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Endothelial Cell Activation Induced by Tumor Necrosis Factor and Lymphotoxin

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Alterations in the morphology and histochemistry of vascular endothelial cells (EC) have been repeatedly observed at sites of chronic inflammation and immune reactions. These changes, which are most prominent in the EC of postcapillary venules present in areas with large lymphocytic infiltrates, include the acquisition of a columnar or cuboidal morphology, the development of ribonuclease-sensitive metachromasia, and an increase in intracellular organelles. Thus, EC at sites of inflammation appear to be activated and to demonstrate increased metabolic activity. This study reports that both tumor necrosis factor-α (TNF) and lymphotoxin (LT) can activate cultured human umbilical vein EC, as measured by: 1) increased adhesiveness for lymphocytes, 2) increased cell metabolism, as measured by RNA and protein synthesis, and 3) increased cell volume. Although gamma interferon (IFN-γ) and interleukin-1 (IL-1) have been shown previously to stimulate EC adhesiveness for lymphocytes, these two cytokines had only marginal effects on EC RNA and protein synthesis, and both caused a decrease in EC volume. These findings suggest that TNF and LT play a role in the type of activation of EC in vivo that leads to the development of tall endothelium and increased lymphocyte emigration. (Am J Pathol 1989, 134:551–560)

Vascular endothelial cells (EC) lining the postcapillary venules (PCV) of the body typically have an elongated, "flat" morphology with relatively little cytoplasm and spindle-shaped nuclei with little dispersed chromatin. EC of PCV present in lymphoid organs and at sites of chronic inflammation, however, often develop a tall, columnar or cuboidal morphology, a large ovoid nucleus with dispersed chromatin and prominent nucleoli, and increased numbers of intracellular organelles.[1–12] Histochemically, these EC exhibit cytoplasmic pyroninophilia, ribonuclease-sensitive metachromasia, and diffuse nonspecific esterase staining.[9–11] These morphologic and histochemical changes have led to suggestions that such EC are activated and have increased metabolic capabilities.[6,8] EC with these characteristics have been reported in experimental cutaneous granulomas,[3,7,11] immunologically-induced experimental synovitis,[4] delayed-type hypersensitivity reactions,[2,6,8] Sjogren's syndrome,[9] Hashimoto's thyroiditis,[7] and rheumatoid arthritis (RA).[5,7,12] In many of these studies, it has been observed that the tall EC tend to be adjacent to densely aggregated lymphocytic infiltrates,[7,9–12] suggesting that they may be sites of active emigration of circulating lymphocytes into the connective tissue. Nightingale and Hurley,[7] and Freemont and Ford,[11] have provided direct evidence for this in kinetic studies of the development of experimental granulomas. Furthermore, it has been demonstrated by this laboratory[13] and by Jalkanen et al[14] that human mononuclear cells preferentially bind to PCV with tall EC in frozen sections of RA synovial membrane.

In lymphoid organs, recirculating lymphocytes preferentially migrate from the blood through specialized PCV similar to those described above, which have been referred to as high endothelial venules (HEV).[1] The EC of lymphoid organ HEV resemble the tall EC of PCV at sites of inflammation not only morphologically, but also in their cytoplasmic pyroninophilia, ribonuclease-labile metachromasia, diffuse nonspecific esterase staining, and increased content of biosynthetic organelles.[15–18] Based on studies using congenitally athymic animals, neonatally thymectomized animals, and animals whose afferent lymphatics have been interrupted, it has been suggested that the development and maintenance of lymph node HEV are regulated by immunologic factors.[18–20] In support of this suggestion is the observation in experimental animals that injection of antigen increases the height of the EC of the PCV of draining lymph nodes.[20,21] In addition, intradermal injection of crude lymphokine preparations has been

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shown to induce both EC hypertrophy and mononuclear cell infiltration.[22] Recent *in vitro* studies with both impure cytokine preparations and purified or recombinant cytokines have further demonstrated that various mononuclear cell-derived soluble factors affect EC morphology and function. Crude mononuclear cell supernatants have been reported to alter the morphology of cultured human umbilical vein EC[23,24] and to increase their motility.[23] Gamma interferon (IFN-γ), a product of activated T cells,[25] causes EC to assume a fibroblasticlike morphology,[26] stimulates their expression of cell-surface HLA class II antigens,[27] enhances their ability to function as antigen-presenting cells,[28] and stimulates their ability to bind B and T lymphocytes.[29] Interleukin-1 (IL-1) and tumor necrosis factor-α (TNF), produced primarily by cells of the monocyte/macrophage lineage, have been reported to alter EC morphology *in vitro,*[30,31] to stimulate EC prostaglandin biosynthesis,[32,33] to induce procoagulant activity on the EC surface,[34-36] and to increase the ability of the EC to bind both lymphocytes[37-39] and polymorphonuclear leukocytes (PMN).[40-43]

In this report, we present data showing that TNF and lymphotoxin (LT), also known as TNF-β, stimulate cultured EC to increase their size, protein synthetic rate, RNA synthetic rate, and T cell binding activity, changes consistent with their development of characteristics of activated EC.

