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Regulation of Arterial-Venous Differences in Tumor Necrosis Factor Responsiveness of Endothelial Cells by Anatomic Context

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We analyzed tumor necrosis factor (TNF) responses of human umbilical artery and vein endothelial cells (HUAECs and HUVECs) in organ and cell culture. In organ culture, TNF induced expression of E-selectin, VCAM-1, and ICAM-1 on HUVECs but only ICAM-1 on HUAECs. Activation of nuclear factor- κ B, c-jun, and ATF2 by TNF was comparable in HUAECs and HUVECs, whereas binding of transcription factors and p300 co-activator to the E-selectin enhancer was lower in HUAECs compared to HUVECs. In cell culture, HUAECs rapidly acquired inducible E-selectin and VCAM-1 whereas ICAM-1 inducibility decreased. Culture of HUVECs rapidly decreased TNF responses of all three genes. By 72 hours in cell culture, TNF-treated HUVECs and HUAECs showed comparable adhesion molecule induction and transcription factor binding to the E-selectin enhancer. Freshly isolated HUAECs expressed higher levels of Kruppel-like factor 2 (KLF2) than HUVECs, consistent with greater KLF2 induction by arterial levels of shear stress *in vitro*. KLF2 expression decreased rapidly in both cell types during culture. Transduction of HUVECs with KLF2 reduced TNF-mediated induction of E-selectin and VCAM-1 while increasing ICAM-1 induction and reduced transcription factor/co-activator binding to the E-selectin enhancer. In conclusion, the differential responses of HUAECs and HUVECs to TNF in organ culture correlate with transcription factor/co-activator binding to DNA and converge during cell culture. Flow-induced expression of KLF2 contributes to the *in situ* responses of HUAECs but not of HUVECs. (Am J Pathol 2008, 172:1088–1099; DOI: 10.2353/ajpath.2008.070603)

Endothelial cell (EC) adhesion molecules for leukocytes, such as E-selectin (CD62E), vascular cell adhesion molecule 1 (VCAM-1, CD106), and intercellular adhesion molecule 1 (ICAM-1, CD54), are expressed in response to tumor necrosis factor (TNF) stimulation and play a key role in the development of inflammatory reactions.¹ There are striking differences in adhesion molecule expression on ECs depending on their position within the vascular bed of a particular tissue.^{2,3} In general, the expression of E-selectin and VCAM-1 is highly restricted to postcapillary venules or veins, the usual sites of leukocyte extravasation, whereas ICAM-1 is more widely expressed. These *in situ* differences are preserved on microvascular ECs in organ culture⁴ but often lost in cell culture.⁵ The molecular mechanisms underlying such differences in arterial versus venous ECs responses to TNF are not clear. Recent work from our laboratory has suggested that arterial-venous differences in EC responsiveness are not fixed by cell fate decisions and instead depend on *in situ* context. Specifically, we showed that human umbilical vein endothelial cells (HUVECs) that have been transduced to express the anti-apoptotic protein Bcl-2 will, on implantation into SCID mice, spontaneously organize into a vascular bed with elements that resemble arterioles, capillaries, and venules, all lined by Bcl-2-transduced human ECs. Remarkably, E-selectin and VCAM-1, but not ICAM-1, expression, which were expressed by all Bcl-2-transduced HUVECs in response to TNF in cell culture, is selectively lost in those ECs that line the arteriolar and capillary segments that developed *in vivo*, consistent with reprogramming of venous ECs by their new anatomical context.⁶

Recently it has been reported that Kruppel-like factor 2 (KLF2), a transcription factor that can be induced in HUVECs by sustained shear stress,^{7–9} exerts a suppres-

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sive effect on TNF-mediated adhesion molecule gene expression.^{9,10} Interestingly, transduced KLF2 did not block nuclear factor (NF)- κ B activation, a key step in TNF-induced adhesion molecule expression. Rather, it was proposed, based on adenoviral overexpression, that KLF2 competes with nuclear NF- κ B for binding of histone acetylases, also known as co-activators such as CREB-binding protein (CBP) or p300, thereby reducing chromatin remodeling and transcription.¹⁰ The interaction of NF- κ B with CBP or p300 depends on the phosphorylation of the p65 subunit of the NF- κ B heterodimer but the effects of KLF2 on phosphorylation of NF- κ B were not examined. A more recent study suggested, based on lentiviral transduction, that KLF2 may selectively prevent phosphorylation of ATF2,¹¹ a transcription factor that heterodimerizes with c-jun to create a variant form of AP-1 that promotes E-selectin transcription.¹² This modification is also believed to affect interaction with co-activators rather than binding to DNA. However, the viral transduction systems used in these experiments can cause gene expression markedly exceeding the normal physiological level so that aberrant protein-protein interactions may result. Nevertheless, these results are consistent with other studies demonstrating that chromatin remodeling and selective histone modification, functions performed by co-activators, are important mechanisms for cell-type-specific regulation of E-selectin expression after TNF treatment.^{13,14}

The present study was undertaken to investigate differences in TNF responses between arterial endothelium (HUAECs) and venous endothelium (HUVECs) and to assess the role that KLF2 may play in such differences. Our new observations support the conclusion that differences between arterial and venous ECs are dependent on *in situ* context rather than being fixed by cell fate decisions because both HUAECs and HUVECs rapidly converge toward a common phenotype in cell culture. Our findings are also consistent with the conclusion that shear stress-induced KLF2 expression, an anatomical context-dependent variable, may contribute to the regulation of TNF responses in arterial ECs *in situ*. However, the responses of venous ECs *in situ* also differ from those of cultured cells and that this change in behavior does not correlate with levels of KLF2 expression.

