Heterogeneity in Endothelial Responsiveness to Cytokines, Molecular Causes, and Pharmacological Consequences

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ABSTRACT

Microvascular endothelial cells play an essential role in inflammatory diseases. Functional heterogeneity between microvascular segments in normal organ homeostasis has been appreciated for a long time, and more recent studies have revealed heterogeneity in endothelial reactivity to inflammatory stimuli as well. This review summarizes the state-of-the-art knowledge regarding endothelial cell responses to the proinflammatory cytokines tumor necrosis factor α, interleukin-1β, and the bacterial product lipopolysaccharide. It focuses on similarities and differences in reactivity between endothelial cell subsets in vitro and endothelial cells in their pathophysiological environment in vivo, and culminates into a mainly theoretical model of possible intracellular control mechanisms that can assist to ultimately explain the molecular causes of endothelial heterogeneity. The last part of this review contains some pharmacological considerations, and, with the aim to further unravel the molecular basis of in vivo endothelial heterogeneity, descriptions of new techniques that will be essential to achieve this.

KEYWORDS: Inflammation, microvascular endothelial cells, endothelial heterogeneity, gene expression control, pharmacology

The vasculature consists of large (conduit) arteries and veins, smaller arterioles and venules, and capillaries, each of them exerting specific functions in controlling whole-body homeostasis and hemostasis. Whereas the various vascular segments differ in architecture and functionality, they all share the fact that endothelial cells (EC) line the inner walls, directly interact with blood and its constituents, and actively engage in pathophysiological processes.

With advancements in cellular, biochemical, and molecular techniques, we have been able to decipher in greater detail the molecular basis of endothelial behavior in health and disease. The general concept describes endothelial cells to actively engage in inflammatory diseases by recruitment of leukocytes from the blood via the production of thrombotic factors, cytokines, chemokines, adhesion molecules, and other proteins. The aim of the current review is to provide an overview of what we have learned in the last decades from in vitro and in vivo studies regarding endothelial cell responsiveness to proinflammatory cytokines, with emphasis on tumor necrosis factor (TNF)α, interleukin (IL)-1β, and

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the bacterial product lipopolysaccharide (LPS). Both intracellular signaling pathways and effects on gene expression and cell behavior are discussed. Next, focus is on the origin of the endothelial cell in relation to its responsiveness and putative molecular mechanisms underlying the heterogenic behavior of different endothelial subsets. The pharmacological consequences of endothelial heterogeneity is briefly addressed because it not only may pose a threat to the one-drug-fits-all concept but also provides a unique opportunity for selective delivery of drugs into diseased endothelial cells using targeted drug delivery strategies. New technological advances and research tools, partly available and partly under development, conclude this review. They are expected to assist in revealing the bigger molecular map that underlies endothelial heterogeneity.

ENDOTHELIAL CELL FUNCTION IN INFLAMMATORY REACTIONS

Leukocyte recruitment is a process that should be executed in any organ upon demand in response to invading organisms or damage. In almost all organs, the preferred site for leukocyte transmigration into the underlying tissue upon an inflammatory challenge is the postcapillary venules, in which the endothelial cells form tight junctions between each other.[1] By this means, a local immune response can be mounted without directly compromising capillary function as part of organ homeostasis. The dimensions of both leukocytes and these first segments of the postcapillary venules furthermore force both cell types to physically interact, allowing efficient intercellular communication. Moreover, vessel wall architecture driven preferential leukocyte transmigration may also play a role, as has been described for neutrophils.[2]

Upon an inflammatory insult, one of the first reactions of the endothelial cells is to exocytose the stored, ready-to-release contents of Weibel-Palade bodies, including the blood coagulation factor von Willebrand factor (VWF) and the adhesion molecule P-selectin.[3] By this means, a rapid interaction among the activated endothelium, platelets, and neutrophils is created that facilitates leukocyte rolling.[4] Directly afterward, the endothelium produces E-selectin, which interacts with the tetrasaccharide sialyl-Lewis X expressed on immune cells, leading to rolling adherence of leukocytes to the endothelium. E-selectin is also implicated in the generation of activated integrin microdomains at the leading edge of neutrophils via E-selectin ligand-1, as a consequence of which erythrocytes and platelets can be captured to create additional inflammatory damage.[5] Firm arrest of leukocytes to the endothelium is next facilitated by adhesion molecules of the immunoglobulin superfamily such as vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, and local production of chemokines and cytokines. The latter molecules rapidly induce integrin activation on the leukocytes via binding to G-protein coupled receptors, prostaglandin, and nitric oxide (NO) production, and actin filament rearrangement leading to permeability changes in the endothelial compartment.[6] The last step is leukocyte transendothelial migration, which is triggered by endothelial integrin adhesion molecule engagement and clustering that affects an array of downstream effector molecules, as recently reviewed by Wittchen.[7]

Because proinflammatory cytokines TNFα, IL-1β, and LPS are best studied for their effects on endothelial cells, these are the main activators discussed in more detail here. Emphasis is on their molecular effects on the endothelium and on our current knowledge regarding endothelial responsiveness in relation to the vascular origin of the endothelial cells.

EFFECTS OF TUMOR NECROSIS FACTOR α ON ENDOTHELIAL CELLS

Human umbilical vein endothelial cells (HUVEC) express both TNFα-receptors TNFR1 (CD120a, p55) and TNFR2 (CD120b, p75), with TNFR1 more abundant than TNFR2. Because TNFR2 protein is located primarily on the cell membrane whereas TNFR1 mainly localizes to the Golgi apparatus,[8] TNFR2-mediated signal transduction would be the preferred route to direct the signal into the endothelial cells. In the intact organ, however, the localization and expression patterns of the receptors were shown to deviate significantly from those observed in vitro. For example, in normal human kidney biopsies, TNFR1 is strongly expressed by the glomerular endothelium, and TNFR2 is profoundly absent from the vascular compartment.[9] In contrast, in normal human myocardium, both TNFR1 and TNFR2 mRNA and protein localize to the vascular endothelial cells.[10] Rat and human lung follow the kidney profile in that TNFR1 is prominently present in the (larger) vessel endothelial cells, and TNFR2 is much less expressed.[11] TNFR1 therefore seems to be the most likely receptor for TNFα to interact with, although a role for TNFR2 in TNFα-induced endothelial cell activation does exist. This was demonstrated in a series of experiments in which aortic endothelial cells from different TNFR knockout mice and blood vessels in the cremaster muscle in vivo were challenged by TNFα.[12]