Materials and Methods

Preparation of Human Umbilical Vein EC

EC were obtained from human umbilical cords by collagenase digestion as described.[44] Cells from different cords were cultured separately in EC growth medium composed of RPMI 1640 supplemented with 15% fetal calf serum (FCS, GIBCO, Grand Island, NY), 10% human type O serum (HS), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B), 20 U/ml heparin (Upjohn Co., Kalamazoo, MI), and 30 μg/ml EC growth factor (ECGS) (Collaborative Research, Inc., Lexington, MA), in tissue culture flasks pretreated with 1% gelatin. At confluence, cells were detached using 0.125% trypsin in Puck's EDTA solution (trypsin/PEDTA). Cells in the monolayer were confirmed to be EC by immunofluorescent staining with an anti-factor VIII serum. EC were used at the third or fourth passage. Less than 1% of the cells at these passages were stained by a monoclonal antimacrophage antibody (63D3), as determined on a fluorescence-activated cell sorter (FACS).

Preparation of Mononuclear Cells

Normal peripheral blood mononuclear cells were separated by centrifugation on Ficoll-Hypaque. After a 60-minute incubation at 37 C on glass petri dishes, the nonadherent cells were passed over a nylon wool column to obtain a T cell-enriched population. This preparation of cells contained more than 94% $T11^+$ cells (T cells) and less than 1% $63D3^+$ cells (monocytes/macrophages) or Leu-14^+ cells (B cells), as determined on a FACS.

^{51}Cr Labeling

Mononuclear cells from 60 to 120 ml blood were suspended in 0.3 ml of assay medium (RPMI 1640 supplemented with 15% FCS, antibiotics, and 10 mM HEPES buffer) plus 0.3 ml saline containing 300 μCi $Na_2\,^{51}CrO_4$. After a 90-minute incubation at 37 C with intermittent agitation, unincorporated ^{51}Cr and dead cells were removed by centrifuging five times through 10 ml of Dulbecco's phosphate-buffered saline (DPBS) containing 5% (wt/vol) bovine serum albumin (BSA). Cell viability was greater than 95% by trypan blue exclusion.

T cell to EC (T–EC) Monolayer Adhesion Assay

EC at confluence were removed from culture flasks with trypsin/PEDTA, centrifuged, and resuspended in EC basic medium (RPMI-1640 containing 15% FCS, 10% HS, and antibiotics). They were then plated out at a confluent concentration of 5×10^4 viable cells/well in flat-bottomed, gelatin-coated, 96-well microtiter plates. After an overnight incubation, the EC were then reincubated with varying concentrations of cytokines in basic medium, for various lengths of time. After washing the EC, 2×10^5 ^{51}Cr-labeled lymphocytes in 0.2 ml assay medium were added per well in quadruplicate and were incubated for 60 minutes at 37 C in 5% CO_2. The microwells were then washed four times with 0.2 ml of warm assay medium to remove nonattached lymphocytes. Two tenths of a milliliter of an aqueous 1% solution of Triton X-100 was then added to each well, and the plate was reincubated for at least 5 minutes to lyse the remaining lymphocytes. The percentage of bound lymphocytes was calculated as follows:

$$\%\ \text{Lymphocytes bound} = \frac{\text{cpm in 0.1 ml lysate}}{\text{cpm in 0.1 ml of original lymphocyte suspension}} \times 100$$

Measurement of EC Protein Synthesis

EC were plated out at a confluent concentration of 3 $\times 10^5$ cells per well in 1 ml of RPMI-1640 medium containing 20% human serum in gelatin-coated 24-well plates, and allowed to adhere overnight. In preliminary experiments, we compared two minor variations of a method for the measurement of protein synthesis using ^{35}S-methionine incorporation. In the first, EC were washed once with methionine-deficient RPMI-1640 medium containing 20% human serum (final methionine concentration of 4 μM, due to the methionine content of the serum) and reincubated with this medium for various periods of time with various concentrations of IFN-γ, IL-1, LT, or TNF. ^{35}S-methionine was added during the last 4 hours of the incubation period in a volume of 0.05 ml to yield a final radioactive concentration of 10 μCi/ml. After this incubation period, the EC were washed once with warm PEDTA, released from the surface of the well with trypsin/PEDTA, and lysed and precipitated with an equal volume of 10% trichloroacetic acid (TCA). The precipitate was then washed three times with 5% TCA, redissolved in 0.5 N NaOH, and counted by liquid scintillation. The results were expressed as the ratio:

$$\frac{\text{cpm incorporated by cytokine-treated EC}}{\text{cpm incorporated by untreated EC.}}$$