Materials and Methods

Cytokines and Antibodies

The concentration of human recombinant TNF (TNF- α ; R&D Systems, Inc., Minneapolis, MN) used to treat HUAECs or HUVECs *in situ* or in culture was 10 ng/ml unless otherwise noted; this concentration was selected based on pilot experiments as optimal but not excessive for the experimental readouts presented here. Adhesion molecule expression was assessed by immunohistochemistry (IHC) of tissues or by immunofluorescence (IF) and fluorescence-activated cell sorting (FACS) analysis of cultured cells using the following primary antibodies: mouse anti-human E-selectin clone H4/18,¹⁵ mouse anti-human

VCAM-1 (R&D Systems), or mouse anti-human ICAM-1 (R&D Systems). Tissues and cultured cells were also analyzed by IF microscopy using mouse anti-human CD31 (DAKO, Carpinteria, CA), mouse anti-phospho-NF- κ B p65 (Ser 276), mouse anti-phospho-c-jun (Ser 63), mouse anti-phospho-ATF2 (Thr71) (all from Cell Signaling, Danvers, MA). Transduced cells were immunostained for exogenous gene expression after permeabilization using mouse anti-hemagglutinin (HA) (Roche Applied Science, Indianapolis, IN). Secondary antibodies used for IHC were biotinylated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). Secondary antibodies used for IF were Alexa Fluor 488- or 594-conjugated goat anti-mouse IgG1 (Molecular Probes, Eugene, OR). Chromatin immunoprecipitation (ChIP) assays used rabbit anti-NF- κ B p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-c-jun (Cell Signaling), rabbit anti-ATF2 (Cell Signaling), and mouse anti-p300 (Millipore, Billerica, MA) for immunoprecipitation. Mouse anti-TNFR1 (Santa Cruz Biotechnology Inc.) and mouse anti-TNFR2 (Cell Signaling) were used for detecting TNF R1 and R2 by immunoblotting or by FACS.

Human Umbilical Cord Organ Culture

Human umbilical cords from anonymized donors were obtained from the Labor and Delivery Services, Yale New Haven Hospital within 24 hours of delivery, in accordance with a protocol approved by the Yale Human Investigations Committee. In each cord, either an umbilical artery or vein was cannulized, rinsed twice with lactated Ringer's solution, and then filled Medium 199 (M199) containing 20% fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mmol/L L-glutamine (all reagents from Invitrogen, Carlsbad, CA), 50 μ g/ml endothelial cell growth supplement (BD Biosciences, Mountain View, CA), and 100 μ g/ml porcine intestinal heparin (Sigma, St. Louis, MO). The medium was further supplemented with TNF or phosphate-buffered saline (PBS) diluent (as a control) as indicated and subjected to organ culture by incubation for 4 hours immersed in lactated Ringers solution at 37°C. Treated vessels were either prepared directly for immunostaining or used for EC harvest. To harvest cells, the cannulized umbilical artery or vein was washed twice with lactated Ringer's solution and filled with 0.1% collagenase solution (Worthington Biochemical Corporation, Lakewood, NJ). After 10 minutes of incubation at 37°C, detached ECs were eluted into a 50-ml Falcon tube and centrifuged at 1000 $\times g$ for 5 minutes. The cell pellet was resuspended and washed with PBS twice by centrifugation. The final cell pellet was resuspended in a 15-ml Falcon tube and ECs were isolated using Dynabeads CD31 (DynaL Biotech, Oslo, Norway) according to the manufacturer's instructions.

Cell Culture

Human ECs were isolated and cultured from umbilical cords from anonymized donors under protocols ap-

proved by the Yale Human Investigation Committee. ECs were dissociated with collagenase as described above. ECs from either three to five umbilical arteries or from three to five umbilical veins were separately pooled and then cultured on 0.1% gelatin (J. T. Baker, Phillipsburg, NJ)-coated tissue culture plastic (BD Biosciences, Bedford, MA) at 37°C in 5% CO₂-humidified air in M199 containing 20% fetal calf serum, 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml endothelial cell growth supplement, and 100 µg/ml of porcine intestinal heparin. Individual cultures of pooled arterial or venous ECs were harvested at different time points, namely after primary overnight culture (~12 to 16 hours), after first passage, after second passage, after third passage, or after fourth passage. First passage cells were typically analyzed at 72 hours after isolation. The time in culture for later passages varied depending on the growth characteristics of the strains. The culture medium was changed at least 24 hours before any cytokine treatment and TNF or PBS diluent was added directly to the cultures without further change of media for the time specified before harvest and analysis. Note that to obtain a sufficient number of early cultured cells for analysis, each time point is derived from a separate pooled strain. A minimum of four separate pooled HUAEC or HUVEC cultures were analyzed for each time point.

In Vitro Flow Experiments

HUVECs were isolated and cultured as described above, and used at subculture 2 for each experiment. Cells were plated at 70,000 cells/cm² on a 0.1% gelatin-coated 95-cm² circular surface, and 24 hours later exposed to arterial or venous shear stress waveforms (derived from a human abdominal aorta and human saphenous vein, respectively) and in a dynamic flow system for 24 hours. Cells were then lysed with Trizol, and RNA was isolated by ethanol precipitation, DNase treatment, and purification on columns as previously described⁹ before quantization by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

RNA Isolation and Quantitative Real-Time qRT-PCR

To quantify transcript levels, mRNA was isolated from HUAECs or HUVECs after organ culture or cell culture as indicated with MicroPoly(A)Purist kit (Ambion Inc., Austin, TX). cDNA was synthesized from mRNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and amplified and quantitated by using SYBR Green PCR reagents (Bio-Rad) according to the manufacturer's instructions. The primers used in this report are listed in Table 1. The PCR reaction mixture (final volume, 25 µl) contained 5 µl of cDNA, 1 µl of 10 µmol/L forward primer, 1 µl of 10 µmol/L reverse primer, 12.5 µl of PCR 2× SYBR Green SuperMix buffer, and 5.5 µl of H₂O. The PCR reaction was performed in triplicate (three wells of C96-well plate). The reaction was amplified with iCycler iQ Multicolor

Table 1. Sequences of the Primers Used for Real-Time Quantitative RT-PCR and ChIP Analysis

Primers	Sequence
Quantitative RT-PCR	
GAPDH	5'-CAACGGATTGTCGTATTG-3' 5'-GATGACAAGCTTCCCCTTCT-3'
E-selectin	5'-GAGGCCAGTGTATTGTCA-3' 5'-TTTGCCTATTGTTGGGTTCA-3'
VCAM-1	5'-GTTGAAGGATGCGGGAGTAT-3' 5'-TTCATGTTGGCTTTTCTTGC-3'
ICAM-1	5'-AGAGGTTGAACCCACAGTC-3' 5'-TCTGGCTTCGTCAGAATCAC-3'
KLF2	5'-AGCCTTCGGTCTCTTCGAC-3' 5'-TCAGATGCCAACTCTTGGTG-3'
TNFR1	5'-TGAGAGGCCATAGCTGTCTG-3' 5'-GCACTTGGTACAGCAAATCG-3'
TNFR2	5'-CTAGGCCACACCATCTCCTT-3' 5'-GCAGACACAAGACTGGCACT-3'
ChIP quantitative PCR Primer	5'-GGCCTCAGCCGAAGTAGTG-3' 5'-CTGCTGCCTCTGTCTCAGG-3'

real-time PCR detector (Bio-Rad) for 40 cycles with melting at 95°C for 30 seconds, an annealing at 58°C for 1 minute, and extension at 72°C for 1 minute in iCycler iQ PCR 96-well plates (Bio-Rad). A standard curve for each gene was established by the iCycler protocol. According to the standard curve, the absolute copy number of each gene was calculated and normalized to GAPDH to yield a copy number parameter.