Endothelial cell exposure to TNFα activates intracellular signaling cascades involving NFκB, the mitogen-activated protein kinases (MAPK) p38, JNK or c-Jun N-terminal kinase, extracellular signal-regulated kinases ERK1/2, and PI3 kinase,[13–17] with reactive oxygen species and ceramide-based lipid second messengers also contributing to the signaling.[18,19] Because both TNFα receptors do not have intrinsic kinase activity, adapter proteins TRAF2 (TNF receptor–associated

factor 2), RIP1 (receptor interacting protein 1) and TRADD (TNF receptor–associated death domain) are recruited upon TNFα binding to relay the signal into the cell's interior.[6] Recently, Pincheira and colleagues reported on the association of TNFR1 with nonreceptor tyrosine kinase proteins Jak and c-Src that function as adapters to direct the message into specific kinase routes. TNFR1/c-Src played a role in activating Akt and NFκB, but not JNK or p38 MAPK; TNFR1/Jak2 relayed the signal to activate p38 MAPK, JNK, Akt, as well as signal transducer and activator or transcription (STAT)-3.[20] Whether such adapter protein-based guidance of signals into specific kinase pathways also takes place in endothelial cells, or in subsets of endothelial cells in different vascular segments, is not known.

Table 1 provides an overview of in vitro studies reporting on TNFα effects on endothelial cells from different vascular sources and the molecular mechanisms involved. Besides affecting expression of adhesion molecules, chemokines and cytokines, and various other proteins involved in cell survival and feedback mechanisms that inhibit intracellular signaling,[21] TNFα activation also affects endothelial permeability. This is partly attributed to the loss of VE-cadherin from the cell membrane in response to TNFα exposure. VE-cadherin is an endothelial-restricted adhesion molecule present in adherent junctions that through its cytoplasmic tail anchors to actin microfilaments via cytoskeletal proteins.[22] Enhanced phosphorylation of VE-cadherin, mediated for example by intracellular oxidant production by NADPHoxidase or by inhibition of phosphatases, and enhanced VE-cadherin internalization and cleavage from the cell membrane can all contribute to this process.[23,24] p38 MAPK, not ERK activation, is in part responsible for the TNFα-induced VE-cadherin loss.[25,26] It is interesting to note that in adult mice, only lung and uterus, and to a lesser extent, ovary vasculature constitutively express low levels of phosphorylated VE-cadherin.[27] These endothelial subsets may experience a microenvironment that contains specific factors that facilitate continuous activation, the functional consequences of which are not known. Another mechanism of enhanced permeability was described in calf pulmonary artery endothelial cells, in which an increase in the rate of α5β1 integrin internalization and recycling to the membrane was observed after TNFα exposure. This modifies cell-matrix interactions and as such can affect monolayer integrity.[28]

INTERLEUKIN-1 EFFECTS ON ENDOTHELIAL CELLS

IL-1 has a lot in common with TNFα with regard to the transcriptional profile launched in endothelial cells, although the upstream signaling pathways are partly dissimilar. IL-1 consists of two major forms, IL-1α and IL-1β, both able to bind IL-1 receptor type I and II. IL-1RI activation leads to IL-1 receptor accessory protein, MyD88, IRAK, and TRAF6 recruitment to form a receptor complex. As a consequence, NFκB becomes activated, as shown in a variety of endothelial cell studies in vitro as well as in vivo in blood vessels of the rat brain exposed to rat IL-1β.[51] Furthermore, p38 MAPK, ERK1/ERK2, AP-1, ATF-2, PKC, and phospholipase A2 become activated by IL-1RI signal transduction.[14,41,52–54] Activation of the PI3K/Akt pathway furthermore inhibits apoptosis, similar to the effect induced by TNFα stimulation.[16]

Molecules reported to be induced by endothelial cells upon IL-1 exposure include E-selectin, VCAM-1 and ICAM-1, MCP-1 (monocyte chemotactic protein 1), IL-8, COX-2 (cyclooxygenase-2), iNOS (inducible NO synthase), and HO-1 (hemoxygenase 1). In a recent study, Williams and colleagues employed microarray analysis to determine the effects brought about in HUVEC by incubation with 0.1 ng/mL IL-1β. Some 2500 genes were differentially expressed. Most were functionally grouped according to gene ontology to apoptosis, cell cycle, NFκB signaling, chemotaxis, and immune response–related events.[55] Of note is the observation that transmigration of PMN only led to a relatively small perturbation of endothelial cells when compared with the effects of proinflammatory cytokine activation. A direct comparison between TNFα and IL-1 activation of HUVEC using a 4000 gene array revealed 58 and 33 genes, respectively, to be significantly regulated, 25 of which were shared between both activators.[56]

These and other studies confirmed that both cytokines, produced early in an inflammatory reaction, show considerable overlap in the nature of endothelial cell activation, although also some differences have been reported. For example, human dermal microvascular endothelial cells only expressed VCAM-1 in response to TNFα, not IL-1. This was related to a dermal microvascular endothelial cell–type specific upstream VCAM-1 gene regulatory region that functions as a transcriptional repressor.[57] In a rat pulmonary artery endothelial cell line, the production of the antioxidant manganese superoxide dismutase (MnSOD) was shown to be regulated differently in response to the two cytokines. Whereas TNFα induced this enzyme via a phospholipase A2 and reactive oxygen species (ROS)-dependent mitochondrial pathway, IL-1β activation led to NFκB-dependent expression of MnSOD.[58] The extent of gene expression induction by TNFα and IL-1β can also be different, as demonstrated in our laboratory. Exposure of HUVEC to TNFα induced adhesion molecule expression to a higher level than exposure to IL-1β, whereas the latter cytokine affected COX-2, IL-6, and IL-8 mRNA levels more extensively.[59] In human pulmonary artery endothelial cells, a similar preference of COX-2 expression upon IL-1β activation was reported. Whereas IL-1β exposure directly activated p38 MAPK, which was

Table 1 Overview of in Vitro Studies addressing the Cellular Consequences of Exposure of Endothelial Cells from Different Sources to Tumor Necrosis Factor α