In the second method, incubation of the EC with the cytokines was performed in normal RPMI medium with 20% human serum (final methionine concentration of 100 μM). After incubation with the cytokines, all nonradioactive methionine was removed by washing with methionine-deficient RPMI containing 20% dialyzed human serum. The EC were then incubated for 4 hours with ^{35}S-methionine in this medium, as described above. Although the cpm were higher using this second method, due to the absence of nonradioactive methionine in the medium during the pulse with ^{35}S-methionine, the two methods gave qualitatively identical results using the various cytokines. We therefore chose to use the first method because it required less perturbation of the EC and permitted the continued presence of the cytokines during the 4-hour pulse with ^{35}S-methionine. That there was no shortage of methionine during the 48-hour incubation period with the cytokines was indicated by the fact that addition of nonradioactive methionine up to a final concentration of 100 μM did not alter the degree of stimulation seen with TNF or LT. In addition, incorporation of the ^{35}S label into TCA-precipitable material was linear during the 4-hour pulse and >80% of the ^{35}S counts added to the EC cultures remained non-TCA-precipitable, ie, not incorporated into protein, during the 4-hour labeling period.

Measurement of EC RNA Synthesis

EC were plated out, checked for confluency, washed and incubated with the cytokines for varying periods of time as described above for the protein synthesis assay. ^{3}H-uridine was added during the last one hour of the incubation period in a volume of 0.02 ml to reach a final concentration of 10 μCi/well. The EC were then washed with PEDTA, removed from the culture surface with trypsin/PEDTA, and lysed and precipitated with TCA as described above. Incorporation of the ^{3}H label into TCA-precipitable material was linear during the 1-hour pulse and >90% of the ^{3}H counts added to the EC cultures remained non-TCA-precipitable. Less than 0.02% of the added counts was precipitable from the EC supernatants with TCA.

Measurement of EC Volume

EC were plated out, checked for confluency, washed, and incubated for varying periods of time with the various cytokines as described above for the protein synthesis assay except that methionine-containing RPMI-1640 medium was used throughout the incubations. Following removal of the EC from the culture well surface with trypsin, they were centrifuged and resuspended in a small volume of medium. EC volumes were then determined, using a Coulter channelyzer apparatus that had been calibrated with spherical beads of known volume (Coulter Corporation, Hialeah, FL).

Cytokines

Recombinant human IL-1α,[45] IL-1β,[46] IFN-γ, and TNF[47] were provided by Dr. Alan Shaw (Biogen; Geneva, Switzerland). Their respective specific activities, as determined by Biogen, were 5 $\times 10^7$ U/mg, 5 $\times 10^7$ U/mg, 3 $\times 10^7$ U/mg and 10^7 U/mg. The assays used to determine the respective specific activities were the murine thymocyte (LAF) assay for the IL-1 preparations, the EMC virus/WISH cell system for the IFN-γ, and a L929 cell cytotoxicity assay for the TNF preparation.[47] The cytokine preparations were all produced in *Escherichia coli,* all were over 98% pure as determined by SDS-PAGE, and all could be specifically neutralized by monospecific rabbit antisera, also provided by Biogen ([39] and unpublished observations). Recombinant human LT (TNF-β), produced by Genentech,[48] was donated by Dr. Gale Granger. It had a specific activity of 3 $\times 10^7$ U/mg, as determined in Dr. Granger's laboratory using an L929 cell cytotoxicity assay.[49]

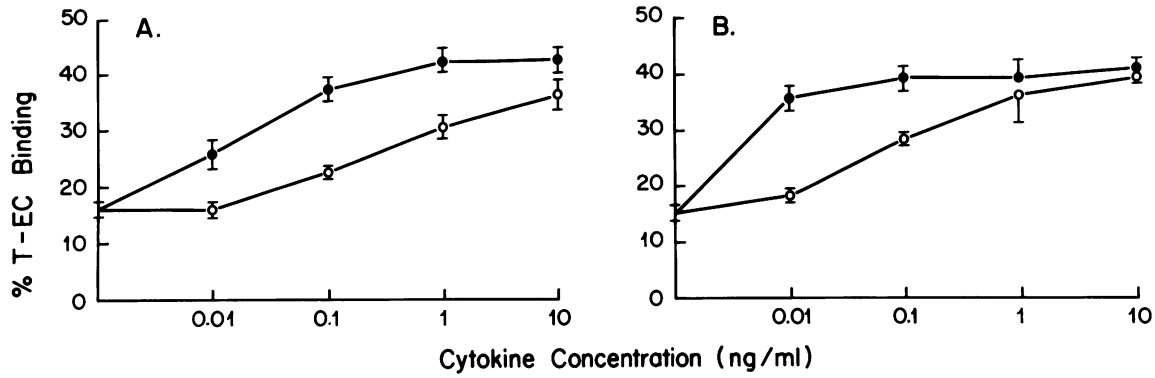

Figure 1. *Stimulation of T-EC adhesion by TNF and LT. EC were preincubated for 4 hours* (A) *or 24 hours* (B) *with the indicated concentrations of TNF (● — ●) or LT (○ — ○). T-EC adhesion was then measured. Similar results were obtained in three other experiments.*