Flow Cytometry

For analysis of cell surface proteins by flow cytometry, HUAECs or HUVECs harvested from organ or cell culture were washed twice with Hanks-balanced saline solution minus Ca²⁺ and Mg²⁺ and incubated with trypsin/ethylenediaminetetraacetic acid for 1 minute. Detached cells were collected by centrifugation at 1000 × g for 5 minutes, washed twice with ice-cold PBS containing 1% bovine serum albumin and 0.1% sodium azide [PBS/bovine serum albumin (BSA)], and incubated with either specific primary antibody or isotype controls in PBS/BSA for 1 hour at 4°C. After two further washes cells were incubated with fluorescently labeled secondary antibodies in PBS/BSA for 1 hour at 4°C, then washed twice, and analyzed by flow cytometry using a FACSort instrument and CellQuest 3.3 software (BD Biosciences). For intracellular immunostaining, cells were detached as above and washed twice with ice-cold PBS before fixation with 2% paraformaldehyde for 15 minutes at room temperature. After a further two washes in PBS, cells were permeabilized for 15 minutes at room temperature in PERM buffer (PBS containing 0.1% saponin, 1% fetal bovine serum, and 0.1% sodium azide), washed once in PERM buffer, and incubated with primary antibody or isotype control. Further washes and incubation with secondary antibody, all in PERM buffer, and flow cytometry were performed as above.

Immunoblotting

Cells were washed twice with phosphate-buffered saline and lysed on ice in lysis buffer [50 mmol/L Tris-HCl, pH 7.5, 1% Nonidet P-40 (v/v), 10 mmol/L NaF, 1 mmol/L vanadate, 1 mmol/L phenylmethyl sulfonyl fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin], and lysates were transferred to an Eppendorf tube and rotated for 45 minutes at 4°C. Lysates were Dounce-homogenized (50 strokes), and insoluble material was removed by centrifugation at $12,000 \times g$ for 10 minutes at 4°C and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferring to polyvinylidene difluoride membrane (Bio-Rad). The membrane was washed with washing buffer (PBS with 0.1% Tween 20) once and blocked by 5% PBS/milk for 1 hour at room temperature with shaking. Then the membrane was hybridized with primary antibody at 4°C overnight. After washing twice with washing buffer at room temperature, the bound primary antibody was detected by secondary antibody and visualized by using the SuperSignal West Pico chemiluminescent substrate kit (Pierce Biotechnology, Inc., Rockford, IL). Signals for specific bands of interest (eg, TNFR1 or TNFR2) were assessed by software Quantity One 4.6.5 (Bio-Rad) for densitometry analysis of exposed X-ray film (Eastman-Kodak, Rochester, NY) and normalized for loaded by comparison to the signal for a control protein (GAPDH).

DNA Constructs and Retroviral Transduction

The Phoenix-Ampho packaging cell line for production of high-titer amphotropic retroviruses was obtained from Dr. G. Nolan (Stanford University, Stanford, CA) and cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The human KLF2 cDNA was cloned into a pcDNA3.1D/V5-His-TOPO vector from a cDNA library of cultured HUVECs exposed to laminar shear stress. Then, by enzymatic digestion, the KLF2 cDNA was removed from this plasmid vector and linked to an HA tag sequence (5'-AGCGTAGTCTGGGACGTCGTATGGGTA-3') on its C terminal. The KLF2-HA cDNA was then subcloned into the LZRS-pBMN-Z retroviral vector. The LZRS-pBMN-Z construct was transfected into Phoenix-Ampho cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Puromycin-resistant cells were selected and used to condition M199 (containing 10% fetal calf serum and 2 mmol/L L-glutamine but without endothelial cell growth supplement, heparin, or antibiotics) for HUVEC transduction. Transduction of HUVECs through four to six rounds of viral infections in the presence of polybrene (8 μ g/ml) (Sigma) was accomplished throughout 2 weeks without drug selection as previously described.^{16,17} Using this protocol the percentage of HUVECs expressing transduced genes is routinely >90%. HUVECs were transduced with a retroviral vector encoding LacZ as control. Transduced HUVECs were used for *in vitro* analysis.

Immunostaining of Organ Culture

For IHC analysis, 5- μ m-thick frozen sections were stained as described.¹⁸ In brief, primary antibody reactive with human E-selectin was used first, and then a biotinylated secondary goat anti-mouse antibody was used to detect the primary antibody. An avidin binding complex and a 3-amino-9-ethyl carbazole detection kit were used for color development (Vector Laboratories, Burlingame, CA). To develop brown-color staining, the ABC-horseradish peroxidase Elite reagent (Vector Laboratories) was used. Slides were coverslipped by using Aqua-Mount (Lerner Laboratories, Pittsburgh, PA) and analyzed using an Axioskop2 plus microscope (Carl Zeiss, Inc., Thornwood, NY). For IF analysis, 6- μ m-thick frozen sections of umbilical cords were fixed with 4% paraformaldehyde for 15 minutes at room temperature. For intracellular staining, the sections were then permeabilized by the addition of 0.2% Triton X-100 for 5 minutes at room temperature. A further blocking step was performed before antibody staining by incubating fixed cells with a buffer containing 1% BSA and 5% goat serum for 1 hour at room temperature. Immunostaining was performed by incubating sections with the indicated primary mouse antibodies overnight at 4°C in a buffer containing 1% BSA and 5% goat serum. After washing twice with washing buffer at room temperature, detection of bound primary antibody was visualized by the addition of specific Alexa Fluor 488- or 594-conjugated goat anti-rabbit secondary antibodies for 30 minutes at room temperature.