Cell Type	TNFα Concentration (ng/mL)	Exposure Time	Major Outcome	Drugs/Inhibitors of Pathways Used	Reference
HUVEC	1–30	1 h	Nur77 expression is induced by TNFα Nur77 directly activates IκBα promoter and gene expression, which attenuates NFκB activation	Ad-dn Nur77	You et al[29]
HUVEC HUAEC	10	4 h	E-selectin, VCAM-1, and ICAM-1 in umbilical cord are more extensively expressed by venous than artery endothelium upon TNFα activation in situ No major differences in NFκB, c-jun, and ATF2 activity was observed, but binding of transcription factors and cp300 coactivator to E-selectin enhancer was lower in HUAEC	—	Liu et al[30]
BAEC HUVEC	2	90 min–3 h	TNFα induces homeobox transcription factor HOXA9, which controls E-selectin expression	—	Bandyopadhyay et al[31]
HUVEC BAEC Mouse EC	5	16 h	HOXA9 inhibits TNFα-induced E-selectin, VCAM-1, and ICAM-1 expression HOXA9 interferes with NFκB-DNA binding HOXA9 expression induces HDAC activity, and Trichostatin rescues HOXA9-mediated NFκB inhibition	HDAC inhibitor Trichostatin	Trivedi et al[32]
HUVEC	0.05–0.02	4 h	2000 ng/mL Ang2 sensitizes EC to low concentration TNFα Both VCAM-1 and ICAM-1 expression are increased by coincubation of Ang2 and TNFα	—	Fiedler et al[33]
HUVEC	50	1–60 min	TNFα-induced expression of eNOS requires Akt This is mediated by neutral sphingomyelinase-2 and sphingosine kinase-1 that generates sphingosine-1-phosphate NO generated via this mechanism inhibits expression of E-selectin	Pertussis toxin (PTx–Gi-coupled receptor inhibitor), LY294002 and wortmannin (PI3K inhibitors)	De Palma et al[19]
HUVEC	10	90 min–6 h	COX-2 expression is induced by TNFα via ROS and p38 MAPK PI3K activation plays basal regulatory role in COX-2 synthesis in HUVEC	NAC, RO31–8220 (PKC inhibitor), fluvastatin (HMGCo reductase inhibitor), p38 MAPK resp. MEK1/ERK inhibitors SB203580 and PD98059, wortmannin	Eligini et al[34]
HUVEC BAEC	10	0.5–4 min–15 h	TNFα inhibits KLF2 expression This is controlled via activation of NFκB and HDAC4 that inhibit MEF2 factors to induce KLF2 expression	Trichostatin (HDAC inhibitor)	Kumar et al[35]
BBMEC	0.1–100	30 min–12 h	TNFα-induced RelA NFκB activation is associated with permeability increase and PGE_2 production	NFκB inhibitors BAY11–7085, CAPE, and PDTC; lactacystin (proteasome inhibitor)	Trickler et al[36]

Table 1 (*Continued*)

Cell Type	TNFα Concentration (ng/mL)	Exposure Time	Major Outcome	Drugs/Inhibitors of Pathways Used	Reference
HUVEC	2	30 min–5 h–16 h	58 of 13,000 genes are upregulated by NFκB and encode proinflammatory, antiapoptotic, and NFκB inhibitory genes Only 13 genes are under the control of p38 MAPK	P38 MAPK inhibitor, SB202190, retrovirus encoding dn IKK2	Viemann et al[21]
HUVEC b.End	5	10 min–6 h	TNFα induces PI3Kδ with downstream kinases Akt and PDK1	PI3Kδ inhibitor IC87114	Puri et al[37]
HAEC HMEC	100 U/mL	4–16 h	TNFα induces cell adhesion molecule expression via Rac1-facilitated, NFκB-mediated transactivation Superoxide production partially affects CAM expression	Ad.N17Rac1 encoding dn Rac1	Chen et al[18]
Human iliac and renal artery EC, BAEC	40	20 min–20 h	Proteasome inhibition blocked TNFα-induced NFκB activation, with consequent inhibition of MCP-1 but enhanced IL-8 expression Proteasome inhibition led to functional AP-1 induction via enhanced c-jun phosphorylation that can transactivate IL-8 gene expression	MG-132 and lactacystin	Hipp et al[38]
BAEC	10	5–60 min/6–20 h	TNFα induced activation of MEK1 and Akt protects cells from apoptosis	MEK1 inhibitor U0126, PI3K inhibitor LY294002	Zhang et al[17]
HUVEC	10	5–60 min	Pre-exposing cells to flow for 10 min inhibited TNFα-induced JNK activation, not ERK1/2 and p38 MAPK Inhibition of JNK is mediated via ERK1/2	PD98059	Surapisitchat et al[15]
HUVEC ECV304	20	15 min–16 h	Ephrin A1 induction by TNFα is controlled via p38 MAPK and JNK, not via ERK1/2 and NFκB	AdmIκBα, Ad.TRAF2.DN, JNK inhibitor DMAP, SB20358, PD98059	Cheng and Chen[39]
HPAEC	100 U/mL	60 min	Critical role for PKC-ζ in TNFα oxidant generation and NFκB activation leading to ICAM-1 expression	Antisense PKC-ζ, PKC-ζ mutants, calphostin, staurosporin	Rahman et al[40]
HUVEC	10–50	5–60 min/18 h	TNFα activates antiapoptotic kinase Akt via PI3K pathway Akt does not affect NFκB pathway but inhibits MAPK pathway	LY294002 and wortmannin	Madge and Pober[16]
HUVEC	500 U/mL	4 h	TNFα induces HO-1 gene expression via PKC phosphorylation, phospholipase A2 (PLA2) activation, and oxidant generation	PLA2 inhibitor Mepacrine, Ca-chelator BAPTA-AM, Ca-ionophore A23–187	Terry et al[41]
HPAEC	100	5–60 min/24 h	TNFα induces IL-8 gene expression via p38 MAPK	SB203580	Hashimoto et al[42]
HUVEC	1–5	10–240 min/12 h	TNFα activates ERK, p38, and JNK MAPKs Raf/MEK/ERK and JNK have no effect on MCP-1 expression; MKK6/p38 MAPK did have an effect	SB203580 and SB202190, PD98059, dominant negative kinase mutants	Goebeler et al[43]

Table 1 (*Continued*)