Other Reagents

^{51}Cr, ^{35}S-methionine, and ^{3}H-uridine were obtained from ICN Radiochemicals (Irvine, CA). Methionine-deficient RPMI-1640 medium was purchased from Flow Laboratories (McLean, VA).

Results

Effects of Cytokines on EC Adhesiveness for Lymphocytes

As previously described by us for TNF-α (TNF),[39] LT also stimulated EC to become more adhesive for T cells in a time and dose-dependent manner (Figures 1 and 2). This finding is consistent with the evidence that LT and TNF bind to the same cell-surface receptor.[50] Although optimal amounts of LT and TNF stimulated T-EC adhesion to an equivalent degree, TNF was approximately 10 times more potent than LT on a weight basis (Figure 1). The effect of LT on EC adhesiveness reached a maximum between 8 and 24 hours and then leveled off (Figure 2). In previous reports, we have shown that IFN-γ[29] and IL-1[37] also stimulate EC to increase their binding of T cells. To confirm that the IFN-γ and IL-1 preparations retained biologic activity toward EC, three further T-EC adhesion assays were carried out. Following preincubation of the EC for 4 hours with 10 ng/ml IFN-γ, 1 ng/ml IL-1β, or 1 ng/ml TNF, T cell adhesion was increased by an average of 46%, 114%, and 108%, respectively. The relatively weak effect of IFN-γ on EC adhesiveness for T cells is consistent with our previous results.[29,37,39]

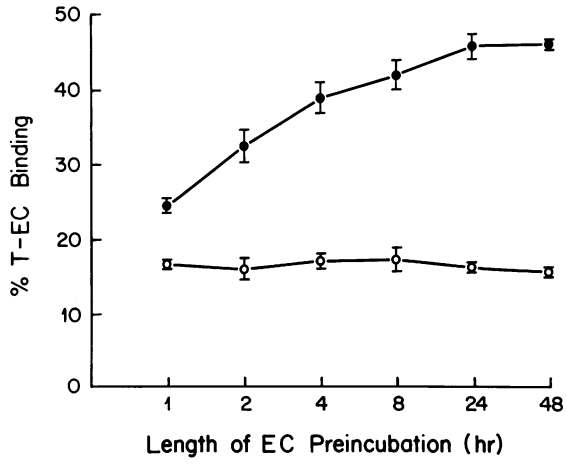

Figure 2. *Kinetics of LT-stimulated T-EC adhesion. EC were preincubated with medium alone (○ — ○) or 3.3 ng/ml LT (● — ●) for the indicated periods of time. T-EC adhesion was then measured. Similar results were obtained in five other experiments.*

Effects of Cytokines on EC Protein and RNA Synthesis

The conversion of flat to tall EC appears to be a process in which the EC are activated. This is indicated *in vivo* by their increased height/base ratio, ribonuclease-labile metachromasia, and increased content of polyribosomes and Golgi apparatus.[11,12,15–17] As measures of EC activation in these experiments, we assayed untreated and cytokine-treated EC for their rates of protein and RNA synthesis, as measured by incorporation of ^{35}S-methionine and ^{3}H-uridine into TCA-precipitable material, respectively.

TNF and LT both strongly stimulated protein synthesis in confluent monolayers of EC in a dose and time-dependent manner (Figures 3 and 4). In contrast, IL-1 and IFN-γ had little effect on EC protein synthesis (Figures 3 and 4). It is of interest that EC adhesiveness for lymphocytes increased more rapidly upon exposure to TNF[39] or LT (Figure 2) than did EC protein synthesis (Figure 4). A 50% increase in T-EC binding was observed within 1 hour after