Chromatin Immunoprecipitation

For ChIP analysis, ECs were treated with 10 ng/ml of TNF for 30 minutes in organ or cell culture. The cells were harvested as above and then cross-linked with 1% formaldehyde at 37°C for 10 minutes. Nuclei were isolated by a nuclear/cytosol fractionation kit (BioVision, Mountain View, CA) and treated with sodium dodecyl sulfate lysis buffer (from a ChIP assay kit, Millipore) for 10 minutes on ice before sonicating with a 12-second sonication pulse from a Sonifier 150 (Branson, Danbury, CT) at the maximum microtip output level. Samples of the sonicated DNAs were evaluated for integrity by reversing the cross-linking and electrophoresis on an agarose gel. The quantity of DNA in each sonicate was assessed with a spectrophotometer (SmartSpec Plus from Bio-Rad) and by real-time PCR. One aliquot of the same amount of DNA from each chromatin preparation was incubated with the indicated specific antibody overnight at 4°C or with no-antibody negative control. Protein A-agarose beads were then added for 1 hour at 4°C with rotation. The collected agarose beads were sequentially washed with low-salt buffer, high-salt buffer, LiCl buffer, and TE buffer before eluting the immunoprecipitate. The cross-linking was then reversed and the recovered DNA was analyzed by real-time PCR using primers listed in Table 1.

Statistics

The data were statistically analyzed in multiple comparisons with Bonferroni correction using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). Differences between groups were considered significant at $P < 0.05$.

Results

Comparison of TNF Responses between HUAECs and HUVECs in Organ Culture

We began our investigation with a comparison of TNF responses in HUAECs and HUVECs, using organ culture to model *in situ* behaviors. Individual vessels in freshly collected cord segments were canulated, flushed with lactated Ringers' solution, and then filled with culture medium supplemented with TNF or PBS diluent control. The cords were maintained at 37°C. After 4 hours of TNF treatment, we could readily observe the induction of E-selectin on the ECs of the umbilical vein, but not on the ECs of umbilical artery by indirect IHC staining (Figure 1).

We then analyzed freshly isolated HUAECs and HUVECs for differences in TNF receptors or signaling components. Both cell types expressed comparable levels of TNF receptor 1 and 2 expression, assessed by qRT-PCR for mRNA (Table 2A) and by immunoblotting for total protein expression (Table 2B). The number of cells collected in these organ culture experiments was too small for measuring surface protein expression by FACS analysis. More extensive surveys of TNF signaling components by qRT-PCR similarly failed to reveal any consistent differences between freshly isolated HUAECs and HUVECs (unpublished observations, M.L.). We next evaluated the TNF-mediated activation of specific transcription factors known to regulate E-selectin in cultured HUVECs, namely NF- κ B^{19–21} and a variant form of AP-1 composed of c-jun and ATF2.^{22,23} To assess the activation of NF- κ B, we performed IF microscopy using an anti-phospho-NF- κ B p65 (Ser276) antibody, which recognizes an activated form of the p65 (Rel A) subunit of this factor. In multiple independent experiments we detected TNF-mediated activation of NF- κ B in the nuclei of both HUAECs and HUVECs and saw no obvious difference in the percentage of positive cells between these

Figure 1. TNF-mediated induction of E-selectin protein on HUAECs and HUVECs in organ culture. Organ cultures of umbilical artery or vein were exposed to PBS diluent control or to TNF for 4 hours, snap-frozen, cryosectioned, and then stained by IHC for E-selectin as described in the Materials and Methods. Note that only TNF-treated venous ECs express E-selectin. Data represent one of four independent experiments with similar results.

Table 2. Expression of TNF Receptor R1 and R2 in ECs

Species	Condition	Cell type	TNFR1	TNFR2
A. mRNA	Organ culture	HUAEC	248.85 ± 21.93	350.82 ± 29.11
		HUVEC	227.58 ± 19.11	103.81 ± 5.07
	Cell culture	HUAEC	112.04 ± 13.29	26.39 ± 6.45
		HUVEC	68.17 ± 6.74	16.10 ± 2.35
	Transduced HUVECs	KLF2	120.77 ± 44.49	16.98 ± 8.93
		LacZ	69.36 ± 25.55	2.83 ± 0.28
B. Total protein	Organ culture	HUAEC	138,340.0	
		HUVEC	116,981.0	
C. Cell surface protein	Cell culture	HUAEC	15.3	24.1
		HUVEC	16.4	21.4
	Transduced HUVECs	KLF2	14.9	25.5
		LacZ	15.1	21.8

A: TNFR1 and TNFR2 transcripts were measured by real-time qRT-PCR and copy numbers normalized to copy numbers of GAPDH ($\times 10^{-4}$) as described in the Materials and Methods. B: TNFR1 expression in organ-cultured HUAECs and HUVECs was measured by immunoblotting and normalized by densitometry to GAPDH. C: TNFR1 and TNFR2 surface protein expression analyzed by FACS. The number indicates the corrected mean fluorescence intensity.

two cell types (Table 3). To assess the activation of c-jun/ATF2, we performed IF microscopy using an anti-phospho-c-jun (Ser63) antibody, which recognizes an activated form of c-jun present in conventional and variant forms of AP-1, or using an anti-phospho-ATF2 antibody. In multiple independent experiments we detected TNF-mediated activation of c-jun in a comparable number of the nuclei of both HUAECs and HUVECs and saw no significant difference in the percentage of positive cells between these two cell types (Table 3). However, there was a small but statistically significant reduction in the activation of ATF2 in HUAECs compared to HUVECs. These observations confirm that HUVECs and HUAECs show dramatic differences in E-selectin inducibility in organ culture, and that this difference is much greater than the small differences in transcription factor activation detected by IF microscopy, although the extent of activation per cell could be underestimated by our approach.

Transcriptional activation of E-selectin not only requires nuclear localization and phosphorylation of the transcriptional activating domains of the relevant factors, but that these factors bind to specific sequences in the E-selectin gene enhancer and that the bound transcription factors then effectively recruit co-activators (ie, his-

tone acetylases) to the enhancer, namely CBP/p300.²⁴ To assess binding of NF- κ B, c-jun, ATF2, and the p300 co-activator to the E-selectin enhancer, we used ChIP assays. As shown in Table 4, there is a large increase in the binding of the p65 (Rel A) subunit of NF- κ B and of ATF2, and a much smaller increase in binding of c-jun to the cytokine regulatory region of the E-selectin enhancer in HUVECs treated with TNF. There is also a large increase in binding of p300 to the E-selectin enhancer in these same cells. Somewhat smaller TNF responses of NF- κ B, ATF2, and c-jun were also observed in HUAECs, but the recruitment of p300 was not detected. In other words, both TNF-induced transcription factor recruitment and especially co-activator recruitment to the E-selectin enhancer are reduced in arterial ECs compared to venous ECs. The all or none differential response of p300 recruitment correlates with the all or none differential response of E-selectin expression.