Cell Type	TNFα Concentration (ng/mL)	Exposure Time	Major Outcome	Drugs/Inhibitors of Pathways Used	Reference
HPAEC	100 U/mL	1–8 h	TNFα induces NFκB activation and resulting E-selectin expression by a pathway that involves ROS production	NAC and PDTC	Rahman et al[44]
HUVEC	100 U/mL	5–120 min	p42 MAPK is activated by TNFα PKC is not involved in this activation Unknown upstream protein tyrosine kinase is involved	PKC inhibitor Ro-31–8220, broad protein tyrosine kinase inhibitors genistein and dadzein	May et al[14]
HMEC-1	100 U/mL	15–120 min	c-fos and c-jun are rapidly and transiently induced by TNFα AP-1 proteins mediate effects of TNFα on VCAM-1 expression via interaction with NFκB factors	AP-1 inhibitors curcumin and NDGA	Ahmad et al[45]
PAEC	5	10–120 min'	Arachidonic acid (AA) blocks degradation of IκB by inhibition of IκB phosphorylation and degradation TNFα-induced E-selectin, ICAM-1, and IL-8 gene expression are concurrently inhibited	AA and stable AA analogue ETYA	Stuhlmeier et al[46]
HSVEC	1000 U/mL	15 min/2–24 h	NO inhibits TNFα-induced VCAM-1 expression NO inhibits NFκB activation; augments IκBα resynthesis and nuclear translocation	NO donors	Spiecker et al[47]
HUVEC	100 U/mL	10 min–6 h	TNFα activates MAPKAPK2 and Hsp27 via p38 MAPK VCAM-1 expression is posttranscriptionally controlled	SB203580	Pietersma et al[48]
HUVEC	0.3–100	4–24 h	Tissue Factor induces tissue factor, E-selectin, ICAM-1, IL-8 expression Effect is not exclusively attributable to TNFR1	TNFR1- and TNFR2-specific antibodies	Paleolog et al[49] Leeuwenberg et al[50]

*The table covers a limited selection of studies with the aim to provide an overview of the diversity of molecular processes being activated upon TNFα-induced endothelial activation, and of the (chemical and biological) compounds that have been used to demonstrate specificity of the processes involved.
Ad, adenovirus; BAEC, bovine carotid artery resp. bovine aortic endothelial cells; BBMEC, bovine brain microvessel endothelial cells; CAPE, caffeic acid phenethyl ester; Dn, dominant negative; HAEC, human aortic endothelial cells; HDAC, histone deacetylase; HMEC-1, immortalized human dermal microvascular endothelial cells; HPAEC, human pulmonary artery endothelial cells; HSVEC, human saphenous vein endothelial cells; HUAEC, human umbilical artery endothelial cells; HUVEC, human umbilical vein endothelial cells; NAC, N-acetyl-L-cysteine; NDGA, nordihydroguatiaretic acid; PAEC, porcine aortic endothelial cells; PDTC, pyrollidine dithiocarbamate; PKC, protein kinase C; ROS, reactive oxygen species.

sufficient to induce the expression of this cyclooxygenase, TNFα was unable to do so on its own.[60]

LIPOPOLYSACCHARIDE EFFECTS ON ENDOTHELIAL SIGNAL TRANSDUCTION

LPS is one of the main components of the cell wall of gram-negative bacteria. In endothelial cells, LPS signals mainly via one of the family members of the toll-like pattern recognition receptors, toll-like receptor (TLR)-4. For this interaction, LPS binding to LPS binding protein (LBP) and subsequent formation of a complex with LPS receptor CD14 is a prerequisite. This facilitates transfer of LPS to the LPS receptor complex that consists of TLR4 and MD2. Because endothelial cells lack the GPI-anchored membrane version of the CD14 molecule, they rely on soluble CD14 for complex formation and subsequent intracellular signaling.[61] The observation that numerous endothelial cells in vitro are responsive to LPS indicates that these cells are likely equipped with the required receptors. As for TNFα and IL-1 receptors, neither of them are restricted to the endothelial compartment.

Endothelial cell exposure to LPS leads to activation of NFκB via PI3K/Akt, and of the MAPKs p38, JNK, and ERK1/2, and they are mediated via adapter proteins that include MyD88, IRAK-1 and -2, and TRAF6 that interact with the intracellular domain of TLR4.[61–65] Induced expression of FLICE-like inhibitory protein (FLIP) and the antiapoptotic proteins A1 and A20 have a role in protecting endothelial cells from LPS-induced apoptosis as well as in creating a suppressive effect on LPS-induced NFκB activation.[66,67] For an overview on the more detailed molecular interactions of these signaling pathways, the reader is referred to two excellent reviews.[61,65]

The functional consequences of LPS-mediated endothelial cell activation are widespread and include engagement in leukocyte recruitment, expression of procoagulant activity, and endothelial sprouting.[68] Exposure of rat thoracic aorta endothelium to LPS for short periods (6 hours) or longer periods (72 hours) regulated metabolic pathways, genes related to proliferation, atherogenesis, inflammation, and NFκB-mediated apoptosis, as well as antiapoptosis and anti-inflammatory and antioxidation genes.[69] Furthermore, LPS exposure induced loss of endothelial barrier function in human dermal microvascular endothelial cells. This was associated with fragmentation of VE-cadherin and loss of claudin 5, and related to a decrease in cAMP levels.[70]

ENDOTHELIAL HETEROGENEITY IN REACTION TO INFLAMMATORY ACTIVATION IN VITRO

To better understand how vascular origin relates to endothelial responsiveness to cytokines and LPS, one could subject the vast number of in vitro studies reported in the literature to a detailed comparison. Several obstacles complicate such a comparison, and include the often limited information provided on cell confluency status and passage number used in the experiments. Moreover, when using endothelial cell lines such as mouse brain endothelial cells b.End3,[71] mouse heart H5V,[72] human umbilical cord vein derived ECV304, EVLC2,[73] and Ea.hy926,[74] human dermal microvascular derived HMEC-1,[75] and hTERT based cell lines,[76] the extensive passage of the cells may have significantly altered the molecular basis of their behavior. An example of this was provided by Viemann and colleagues, who compared TNFα responsiveness of HUVEC and HMEC-1.[77] The upregulated genes in HUVEC that did not respond in HMEC-1 mainly included those encoding for adhesion molecules, cytokines, and chemokines, molecules expected to be regulated in endothelial cells in proinflammatory conditions. In contrast, in the HMEC-1 cell line the differentially upregulated genes represented signaling, transcription factor, apoptosis, and proliferation-associated genes. Another factor that complicates direct comparison of published studies is that readout technology such as real-time transcriptase polymerase chain reaction (RT-PCR), fluorescent-activated cell sorting (FACS) analyses, Western blotting, and array-based assays are often executed using at the beginning and end of standard operating procedures yet employ different antibodies, amplification conditions, housekeeping genes, and normalization procedures. As a consequence, only those studies reporting on a direct comparison between endothelial cells from different vascular origins can readily assist in creating a better understanding of endothelial heterogeneity in response to an inflammatory stimulus. The following sections therefore mainly focus on these direct-comparison-investigations.