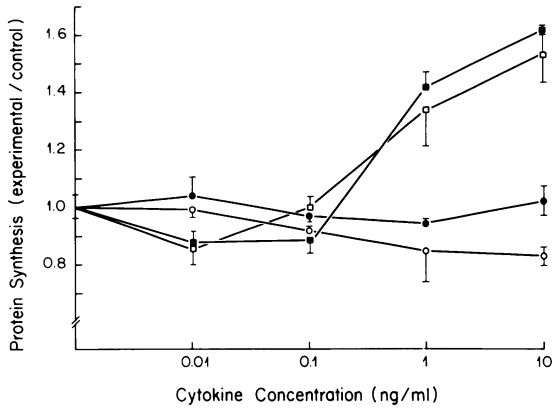

Figure 3. *Dose-response curves for cytokine-stimulated EC protein synthesis. EC were incubated in methionine-deficient medium for 48 hours in the presence of the indicated concentrations of IFN-γ (○ —○), IL-1 (● —●), TNF (■ —■) or LT (□ —□). ^{35}S-methionine was added during the last 4 hours. The mean cpm ± SD in the control EC cultures (no cytokines added) were 16,213 ± 834. Similar results were obtained in three other experiments.*

the addition of TNF[39] or LT (Figure 2) to EC, while a similar increase in protein synthesis required 24 hours (Figure 4). Because under the conditions of these experiments, TNF and LT inhibited the incorporation of tritiated thymidine (^{3}H-TdR) by the EC (Table 1), and had no effect on EC number or viability (data not shown), the increased incorporation of ^{35}S-methionine into TCA-precipitable material was not due to an increase in EC number.

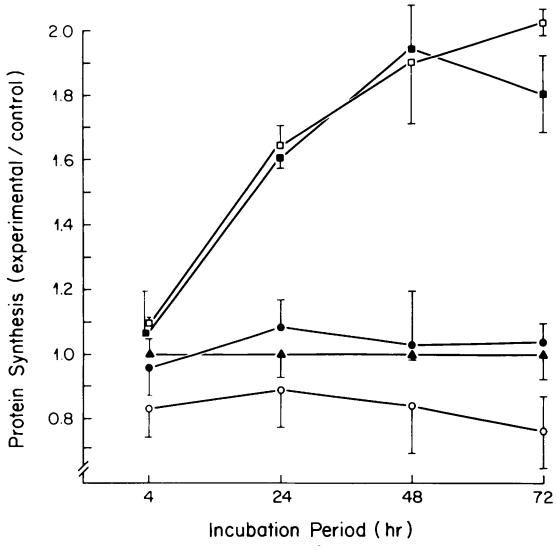

Figure 4. *Kinetic study of effects of cytokines on protein synthesis. EC were incubated in methionine-deficient medium for the indicated periods of time in the presence of medium alone (▲ —▲), 3 ng/ml IFN-γ (○ —○), 3 ng/ml IL-1 (● —●), 3 ng/ml TNF (■ —■), or 10 ng/ml LT (□ —□). ^{35}S-methionine was added during the last 4 hours of the incubation period. The mean cpm ± SD in the control EC cultures (no cytokines added) at 4, 24, 48, and 72 hours were, respectively, 14,154 ± 926, 14,578 ± 1068, 18,380 ± 180, and 19,120 ± 1350. Similar results were obtained in four other experiments.*

Table 1. *Comparative Effects of Cytokines on EC Protein and DNA Synthesis**

		EC metabolism	
Cytokine	Concentration (ng/ml)	Protein synthesis (cpm × 10^{-3})	DNA synthesis (cpm × 10^{-3})
–	–	20.8 ± 0.1	42.8 ± 1.9
IFN-γ	1	19.6 ± 0.3	43.6 ± 6.9
IL-1	1	20.9 ± 0.4	45.4 ± 6.6
LT	10	43.4 ± 1.5	16.0 ± 1.8
TNF	1	38.6 ± 0.9	21.8 ± 1.0

* EC were plated out at a confluent concentration (3 × 10^5 cells/well) in RPMI containing 20% HS and incubated overnight. After one wash with methionine-deficient RPMI/20% HS, the EC were incubated for 48 hours in methionine-deficient medium with the indicated concentrations of cytokines. EC DNA and protein synthesis were measured using a 16-hour pulse of ^{3}H-TdR and a 4-hour pulse of ^{35}S-methionine, respectively.

Similar to their effects on protein synthesis, both TNF and LT, but not IFN-γ, stimulated EC RNA synthesis in a dose-dependent manner, as measured by the incorporation of ^{3}H-uridine (Figure 5). In this experiment, IL-1 had a weak stimulatory effect on RNA synthesis, which was only 21% of the increase seen with TNF. In six similar experiments, 10 ng/ml IL-1 stimulated EC RNA synthesis by an average of 29% whereas an equivalent amount of TNF caused an average increase of 137%. In four of those experiments, 10 ng/ml LT was also tested and resulted in an average stimulation of 165%. As expected, increased RNA synthesis due to TNF or LT peaked before the peak of protein synthesis, reaching a maximum at 24 hours and declining toward baseline levels at 48 hours (data not shown).