Comparison of TNF Responses between HUAECs and HUVECs in Cell Culture

Having established that HUAECs and HUVECs differ in the binding of transcription factors and especially co-

Table 3. TNF-Mediated Activation of NF- κ B and AP-1 in ECs

Cells	Activated transcription factors (percent positive nuclei ± SEM)		
	Phospho-p65	Phospho-c-jun	Phospho-ATF2
A. In organ culture			
HUAECs	54.2 ± 3.8	52.3 ± 2.8	53.7 ± 3.6
HUVECs	53.7 ± 2.8	53.5 ± 1.3	62.8 ± 5.6
B. In cell culture			
KLF2-HUVECs	92.5 ± 3.5	89.2 ± 0.3	91.7 ± 2.2
LacZ-HUVECs	93.9 ± 0.2	91.7 ± 0.6	93.3 ± 0.8

A: umbilical cord organ cultures were treated with TNF for 30 minutes and then double stained by IF for activated NF- κ B (phospho-p65) or activated AP-1 (phospho-c-jun) or activated ATF2 (phospho-ATF2) and for CD31. The data represent the percentage of the positive nuclei in CD31-expressing cells. B: As in A except staining was performed on TNF-treated HUVEC transductants in cell culture. Data are pooled for three or four independent experiments in organ and cell culture, respectively. Only ATF2 was significantly ($P < 0.01$) different between HUAECs and HUVECs and no significant differences were observed between KLF2 and LacZ transductants.

Table 4. Chromatin Immunoprecipitation

Factors	Cell source	Determination no.		
		1	2	3
p65	HUAEC organ culture	7.64	1.66	3.91
	HUVEC organ culture	53.82	2.38	9.51
	HUAEC cell culture	16.00	2.46	6.35
	HUVEC cell culture	2.24	1.46	1.82
	KLF2 HUVEC	4.92	1.41	nd
	LacZ HUVEC	11.06	4.19	nd
c-jun	HUAEC organ culture	0.70	1.00	nd
	HUVEC organ culture	1.40	1.22	nd
	HUAEC cell culture	1.93	1.33	nd
	HUVEC cell culture	4.29	5.28	nd
	KLF2 HUVEC	3.32	17.55	nd
	LacZ HUVEC	1.16	2.24	nd
ATF2	HUAEC organ culture	5.92	0.83	0.92
	HUVEC organ culture	14.25	2.52	1.82
	HUAEC cell culture	12.13	8.88	nd
	HUVEC cell culture	6.50	2.38	nd
	KLF2 HUVEC	1.29	0.62	0.30
	LacZ HUVEC	1.87	1.66	1.38
p300	HUAEC organ culture	0.81	0.55	0.93
	HUVEC organ culture	4.81	4.54	11.58
	HUAEC cell culture	2.05	1.91	2.24
	HUVEC cell culture	2.14	1.02	2.30
	KLF2 HUVEC	1.78	0.51	0.46
	LacZ HUVEC	2.7	1.52	1.04

Chromatin samples from freshly isolated artery and vein ECs, cultured ECs after three passages, and LacZ- and KLF2-transduced HUVECs were harvested after stimulation with 10 ng/ml of TNF for 30 minutes or after mock stimulation. The chromatin was precipitated with anti-p65 (p65), anti-c-jun (c-jun), anti-ATF2 (ATF2), and anti-p300 (p300) antibodies. After DNA recovery the precipitates were evaluated by real-time PCR for the level of enrichment over the mock-treated control with primers of promoter regions of E-selectin gene. Each column represents one of three independent experiments with similar outcomes and expressed as a ratio of TNF to mock-treated (control) cells. The various determinations of each factor were performed on different samples. nd, not done.

activator to the E-selectin enhancer in organ culture, we next examined if these differences in TNF responsiveness between HUVECs and HUAECs are stable properties of these two cell types as expected for cell fate-determined responses or are anatomical context-dependent and would be diminished by cell culture. To assess this, we compared the TNF responses of freshly harvested ECs with ECs subjected to cell culture for varying time periods. The experimental groups were: 1) HUAECs or HUVECs within intact vessels treated with culture medium containing 10 ng/ml of human TNF or PBS diluent for 4 hours in organ culture before collagenase harvest and analysis; 2) HUAECs or HUVECs cultured overnight and treated with 10 ng/ml of human TNF or PBS for 4 hours the next morning before trypsin harvest and analysis; 3) HUAECs or HUVECs cultured for 72 hours, during which the primary ECs were passaged once and then treated with 10 ng/ml of TNF or control PBS diluent for 4 hours before trypsin harvest and analysis; and 4) HUAECs or HUVECs cultured for 2 weeks or more, at which time the ECs were treated with 10 ng/ml of TNF or control PBS diluent for 4 hours before trypsin harvest and analysis. Cells were analyzed by real time qRT-PCR and flow cytometry for TNFR and adhesion molecule mRNA and for surface protein expression, respectively. Consistent with the

organ culture immunostaining data, mRNA expression showed that HUAECs treated in organ culture were less responsive to TNF, displaying no significant induction of E-selectin or VCAM-1, but did display induction of ICAM-1 (Figure 2). In contrast, HUVECs in organ culture strongly up-regulated transcripts encoding all three adhesion molecules. HUAECs that were cultured overnight began to show some induction of E-selectin and VCAM-1 in response to TNF and further increased responsiveness after 72 hours of culture. During the same time period, inducibility of ICAM-1 declined. In contrast, HUVECs began to lose responsiveness of all three genes on overnight culture and were even less responsive after 72 hours. TNFR mRNA expression was reduced in both cell types with culture (Table 2A). Cultured HUVEC TNFR mRNA levels were lower than those of HUAECs (Table 2A), but FACS analysis for surface protein expressions was comparable (Table 2C). The patterns of responsiveness to TNF established in cultured HUAECs and HUVECs by 72 hours were primarily unchanged through four passages (data not shown). Statistical analysis confirmed that the increased inducibility of E-selectin and the decreased inducibility of ICAM-1 in response to TNF on HUAECs was significant ($P < 0.05$), and the decreased inducibility of all three adhesion molecules in response to TNF on HUVECs was significant ($P < 0.05$). The change of VCAM-1 inducibility at the mRNA level in HUAECs failed to reach statistical significance because of the higher variability in the VCAM-1 than the E-selectin response of the cultured cells, but showed a general tendency to increase.