The extent of quiescent endothelial diversity was for the first time described by Chi and colleagues, who used DNA microarrays to analyze 53 cultured endothelial cell types isolated from different origins in the body. They uncovered persistent differences in gene expression profiles that distinguish large vessel endothelium from endothelium located in the microvasculature, groups of genes specific for arterial and venous endothelium, and tissue-specific expression patterns in different endothelial subsets.[78] One of the earlier studies that directly compared responsiveness of endothelial cells from different vascular origins to TNFα and IL-1 investigated VCAM-1 expression. Although both cytokines induced VCAM-1 in HUVEC, human arterial endothelial cells (HAEC) only expressed this adhesion molecule upon exposure to IL-1.[79] Another study compared TNFα responsiveness of human endothelial cells derived from glomeruli (GEC), dermal microvasculature (MVEC), and umbilical vein (HUVEC). All three endothelial subsets exhibited a reduction in the expression

of CD31 and strong induction of E-selectin, yet VCAM-1 was only (moderately) upregulated in GEC and HUVEC.[80] Using rat pulmonary microvascular and rat pulmonary arterial endothelial cells, a major difference was observed between the two cell types in reaction to TNFα and neutrophil adherence. Both exerted a similar increase in ICAM-1 expression and redistribution upon ICAM-1 cross-linking by exposure to neutrophils or antibodies. However, only the microvascular endothelial subset responded with induced p38 MAPK phosphorylation, ROS production, and F-actin formation.[81]

In an attempt to better understand whether molecular differences in responsiveness to atherogenic stimuli underlie arterial susceptibility to atherosclerosis, Deng and colleagues compared human saphenous vein endothelial cells (HSVEC) and human coronary artery endothelium (HCAEC) gene expression profiles in quiescent conditions and in reaction to oxidized LDL, TNFα, and IL-1β. In quiescent conditions, HSVEC expressed 285 genes to a higher extent than the artery endothelium. These genes were mainly related to anti-inflammatory responses, cell growth, and homeostasis functions. Atherogenic oxidized LDL preferentially induced cellular proliferation and adhesion pathways in CAEC, whereas in HSVEC, focal adhesion, inflammatory response, and apoptosis and NFκB pathway genes were downregulated. TNFα and IL-1β activation, in contrast, induced apoptosis and downregulated anti-inflammatory genes in CAEC, whereas in HSVEC both antiapoptotic and antiatherogenic genes were induced.[82]

Organ-encoded differences in response to inflammatory stimuli are already visible in endothelial cells derived from fetal tissues.[83] TNFα exposure activated the heart, kidney, and aorta endothelium derived from 12-week-old human embryos to express E-selectin, whereas IL-1β treatment only induced this adhesion molecule in fetal kidney and aorta endothelium. ICAM-1 expression was most pronounced in heart, liver, and lung endothelium when exposed to IL-1β and induced to a limited extent in lung endothelium when exposed to TNFα. VCAM-1 induction was lacking in fetal brain–derived EC, irrespective of the stimulus, although it was a late event in all but the heart-derived endothelium upon TNFα exposure. Its expression was only increased by IL-1β in fetal heart EC at a later time point.[83]

In a study by Methe et al, HSVEC and HCAEC were exposed to venous and coronary artery flow patterns, respectively 2.2 dyne/cm^2 (static) and 17 dyne/cm^2 (at 1 Hz) in combination with TNFα challenge.[84] Under static conditions, an induced expression of VCAM-1 could only be observed in the venous endothelial cells in response to TNFα treatment. This difference in expression induction between the cell types was not observed for E-selectin and ICAM-1. In the saphenous vein endothelium, exposure to both venous and artery flow conditions increased TNFα-induced E-selectin and ICAM-1 expression, whereas only coronary artery flow affected TNFα-induced VCAM-1 upregulation. In contrast, venous and coronary artery flow created a completely different effect on the coronary artery endothelium. Although either flow type attenuated TNFα-induced E-selectin and VCAM-1 expression, TNFα-induced ICAM-1 expression was only increased further by coronary artery flow, not by venous flow.[84]

Other inflammation-related endothelial activators have been studied with regard to endothelial subset specific responsiveness. These studies include protein kinase C activation,[85] nucleotide-induced P2Y(2) receptor desensitization,[86] and angiopoietin-1 and -2 activation of Tie-2,[87] which are not discussed further because they are beyond the scope of this review.

ENDOTHELIAL HETEROGENEITY IN ADHESION MOLECULE EXPRESSION IN VIVO

In vivo, endothelial heterogeneity in adhesion molecule expression is already visible in quiescent microvascular segments. For example, Eppihimer and colleagues showed that E-selectin protein expression is completely absent throughout the vasculature in healthy cytokine-naive mice,[88] an observation that was corroborated at the mRNA level.[89] At the same time, P-selectin is expressed to a significant extent in the lungs, mesentery,

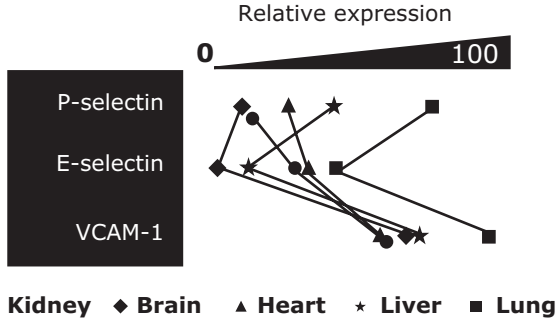

Figure 1 Interorgan differences in endothelial restricted adhesion molecule expression in quiescent conditions. Semi-quantitative representation of mRNA levels of P- and E-selectin, and VCAM-1 in C57bl/6 mouse organs as assessed by quantitative RT-PCR and related to housekeeping gene GAPDH. Lowest and highest expression levels were set at 0 and 100, respectively; others were calculated accordingly. The graph shows that brain, liver, and lung exert a comparable profile, with E-selectin expression the lowest of the three. In kidney and heart, in contrast, E-selectin mRNA levels are higher than those of P-selectin, the functional meaning of which is not known (Kuldo and Molema, unpublished data).

small intestine, and pancreas.[88] Northern Blot analysis furthermore demonstrated that, especially in the lungs, both VCAM-1 and ICAM-1 are constitutively expressed.[89] With the advent of RT-PCR technology, one can now obtain a more quantitative view on basal expression profiles of adhesion molecules in the different organs and the heterogenic character thereof (Fig. 1), the functional meaning of which remains to be established. Even more striking from a heterogeneity point of view is the observation that within one organ, different expression levels of adhesion molecules prevail in different vascular segments in healthy conditions. For example, quiescent mouse kidney, arteriolar and peritubular endothelium (EC) express significant levels of VCAM-1 protein, while at the same time glomerular endothelium is completely devoid of this adhesion molecule (Kuldo and Molema, manuscript in preparation).