Effects of Time at Confluence and Serum Concentration on EC Protein Synthesis

Others have demonstrated that monolayers of cultured human EC become contact-inhibited when confluence is

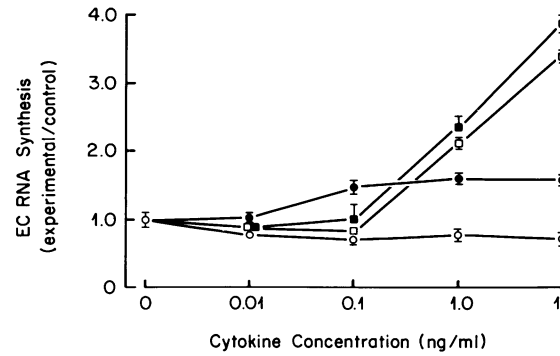

Figure 5. *Dose-response curve for cytokine-stimulated EC RNA synthesis. EC were incubated for 24 hours in the presence of the indicated concentrations of IFN-γ (○ —○), IL-1 (● —●), TNF (■ —■), or LT (□ —□). ^{3}H-uridine was added during the last 60 minutes. The mean cpm ± SD in the control EC cultures (no cytokines added) were 11,113 ± 1247. Similar results were obtained in two other experiments.*

Table 2. *Effect of Time at Confluence on EC Protein Synthesis**

Cytokine	Concentration (ng/nl)	Cytokines added 1 day after plating: Mean cpm ($\times 10^{-3}$) ±SD	SI†	Cytokines added 3 days after plating: Mean cpm ($\times 10^{-3}$) ±SD	SI
-	-	16.8 ± 1.6	1.00	23.4 ± 2.3	1.00
IFN-γ	0.1	16.0 ± 1.0	0.95	21.1 ± 3.7	0.90
IFN-γ	1	12.6 ± 0.2	0.75	15.5 ± 0.4	0.66
IFN-γ	10	12.9 ± 1.1	0.77	18.4 ± 1.7	0.79
IL-1	0.1	18.4 ± 2.1	1.09	20.8 ± 0.4	0.89
IL-1	1	16.9 ± 0.1	1.01	19.3 ± 0.5	0.83
IL-1	10	18.4 ± 1.0	1.10	21.6 ± 0.8	0.92
TNF	0.1	20.3 ± 0.0	1.21	29.7 ± 1.0	1.27
TNF	1	25.1 ± 0.8	1.49	32.9 ± 0.8	1.41
TNF	10	37.4 ± 1.1	2.22	38.9 ± 0.5	1.67

* Two plates of EC were prepared and incubated overnight. The indicated concentrations of cytokines were added to one of the plates the following day and added to the second plate 2 days later. Protein synthesis assays were performed 48 hours after the addition of the cytokines. Similar results were obtained in two other experiments.

† SI, stimulation index = $\frac{\text{cpm in cultures with cytokine}}{\text{cpm in cultures without cytokine}}$.

reached[51,52] and, unlike fibroblasts, cannot be stimulated by the addition of fresh serum to reenter the cell cycle.[52] Although our assays were performed on confluent monolayers, it was possible that not all of the cells were growth-arrested because they had been cultured in the absence of ECGS and heparin for only 24 hours before the additions of the cytokines. Therefore, two additional types of experiments were performed. In the first, protein synthetic rates in cultures in which the cytokines were added 1 day after the EC were plated out were compared with the rates in cultures in which the cytokines were added 3 days after plating. As shown in Table 2, very similar effects of the cytokines were observed. In the second type of experiment, two sets of EC were plated out in medium containing 20% HS and allowed to form confluent monolayers overnight. The medium in one set of plates was then replaced with medium containing 20% serum while the medium in the other set of plates was replaced with medium containing 5% serum. After a second overnight incubation, the cytokines were then added in medium containing the identical serum concentration and the protein synthesis assay was carried out 48 hours later. No significant differences were observed between the EC cultures in 5% and 20% serum (data not shown).