These changes observed in adhesion molecule mRNA levels were reflected in the surface protein level assessed by flow cytometry (Figure 3). The striking result is that as a consequence of the opposing changes in the arterial versus venous ECs in culture, HUAECs and HUVECs became essentially indistinguishable in their response to TNF by 72 hours and remained so thereafter. ChIP analyses of serially cultured HUVECs and HUAECs also suggested that organ culture differences in transcription factor binding to the E-selectin promoter also had changed. TNF-induced recruitment of p65 and of ATF2 was actually somewhat higher in arterial ECs whereas c-jun was higher in venous ECs, but both cell types showed TNF responses. TNF-induced binding of p300 was observed but was generally lower in both cultured cell types compared to HUVECs *in situ* consistent with the reduced responsiveness of cultured ECs (Table 4). The alterations in adhesion molecule expression and in transcription factor/co-activator binding observed in ECs upon isolation and cell culture suggested that differences in adhesion molecule inducibility between arterial and venous ECs are most likely imposed by local context and not by irreversible cell fate decisions since both cell types rapidly change their pattern of responses as they move from the vessel wall to cell culture, approaching a common EC culture phenotype.

Figure 2. Comparative analyses of transcript levels in HUAECs and HUVECs. **A–C:** TNF-mediated induction of E-selectin (**A**), VCAM-1 (**B**), and ICAM-1 (**C**) transcripts in HUAECs (striped bars) and HUVECs (stippled bars). Cells were treated with TNF for 4 hours before extraction of RNA. Each bar represents data pooled from a minimum of four independent experiments using freshly harvested ECs, ECs subjected to overnight (O/N) cell culture, or ECs maintained in cell culture for 72 hours. Data show mean $\pm$ SE of transcript copy numbers normalized to copy numbers of GAPDH, measured by real-time qRT-PCR as described in the Materials and Methods. Note that venous ECs show higher levels of all three transcripts and all three transcript levels fall with cell culture. In contrast, arterial ECs show increasing levels of E-selectin and VCAM-1 but declining ICAM-1 transcript levels with cell culture. At 72 hours of cell culture, HUAECs and HUVECs are indistinguishable. *The difference of HUAECs or HUVECs between organ culture condition and their corresponding cell culture condition is statistically significant ($P < 0.05$). **D:** Basal expression of KLF2 transcripts in HUAECs (striped bars) and HUVECs (open bars). Each bar represents data pooled from a minimum of four independent experiments using freshly harvested ECs, ECs subjected to overnight (O/N) cell culture, or ECs maintained in cell culture for 72 hours. Data show mean $\pm$ SE of transcript copy numbers normalized to copy numbers of GAPDH, measured by real-time qRT-PCR as described in the Materials and Methods. Note that HUAECs express much higher levels of KLF2 transcript than HUVECs *in situ*, but that the levels of transcript falls in both cell types as a consequence of cell culture. #The difference between HUAECs and HUVECs in organ culture is statistically significant ($P < 0.05$). *The difference of HUAECs or HUVECs between organ culture condition and their corresponding cell culture condition is statistically significant ($P < 0.05$).

KLF2 Gene Expression and Function in HUAECs and HUVECs

As noted in the Introduction, KLF2 is a shear stress-induced transcription factor that has been implicated in suppressing certain arterial EC responses to TNF.^{9–11} We therefore investigated if differential KLF2 expression

can explain the differences in TNF responses of HUAECs and HUVECs *in situ*. KLF2 gene expression was assessed at the mRNA level by real-time qRT-PCR throughout the time course of culture (Figure 2). KLF2 expression in freshly isolated HUAECs is approximately four times higher than in freshly isolated HUVECs. This difference is consistent with the observation that arterial levels of shear

Figure 3. TNF-mediated induction of E-selectin, VCAM-1, and ICAM-1 on HUAECs and HUVECs in cell culture. Cells were cultured for 2 weeks, subjected to TNF (solid lines) or PBS diluent control (dotted lines) for 4 hours, harvested, stained, and subjected to flow cytometry. Note that basal expression and TNF-induced expression levels are similar between both cell types for all three adhesion molecules. Data represent one of four independent experiments with similar results.

Figure 4. Flow-dependent expression of KLF2. HUVECs were cultured under static (no flow), venous, or arterial flow conditions for 72 hours, and KLF2 mRNA expression was measured by RT-PCR ($n = 3$; mean $\pm$ SD). * $P < 0.05$. Note that arterial flow conditions induce much higher levels of KLF2 expression than do venous flow conditions.

stress induce higher expression of KLF2 in cultured HUVECs than do venous levels of shear stress, but both types of stimuli increase expression above that of cultured HUVECs maintained in static cultures (Figure 4). Furthermore, the level of KLF2 mRNA falls in both types of ECs when freshly isolated cells are placed in cell culture, reaching similar levels of expression by 72 hours (Figure 2). These observations suggest that a decrease of KLF2 expression levels may be correlated with increased responsiveness of HUAECs in culture, but a decrease in KLF2 expression also occurs concomitantly with a reduction of responsiveness in HUVECs in culture.

To better assess whether arterial levels of KLF2 expression do underlie *in situ* arterial responses to TNF, we used retroviral transduction to express genes at physiological levels in cultured HUVECs. Specifically, we prepared HUVEC transductants using LacZ control, and HA-tagged KLF2 transductants. FACS analysis confirmed more than 90% efficiency of transduction (Figure 5A), KLF2 mRNA levels in the KLF2 transduced HUVECs are comparable to that observed in freshly harvested HUAECs (Figure 5B). (We are unable to measure KLF2 protein in freshly isolated cells because of the lack of a suitable antibody.) KLF2 transduction does not reduce the expression of TNFR1 or TNFR2 mRNA or surface protein expression (Table 2, A and C). We then analyzed the TNF responses of our transductants. By IF, there were no statistically significant differences in the TNF-mediated activation of NF- κ B or c-jun and only a small reduction in the activation of ATF2 in KLF2-HUVECs compared to LacZ-HUVECs as shown by nuclear staining for activated factors of these transcription factors (Table 3). These observations confirm and extend a previous report that KLF2 does not suppress the activation of NF- κ B when transduced into HUVECs.¹⁰ Although our data do not exclude an effect of KLF2 on the phosphorylation of ATF2, as reported by others,¹¹ they suggest that such an effect, if it occurs, must be partial. In the same cell

