seen in doubly treated cultures. TNF and IFN-γ in combination do not appear to alter HEC surface dynamics simply by decreasing the number of cell–cell contacts. Other organizational features of doubly treated cultures may, however, lead to changes in cell surface dynamics.

Cytokines may act through other mechanisms to alter endothelial cell surface dynamics. For example, cytokine-induced changes in lipid composition or protein concentration could increase membrane viscosity or induce lipid domain formation, leading to a decrease in lateral mobility (Saxton, 1982; Metcalf et al., 1986; Yechiel and Edidin, 1987; Jacobson et al., 1987; Golan et al., 1988). Alternatively, cytokines could restrict the mobilities of class I protein, gp96, and Fl-PE by promoting interactions with immobile or slowly diffusing structures in the plasma membrane. Finally, changes in the extracellular matrix may act to restrain protein and lipid mobility (Nakache et al., 1985; Wier and Edidin, 1986; Edidin and Wier, 1987). Matrix components of interest in HEC cultures include fibronectin and glycosaminoglycans. HEC synthesize and deposit a lacy, fibrillar meshwork of fibronectin beneath the cell monolayer (Jaffe and Mosher, 1978). TNF and IFN-γ induce a loss of fibronectin in the extracellular matrix of cultured HEC and have the same effect whether used singly or in combination (Stolpen et al., 1986). These findings make it unlikely that extracellular fibronectin is responsible for specific restriction of protein and lipid mobility in doubly treated cultures. HEC cultures treated with crude lymphokine preparations produce a dense, glycosaminoglycan-rich extracellular matrix (Montesano et al., 1984). Interactions with extracellular glycosaminoglycans may restrict the lateral mobility of class I protein, gp96, and Fl-PE in HEC treated with TNF and IFN-γ in combination. If such interactions occur they must be relatively weak and noncovalent, because class I protein,

gp96, and Fl-PE all have fractional mobilities of >80% in doubly treated HEC.

Is the diminished lateral mobility of membrane macromolecules observed in cultured endothelium in response to TNF and IFN-γ likely to occur in vivo? Examination of endothelial cells in situ suggests that the vascular lining is, in fact, exposed to a variety of cytokines, including TNF and IFN-γ. Blood vessels at sites of immune and inflammatory reactions express the same activation antigens that are induced by TNF and IFN-γ in vitro (Cotran and Pober, 1988). Furthermore, many of the specialized morphologic features of the endothelial lining at sites of delayed hypersensitivity reactions and at lymph node (i.e., "high endothelial") venules (Willms-Kretschmer et al., 1967; Graham and Shannon, 1972; Dvorak et al., 1976; Polverini et al., 1977; Freemont and Ford, 1985) may be reproduced in HEC in vitro by addition of IFN-γ and TNF to the culture medium (Stolpen et al., 1986) and in vivo in baboons by intradermal injection of IFN-γ and TNF (Munro et al., 1988). Concomitant exposure to more than one cytokine appears likely to occur in vivo because IFN-γ and lymphotoxin (and possibly TNF) are produced by the same subpopulations of antigen-stimulated T lymphocytes (Tite et al., 1985), and secretion of these cytokines may well be coupled. Thus, cytokines may act on endothelial cells in vivo, as they do in vitro, to slow the mobility of plasma membrane proteins and lipids. Might retardation of lateral diffusion have functional consequences? In allogeneic graft rejection, the vascular lining has been shown to be a primary site of attack by cytolytic T lymphocytes (CTL) (McCluskey, 1980). Studies by Herrmann and Mescher (1981) suggest that CTL responses are augmented by immobilization of class I protein on the target cell. Vascular endothelial cells may also serve as immune accessory cells, presenting antigen in an MHC-restricted manner to T helper cells (reviewed by Pober et al., 1986*b*). Watts et al. (1984) showed that the efficiency of foreign antigen presentation to cloned T cells is increased 10-fold by immobilizing class II protein. These findings suggest that endothelial cell–lymphocyte interactions are perturbed by changes in MHC protein mobility. Furthermore, these findings suggest that TNF (and possibly lymphotoxin) may augment the capacity of IFN-γ-stimulated (i.e., class II antigen expressing) endothelium to present antigen to circulating T helper cells.

In summary, we have shown that TNF and IFN-γ act in concert to slow the lateral diffusion of class I MHC protein, gp96, and Fl-PE in cultured HEC. The decrease in mobility is not caused by anisotropic diffusion barriers, perturbation of surface planarity, or loss of cell contact. Our observations are most consistent with the hypothesis that cytokines act directly on endothelium to decrease the lateral diffusion of surface macromolecules. We suggest that such changes could influence the immune accessory functions of the cytokine-activated endothelial surface in situ.

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