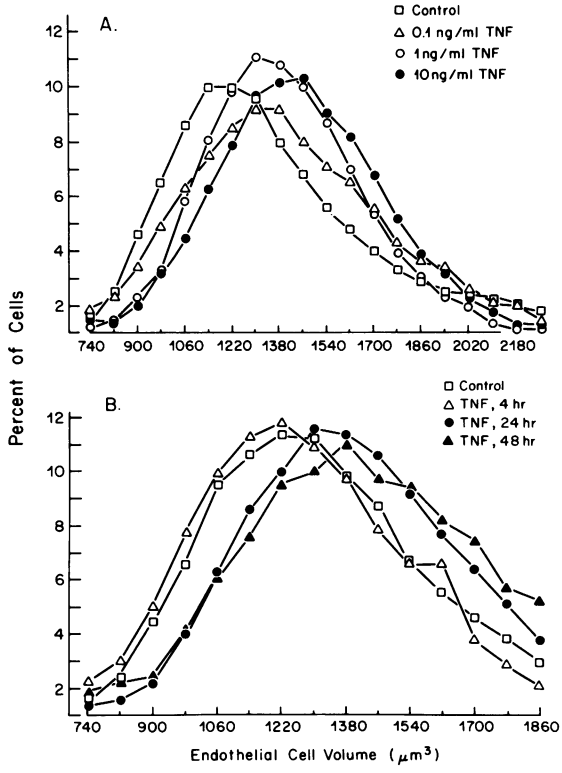

Figure 6. *TNF-induced increase in EC volume. EC volumes were determined using a Coulter channelyzer apparatus after incubations as follows:* **A:** *Incubation for 24 hours with the indicated concentrations of TNF.* **B:** *Incubation with 10 ng/ml TNF for the indicated periods of time. The results shown are the mean of duplicate cultures.*

Effects of Cytokines on EC Volume

In view of the increased height of the EC of HEV in comparison with those of other PCV, we investigated the effects of cytokines on EC volume, using a Coulter channelyzer. As shown in Figure 6, treatment of EC with TNF resulted in a dose and time-dependent increase in EC volumes compared to untreated EC. Similar results were obtained with LT (data not shown). IFN-γ and IL-1, in contrast, both decreased EC volume (Table 3). As with EC

protein synthesis (Figure 4), the increase in EC volume due to TNF (Figure 6) appeared to develop more slowly than the increase in EC adhesiveness for lymphocytes.

Discussion

Lymphocyte emigration from the blood into both lymphoid organs and chronic inflammatory sites occurs most actively through PCV lined by tall, cuboidal EC. These tall EC appear to arise as a result of a reversible transition from relatively flat EC.[11,19,20] Because both *in vivo*[18,20,22] and *in vitro*[23,24,30,31] experiments have suggested that immune factors can alter EC morphology, we initiated experiments to determine whether cytokines known to affect EC *in vitro* could induce tall EC-like properties in cultured EC.

LT and TNF were found to stimulate the development of three properties of human umbilical vein EC that resemble those of tall EC *in vivo:* 1) increased adhesiveness for lymphocytes, 2) increased cell metabolism, as measured by protein and RNA synthetic activities, and 3) increased cell volume. Although this laboratory has demonstrated previously that IFN-γ and IL-1 can stimulate EC adhesiveness for lymphocytes,[29,37] these cytokines had only marginal effects on EC RNA and protein synthesis and both caused a decrease in EC volume. The latter findings suggest that increased EC adhesiveness for lymphocytes is not directly linked to the metabolic state or size of the EC. In addition, these data establish a clear distinction between the actions of two monokines, TNF and IL-1, on EC, in that TNF increased EC adhesiveness, metabolic activity and size, whereas IL-1 stimulated EC adhesiveness in the absence of effects on RNA and protein synthesis, and also decreased EC volumes. The differing effects of IL-1 and TNF on EC metabolism and size are particularly interesting in view of our previous observations suggesting that the two cytokines appear to stimulate EC adhesiveness for T cells by the same mechanism. Although T cell adhesion to unstimulated EC is greatly inhibited by monoclonal antibodies (MAbs) to the lymphocyte-function-associated (LFA)-1 antigen,[53] such MAbs have little or no inhibitory effect on T cell binding to either IL-1-treated EC[53] or to TNF-treated EC.[39] In addition, panning T cells on IL-1-treated or TNF-treated EC monolayers results in the depletion of T cells capable of binding to EC preincubated with either cytokine.[54]

One possible explanation for the present results could be that the cytokines stimulated the proliferation of the EC, leading to increases in their RNA and protein synthesis. However, the culture conditions for these experiments (confluent EC monolayers, and medium without ECGS or heparin) were specifically chosen to favor contact inhibition and growth arrest. As reviewed by Hormia and Virtanen,[55] it has been shown that such monolayers are extremely quiescent, and that the rate of cell turnover is very slow, only 2–4% of the EC taking up ^{3}H-thymidine, as measured by autoradiography.[51,52] When such confluent monolayers are mechanically "denuded" in a small area, however, there is a localized stimulation of ^{3}H-thymidine incorporation and the nearby EC undergo mitosis and spread to cover the "lesion."[51] As pointed out by Gimbrone,[51] these properties of cultured EC fulfill the definition of contact inhibition of growth and are similar to vascular endothelium *in vivo.* Furthermore, such confluent monolayers cannot be stimulated to reenter the cell cycle by the addition of fresh serum.[52] It is reasonable, therefore, to assume that our confluent monolayers of EC were in a growth-arrested state during these experiments. Furthermore, as noted in Table 1 and as described by others,[56,57] TNF inhibits EC proliferation *in vitro.* Thus, the stimulatory effects of TNF on EC RNA synthesis, protein synthesis, volume, and adhesiveness for T cells cannot be explained on the basis of a stimulation of EC division. It should be added that we observed similar stimulatory and inhibitory effects of TNF and IL-1, respectively, on EC RNA and protein synthesis under two other experimental conditions favoring growth arrest: 1) preincubation of the EC at confluence for 3 days before the addition of the cytokines (Table 2), and 2) preincubation of the EC for 24 hours in medium containing only 5% serum, the minimal concentration of serum which we have observed to maintain the integrity of confluent EC monolayers for 48 hours.