Figure 5. Characterizations of transduced HUVECs. **A:** Expression of transduced proteins in HUVECs assessed by flow cytometry of permeabilized cells. Shown are staining for HA-KLF2 in HUVEC cultures transduced for HA-KLF2 (solid lines). **B:** Comparison of KLF2 transcript levels in freshly isolated HUAECs and HUVECs (left) and in transduced HUVECs (right). Data represent mean $\pm$ SE for KLF2 copy number normalized to GAPDH copy number for four isolates analyzed by real-time qRT-PCR as described in the Materials and Methods. Note that KLF2-transduced HUVECs express comparable transcript levels to freshly isolated HUAECs. *The difference of KLF2 expression levels between freshly isolated arterial and venous ECs or KLF2- and LacZ-transduced HUVECs is statistically significant ($P < 0.05$).

populations, qRT-PCR analysis of KLF2-HUVECs showed that, compared to control LacZ-HUVECs, TNF-induced E-selectin mRNA expression is decreased by $\sim 70\%$, and VCAM-1 mRNA expression is reduced by $\sim 25\%$ after 4 hours of TNF treatment. In contrast, TNF-induced ICAM-1 mRNA expression in KLF2 transductant is increased by 67% over the control cell at the same time of treatment (Table 5). Consistent with these mRNA measurements, flow cytometry measurements of surface protein expression showed that, after 4 hours of TNF treatment, E-selectin expression is decreased by 11 to 29%, VCAM-1 expression is reduced by 12 to 28%, but ICAM-1 expression is increased by 33 to 46% (Figure 6).

Table 5. Effects of KLF2 on Adhesion Molecule Transcript Induction

Transcript	Transductants		<i>P</i> value
	LacZ	KLF2	
E-selectin	0.17 $\pm$ 0.02	0.05 $\pm$ 0.01	<0.05
VCAM1	0.12 $\pm$ 0.01	0.09 $\pm$ 0.01	n.s.
ICAM1	0.06 $\pm$ 0.01	0.10 $\pm$ 0.01	<0.01

Cells were treated with TNF for 4 hours. Transcripts were measured by real-time qRT-PCR and copy numbers normalized to copy numbers of GAPDH ([times]10⁻⁴) as described in the Materials and Methods. Note that KLF2 reduces E-selectin and VCAM-1 but enhances ICAM-1 induction. Data represent mean $\pm$ SEM from three independent experiments. n.s., difference not significant by statistical analysis.

Figure 6. TNF-mediated induction of E-selectin, VCAM-1, and ICAM-1 in transduced HUVECs. HUVEC cultures transduced with Lac Z (control) or KLF2 were treated with TNF (solid line) or PBS diluent (dashed line) for 4 hours, harvested, stained, and subjected to flow cytometry as described in the Materials and Methods. The number indicates the mean fluorescence intensity. Note that KLF2 transduction reduces E-selectin and VCAM-1 expression while increasing ICAM-1 expression. Data represent one of three independent experiments with similar results.

In a final set of experiments, we performed ChIP analyses of the E-selectin promoter to compare TNF responses between KLF2- and LacZ-transduced HUVECs (Table 4). These data indicate that KLF2 expression at arterial levels can reduce TNF-mediated binding of NF- κ B p65 and of ATF2 as well as that of p300 while enhancing that of c-jun to the E-selectin promoter in serially cultured HUVECs, more closely resembling but not fully recapitulating the responses of HUAECs in organ culture. Once again, there is a strong correlation of E-selectin expression with p300 recruitment to the E-selectin enhancer.

Discussion

In this study, we investigated the differential expression of adhesion molecules in ECs of arteries or veins. Using organ culture to model *in situ* responses, we observed that TNF is able to activate NF- κ B and AP-1 in both HUAECs and HUVECs, but only HUVECs display induction of E-selectin and VCAM-1. ICAM-1 is induced in both cell types, but at a higher level in venous than arterial ECs. This difference between venous and arterial ECs disappears as early as 72 hours in cell culture. The equivalence developed because HUAECs gained the responsiveness to TNF whereas HUVECs reduced their potential to respond to TNF as assessed by induction of E-selectin and VCAM-1. Both cell types lost expression of ICAM-1 as a result of cell culture. The inducibility of E-selectin correlates with transcription factor and especially p300 co-activator binding to the enhancer region of the E-selectin gene assessed by ChIP and less well to the activation of transcription factors as assessed by IF. Arterial ECs express higher levels of KLF2 than do venous ECs and a fall in KLF2 expression correlates with the gain in TNF responsiveness seen when arterial ECs are placed in cell culture. However, changes in KLF2 expression do not correlate with the changes in venous ECs that occur in cell culture.

In the present study, we have used two rather atypical neonatal vessels, namely umbilical artery and vein. We made this choice because these vessels are readily available from human discarded tissues and represent

the only human tissue source from which freshly isolated ECs can be obtained in sufficient numbers for ChIP studies. It is worth noting that cultured HUAECs and HUVECs fit into the transcriptional profiling patterns that have been described as defining arterial and venous ECs, respectively.²⁵ More importantly, the organ culture systems used here do show characteristic differences in TNF responsiveness. Studies of pathological inflammation of the human umbilical cord, known as funisitis, have been described as showing restricted involvement of the vein (phlebitis) in mild cases and involvement of the artery (arteritis) in only the more severe cases.^{26,27} Thus, although vessels in distinct anatomical locations may have distinct properties, umbilical vessels are a reasonable choice for comparing the inflammatory responses of artery versus vein as analyzed in this report. Extending these findings to other vascular beds will be necessary to determine how general these findings may be. A second experimental limitation worth noting is that organ culture does not produce conditions identical to the true *in situ* environment. It removes hemodynamic and metabolic variables. Cytokine treatments in organ culture lack vectorial properties, ie, whether the cell sees cytokine on its luminal or abluminal surface. The rationale for using organ culture is that it preserves some of the anatomical context lost in cell culture systems, and that the effects on cell behavior caused by removing a blood vessel cell from its *in vivo* setting may decay slowly, allowing fresh tissues to better recapitulate true *in situ* behaviors. Both points are supported by our empirical observations about the gradual approximation of HUAECs and HUVECs to a common phenotype throughout a mere 3 days of cell culture.