Considering the heterogeneity in TNFR expression within the quiescent vasculature, it is not surprising that activation of these receptors results in variable responses in the different vascular segments. Using competitive RT-PCR, it was shown that intravenous injection of 3000 U TNFα in mice mainly induced E- and P-selectin expression in heart, lung, kidney, liver, and brain but not or to a lesser extent in spleen.[90] A similar difference in microvascular reactivity was reported by Tamaru and colleagues in response to IL-1β. Using Northern blot it was shown that adhesion molecule induction after IL-1β administration was mainly present in heart and lung, whereas it was less prominent in liver and kidney and even lower or absent, respectively, in brain and skin.[89] Using RT-PCR, a quantitative representation of the complexity of organ-specific microvascular reactions to inflammatory cytokines such as TNFα and IL-1β can be created (Fig. 2).

In addition to organ-specific control of gene expression, Tamaru and colleagues also revealed the existence of intraorgan variation in endothelial responsiveness by showing that systemic IL-1β administration mainly induced VCAM-1 mRNA in the larger vessels of the lungs and the heart, whereas small

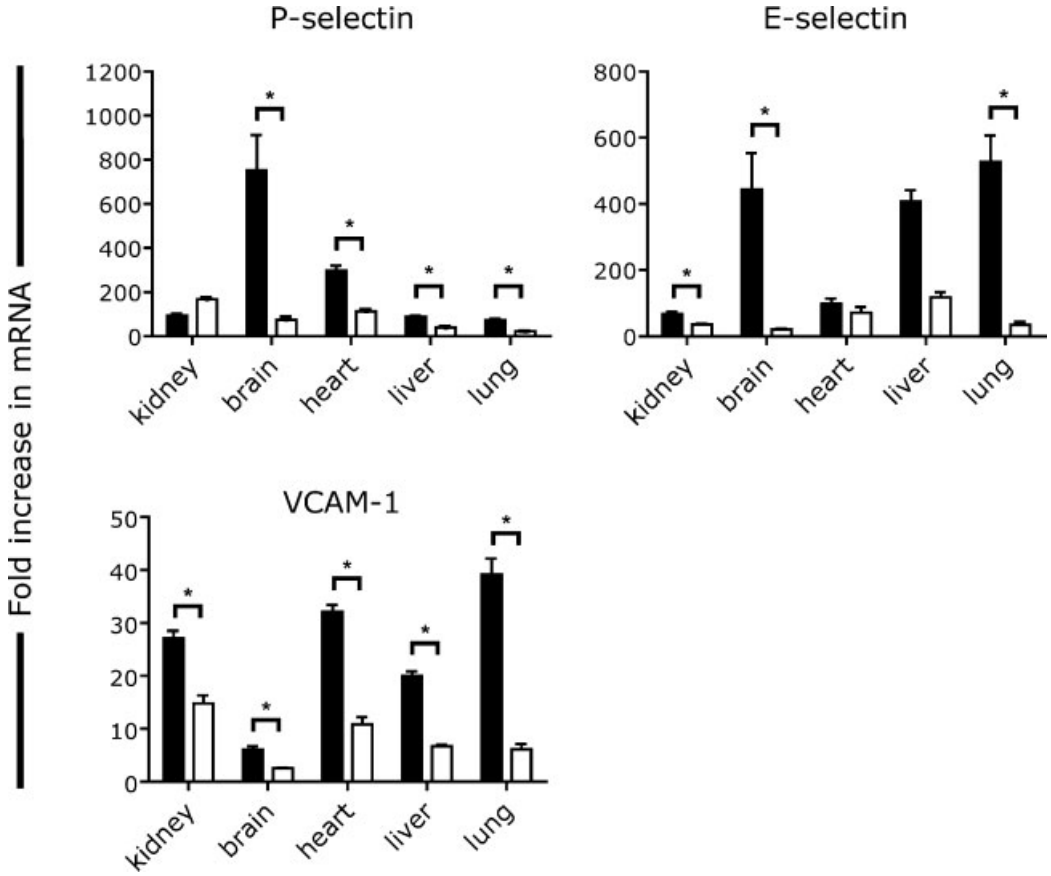

Figure 2 Interorgan differences in endothelial responsiveness to systemic tumor necrosis factor (TNF)α and interleukin (IL)-1β administration. C57bl/6 mice were injected intravenously with 200 ng TNFα (black bars) or IL-1β (white bars) per mouse and sacrificed 2 hours later. mRNA isolated from snap-frozen organs was analyzed by quantitative reverse transcriptase polymerase chain reaction with GAPDH as the housekeeping gene. Mean values ± standard error of mean. * Statistically significant, <0.05. The endothelial cells in all organs responded to the inflammatory cytokines, although in general TNFα administration induced higher adhesion molecule expression than IL-1β. Each organ displayed its unique pattern of extent of adhesion molecule induction, with liver and lung displaying the most comparable response. VCAM, vascular cell adhesion molecule. Data from Kuldo and Molema (unpublished).

capillaries were devoid of such an upregulation.[89] Other systemic inflammation models have also revealed intraorgan microvascular segment specific reactivity to inflammatory stress. For example, we showed that induction of hemorrhagic shock by withdrawal of blood to 30 mm Hg and maintenance thereof for 90 minutes led to expression of E-selectin in glomerular endothelial cells in the kidney, whereas it was much less pronounced or even absent in the endothelium of afferent and efferent arterioles, and in the peritubular and postcapillary venule segments. In contrast, VCAM-1 expression was induced in all vascular segments except in glomerular endothelium.[91]

Intravenous or intraperitoneal administration of LPS leads to a rapid induction of systemic TNFα levels besides having direct effects on cells via TLR family members. In the microvasculature of main organs such as the heart, lung, brain, and liver, the expression of E- and P-selectin and ICAM-1 are all significantly induced in response to systemic LPS treatment. Microvascular heterogeneity in response to LPS was, however, observed with regard to the regulation of VCAM-1 because both brain and liver microvasculature did not upregulate this adhesion molecule.[92,93] Interestingly, in this model, the skin microvasculature showed a rather aberrant gene induction profile compared with the other organs,[93] thus questioning the validity of using skin biopsies as surrogate readout of endothelial activation in other tissues. In mouse cremaster venules, LPS induced enhanced thrombosis that was mediated by von Willebrand factor (VWF).[94] Because VWF is highly heterogenically expressed throughout the vascular bed,[95] it is conceivable that LPS-induced enhancement of thrombotic events mediated by VWF is a vascular segment restricted feature.