It is of interest that TNF was more potent than LT, on a weight basis, in stimulating EC adhesiveness for T cells in view of the evidence that these two cytokines appear to bind to the same cell-surface receptor with similar affinities.[50] In spite of their difference in ability to stimulate EC adhesiveness, no difference was observed between these two cytokines in their capacity to stimulate EC RNA and protein synthesis, again suggesting that EC protein synthesis and adhesiveness for lymphocytes may be reg-

Table 3. *Comparative Effects of Cytokines on EC Volumes**

Cytokine	Number of experiments	Modal EC volume (mean ± SEM)	
		Control EC	Cytokine-treated EC
IFN-γ	12	1210 ± 24	1120 ± 24
IL-1	8	1185 ± 23	1055 ± 33
LT	8	1190 ± 21	1315 ± 28
TNF	20	1207 ± 20	1329 ± 26

* EC were plated out at confluence (3×10^5 cells/well) in RPMI/20% HS. After an overnight incubation, the EC were washed and reincubated for 24 or 48 hours with the optimal amounts of the indicated cytokines and the EC volumes then determined. In each experiment, the modal EC volume was determined for both untreated (control) EC and cytokine-treated EC. Mean ± SEM of the modal EC volumes for the indicated number of experiments are shown. For each cytokine, the mean of the modal EC volumes was significantly different from the controls ($P < 0.02$).

ulated independently. The onset of increased adhesiveness of EC for T cells before the increase in their rate of protein synthesis also points to the independent nature of these two phenomena. It should also be noted that others have reported that TNF is more potent than LT in stimulating EC adhesiveness for PMN, EC growth factor production[58] and release of IL-1.[59] In addition, Pober et al have reported that LT and TNF have similar effects on the expression of a number of different EC surface antigens, and on EC morphology.[60]

Results of animal studies have suggested that the development of tall EC may be a consequence, and not a cause, of lymphocyte emigration. Thus, Nightingale and Hurley showed that hypertrophic EC developed in granulomas and evolving Peyer's patches subsequent to the appearance of lymphocytic infiltrates.[7] The same conclusion was reached by Freemont and Ford in their kinetic study of the development of BCG-induced granulomas in rats.[11] Also, flattening of EC in lymph node HEV occurs following lymphocyte depleting events such as neonatal thymectomy,[18,21] chronic drainage of the thoracic duct,[18] or interruption of afferent lymphatic vessels.[19,20] The observation by Hendriks et al that intranodal injection of a T cell-dependent antigen (sheep erythrocytes), but not a B cell mitogen (LPS), was sufficient to induce the reappearance of both HEV and immigrating lymphocytes in nodes deprived of their afferent lymphatics[20] was interpreted to suggest that mediators released by stimulated T cells and/or macrophages may activate nearby EC and stimulate their ability to facilitate lymphocyte emigration.[20] Our finding that incubation of EC with LT or TNF led to an increase in EC adhesiveness for lymphocytes before increases in either protein synthesis or cell volume is consistent with all of these observations. It suggests that cytokine-stimulated lymphocyte adhesion is an early event in the development of the tall EC and that the hypertrophy of the cell, which appears to be accompanied by increases in RNA and protein synthesis, occurs later in the process.

From the above results, it would appear that an initial stimulation of EC by cytokines secreted by resident antigen-stimulated macrophages and/or sensitized lymphocytes migrating through the connective tissues, such as IFN-γ, IL-1, TNF, and LT, may cause an early increase in EC adhesiveness for lymphocytes. The subsequent increases in protein synthesis and cell size induced by LT or TNF, but not by IFN-γ or IL-1, may determine whether the initial infiltrate develops into the more densely aggregated lymphocyte-rich areas commonly seen around tall endothelial venules.[7,9–12] The results of these studies suggest that cytokines secreted by cells of the immune system may play an important role in the regulation of lymphocyte traffic at inflammatory sites through their actions on the vascular endothelium.

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