Our data favor the interpretation that the differences in TNF responses observed *in situ* between arterial and venous ECs are primarily determined by the microenvironmental context in which these cells are located rather than determined by cell fate decisions that likely arise from epigenetic changes. Epigenetic changes in differentiated cells are generally stable, which is why primary cultures of different cell types retain many of the phenotypic features that the cell type of origin displayed *in situ*. Most epigenetic changes occur during embryonic devel-

opment. For example, studies in zebra fish have suggested that arterial-venous cell fate decisions are made early in development, before the establishment of blood flow, and arise from proximity to other structures, such as the notochord.²⁸ Arteries and veins are structurally different vessels and are functionally defined by the direction of blood flow that they carry. In general, arterial ECs are exposed to significantly greater levels of shear stress, stretch, and pressure. In many vessels, arterial blood is more oxygenated than venous blood, but this is reversed in umbilical vessels during fetal development, when gas and nutrition exchange occurs in the placenta. In some tissues, arterial and venous ECs are also seated on morphologically distinct basement membranes and are surrounded by differently organized smooth muscle cells.²⁹ In other words, these cell types are exposed to a distinctly different *in situ* context. KLF2 is a transcription factor recently discovered as a shear stress-inducible suppressor of adhesion molecule expression.⁷ We found that KLF2, which was initially expressed at substantially higher levels in HUAECs than HUVECs, fell in both cell types during the transition to culture. Retroviral transduction of KLF2 in cultured HUVECs reduced responses of E-selectin and VCAM-1 but increased that of ICAM-1. It also affected TNF-induced binding of transcription factors and co-activator to the E-selectin promoter, making transduced HUVECs appear more like HUAECs with respect to the responses studied here. Based on these data, we conclude that KLF2 likely contributes to the arterial endothelial pattern of TNF responses *in situ*. It is of note that the pattern of HUAEC responses to TNF was not completely recapitulated in cultured cells by transduction of HUVECs to produce arterial levels of KLF2 expression. For example, ATF2 activation did not appear to be inhibited when assessed as percent positive nuclear staining for phosphorylated ATF2, although we cannot exclude a quantitative decrease in the level per cell. This raises the possibility that other factors are also involved. Although we did match mRNA expression levels to be found in freshly isolated HUAECs, it is quite possible that the time lapsed between the termination of circulation through the umbilical artery (ie, birth) and the time of cell harvest leads to a fall of KLF2 expression. If this speculation is true, then it also remains possible that yet higher levels of exogenous KLF2 could more fully recapitulate arterial EC behaviors, although such levels cannot be achieved with retroviral vectors. It is also unclear if the differences in KLF2 expression observed in large vessels also occur in the microvasculature where arterial and venous differences in TNF responses were first observed. The lack of useful antibodies makes this determination difficult, but preliminary *in situ* hybridization studies have revealed heterogeneity of KLF2 expression in the microvessels of mouse heart (G. Villarreal and G. Garcia-Cardena, unpublished observations). Studies are in progress to determine whether this heterogeneous expression correlates with arteriolar-venular differences.

The mechanisms by which KLF2 inhibits the induction of E-selectin and VCAM-1 but enhances ICAM-1 induction in arterial ECs are unclear. Transcription of all three genes depends on the activation of NF- κ B. NF- κ B tran-

scriptional activity in ECs depends on chromatin remodeling mediated by recruitment of histone acetylases such as p300/CBP.²¹ The recruitment of these enzymes depends on the phosphorylation of Rel A (p65 subunit of NF- κ B) at Ser residue 276.^{30,31} As we have shown, NF- κ B phosphorylation at this residue occurs in HUAECs in organ culture and is not inhibited by KLF2 transduction. It has been proposed that KLF2 can bind p300 and compete with phosphorylated NF- κ B p65 antagonizing NF- κ B transcriptional activity.^{8,10,32} Our ChIP data are not fully consistent with this proposal, because a putative competition for histone acetylases does not explain why NF- κ B binding to the E-selectin enhancer is also reduced in arterial versus venous ECs in organ culture or in KLF2 transductants. However, the difference between HUAECs and HUVECs *in situ* in the TNF-induced binding of p300 does appear more profound than the difference in NF- κ B binding to the E-selectin enhancer. Inhibition of NF- κ B recruitment of p300 by KLF2 also cannot explain why ICAM-1 transcription is enhanced as a result of KLF2 transduction. A more recent study has suggested that KLF2 may selectively interfere with the phosphorylation of ATF2.¹¹ By means of IF analysis, we see only a small KLF2 effect on the percentage of cells that show phospho-ATF2 nuclear staining in response to TNF. This does not preclude the possibility that fewer copies of the factor are activated per cell or that some other change involved in transcriptional activity is being targeted. It is not clear how nuclear localization correlates with the activity of ATF2. However, our ChIP data also show an effect at the level of ATF2 binding to DNA, which should not be affected by ATF2 phosphorylation. It is also unclear if phosphorylation of ATF2 actually affects E-selectin transcription as overexpression of a truncated version of ATF2 lacking the N-terminal sites for phosphorylation of this factor did not inhibit TNF-induced E-selectin expression whereas a nonphosphorylated form of c-jun was profoundly inhibitory.³³

A significant new question raised by our study is what accounts for the high level of TNF responsiveness manifested by HUVECs in organ culture compared to cell culture? As we have noted, HUVECs, unlike other venous systems, actually see oxygenated, nutrient-rich blood cleansed of waste and CO₂ by exchange within the placenta, so the metabolite levels in the blood are artery-like and unlikely to contribute to a venous phenotype in this case. HUVECs, like other venous ECs, do see limited shear force and this may account for the low level of KLF2 expression noted in freshly isolated HUVECs that is lost in cell culture. However, in contrast to HUAECs, HUVECs show decreased TNF responsiveness in culture as KLF2 levels fall. Thus, repression by KLF2 cannot explain the decline in the level of induced adhesion molecule expression noted on venous ECs as they are subjected to cell culture. Instead, we hypothesize that other microenvironmental cues of the vein contribute to EC behavior *in situ*. Previous analysis of skin microvessels revealed that the basement membrane of venular ECs in skin have a distinct appearance, showing a lamellated structure lacking in arteriolar and capillary ECs.²⁹ Interestingly, in psoriatic skin, the capillary loops within the dermal papillae be-

come venularized, acquiring both a venule-like basement membrane and a venule-like responsiveness to TNF.⁵ It is thus possible that a basement membrane component contributes to the capacity of venous ECs to respond to TNF, and that this stimulus is lost when venous ECs are detached during collagenase treatment. Such a regulator could be produced by the ECs themselves or by a supporting mural cell. Little is known about differences in the structure or composition of basement membrane in umbilical artery versus vein and further investigation will be needed to prove or disprove this model.

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