Another interesting example of intraorgan endothelial heterogeneity in reaction to LPS was reported by Carrithers and colleagues. In CD31-deficient mice, LPS administration selectively induced microvascular endothelial cell apoptosis in the peritubular capillaries of the kidney while not affecting any of the other microvessel endothelial cells.[96] This effect was not seen in CD31-competent mice, implying that only the peritubular endothelial cells in the kidney rely on a CD31-mediated survival program when being exposed to LPS.

Long-term exposure to systemic disease conditions can also alter the basal gene expression pattern and functional behavior of endothelial cells in an endothelial subset specific manner. Using the type 2 diabetic Goto-Kakizaki rat model, Wang et al demonstrated that myocardial microvascular endothelial cells expressed decreased protein levels of VEGF (vascular endothelial growth factor), VEGF receptor 1 (VEGFR1), and VEGFR2, and exhibited decreased phosphorylation of the receptors compared with their healthy controls, whereas aortic endothelial cells from the diabetic rats did not exhibit such an altered phenotype.[97]

MECHANISMS UNDERLYING HETEROGENIC ENDOTHELIAL RESPONSIVENESS

Microenvironmental conditions including shear stress, biomechanical forces, leukocyte-endothelial cell interactions, and local production of specific factors by resident cells of the tissues and the microvascular walls can all influence in vivo endothelial cell behavior. How they in concert affect endothelial molecular makeup, to explain heterogenic responsiveness to inflammatory stimuli, is almost exclusively a hypothetical exercise at this moment because only a limited number of studies have addressed this issue. It is nevertheless challenging, and rewarding at the same time, to draw a picture of the molecular steps that lie in between the initiation of cell activation, induced by receptor activation, and the final outcome, changes in expression of genes and proteins. These relationships makes one realize that an integrated approach will be needed to answer the question of how this heterogeneity is brought about.

The first stage of cell activation is at the level of receptor expression and the local concentration of the ligand, which can be different for different vascular segments. In normal kidney, for example, TNFR1 is only expressed in the glomerular and peritubular capillary endothelium.[98] In contrast, in the normal human heart, TNFR1 and TNFR2 are both expressed by the vascular endothelial cells, whereas TNFα mainly localizes to microvessels.[10] Disease conditions such as allograft rejection markedly altered these expression patterns, with TNFR1 expression being downregulated and TNFR2 and TNFα expression being upregulated.[10,98]

The next level of control lies in the signaling pathways employed. Kinases may be differentially expressed in different endothelial subsets, as may be the scaffolds that guide the kinases involved in final transcription factor activation. An example of such a differentially employed signaling pathway was recently provided in a study that investigated endothelial responsiveness to chemokine receptor CXCR2 ligation by macrophage inflammatory protein (MIP)-2. Although both mouse aortic and pulmonary artery endothelial cells expressed comparable levels of the receptor, only in aortic endothelial cells did exposure to MIP-2 result in RhoA activation and enhanced chemotaxis.[99]

The outcome of signal transduction is activation of transcription factors. For many constitutively expressed endothelial restricted genes such as VWF, eNOS, VE-cadherin, and Tie-2, their promoter regions are enriched for Ets and GATA family members, Sp1 family members, AP-1 and Octamer transcription factors, which (by not fully understood mechanisms) lead to endothelial restricted expression.[100,101] The homeobox gene HOXA9 also belongs to this group of transcription factors that have a preferential function in endothelial gene expression control.[102] Because

HOXA9 is obligatory in mediating cytokine induced E-selectin expression,[31] the described heterogenic E-selectin expression in response to systemic TNFα administration or hemorrhagic shock can possibly be explained by vascular segment restricted expression of HOXA9. Liu et al furthermore revealed by in situ exposure of umbilical cord artery and vein to TNFα that the induction of adhesion molecule expression was remarkably restricted to the vein segment.[30] This difference could not be explained by differences in TNFR expression levels nor in the extent of activation of NFκB and AP-1, as assessed by phospho-p65 and phospho-c-jun immunohistochemistry. Possibly, the higher levels of phospho-ATF2 in the vein endothelial cells (in part) contribute to the differential adhesion molecule expression patterns observed.

Differences in epigenetic regulatory mechanisms, affecting methylation status of gene promoters and posttranslational histone modifications such as acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation, may partially explain endothelial subset preference of gene expression. That epigenetic marks are not a static feature of endothelial cells but can dynamically vary in time was recently shown for eNOS, whose histone marks were reset due to hypoxia, thereby affecting gene expression.[103] Also, DNA sequences proximal and distal from the promoter region significantly affect gene expression, and they can in theory be epigenetically controlled to a variable extent in endothelial subsets along the vascular tree.

Noncoding RNAs add an additional level of control of gene expression and VCAM-1 expression[104–106] and miR-296 regulates were shown to represent a missing link for some hitherto difficult to explain observations. Several microRNAs have been reported to be restrictedly expressed in the endothelial compartment and/or to exert a function in the vasculature. For example, miR-126 regulates angiogenesis, vascular integrity, and VCAM-1 expression;[104–106] miR-296 regulates the expression of various growth factors in angiogenic endothelial cells.[107] Suárez and colleagues reported that TNFα-induced miR-31 and miR-17–3p can provide negative feedback control of inflammation by affecting respectively E-selectin and ICAM-1 protein expression.[108] More recently, the large intervening noncoding, or lincRNAs, entered the stage.[109] Whereas small miRNAs hybridize with their target mRNAs to either suppress or completely silence gene expression at the posttranscriptional level, lincRNAs, transcriptionally activated by among others p53 and NFκB, regulate the expression of neighboring protein-coding genes at the level of chromatin modification, transcription, and posttranscriptional processing.[109–111]

Another mode of posttranscriptional control that has been described for the MAPK family member MAPK-activated kinase-2 (or MK2 or MAPKAP-K2) deals with compartmentalization of proteins in the cell. In conjunction with heat-shock protein 27 (Hsp27), MK2 prevented nuclear retention of p38 MAPK by sequestering it in the cytosol. As a result, MK2 inhibited excessive mitogen- and stress-activated protein kinase, or MSK-1, and NFκB phosphorylation, and hence IκB production, thereby preventing IκB-driven NFκB transport out of the nucleus and regulating NFκB transcriptional activity.[112]

For many proteins, once formed they will shuttle to the desired cellular compartment or be exocytosed for extracellular function. Some proteins that need to be rapidly active in situations of stress are continuously produced but almost immediately afterward are subjected to proteasomal degradation, creating a situation in which mRNA levels are significant yet protein levels minor. Upon exposure to a stressor, proteasomal degradation is instantly halted as a consequence of which the protein becomes instantly available.

Fig. 3 summarizes at which levels within endothelial cells heterogenic behavior may be controlled. Only a few studies have addressed these issues so far, and only to a limited extent. Future research is expected to shed light on their significance in controlling endothelial heterogenic behavior in either quiescent conditions or in response to inflammatory stimuli.

OTHER FACTORS ADDING TO THE COMPLEXITY OF HETEROGENIC RESPONSIVENESS

This review has so far summarized a selection of studies that have addressed endothelial responsiveness to proinflammatory cytokines in vitro and in vivo. It has focused on TNFα, IL-1, and LPS, and thus neglected other important proteins that can also strongly affect endothelial behavior due to their presence in the microenvironment within vascular segments in vivo. Such proteins are VEGF and its receptors, known for their widespread function in vascular homeostasis and angiogenic sprouting of blood vessels (e.g., in tumor growth), and that have now also been extensively studied for their role in inflammatory reactions. One effect of VEGFR2 signaling is the induction of VE-cadherin phosphorylation, with consequent changes in vascular permeability, as elegantly shown by Weis in an in vivo model of myocardial infarction.[113] In addition, membrane-bound TNFα was shown to sensitize endothelial cells for VEGF-induced permeability.[114]

Combinations of proteins can furthermore strongly affect each other's receptor and intracellular signaling dynamics. Adiponectin, for example, binds in a saturable manner to endothelial cells and selectively inhibited TNFα-induced NFκB activation without affecting JNK, p38 MAPK, and Akt.[115] Moreover, cytokine activation can lead to the production of new

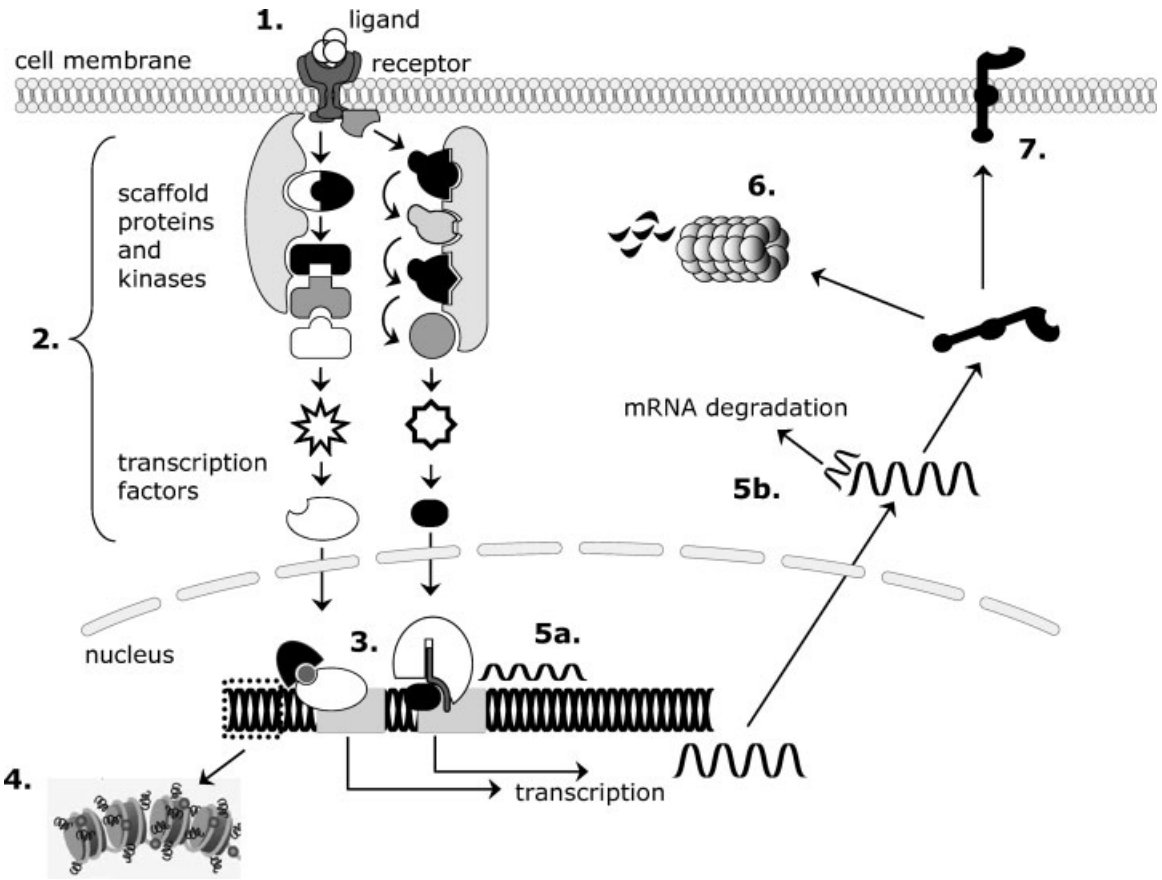

Figure 3 Simplified scheme of cellular events that may be differentially regulated in endothelial subsets and can contribute to endothelial heterogeneity in response to inflammatory stimuli. Microvascular endothelial cells are exposed to microenvironmental factors that differ from one vascular segment to the other, as a consequence of which their basic phenotype may vary. The following levels of control can in theory be involved in the heterogenic response of endothelial subsets to proinflammatory cytokines such as TNFα or IL-1β. **1.** Variation in local levels of cytokines and endothelial expression levels of the receptors for the cytokines. **2.** Differences in expression levels of adapter, scaffold, and kinase proteins that lead to activation of a preferred intracellular signal transduction cascade. **3.** The route of signaling may affect the nature of the transcription factors that become activated and thus the type of genes that will be expressed. **4.** Epigenetic marks such as histone modifications and DNA methylation may vary between endothelial cell subsets to explain differences in gene expression. **5.** Noncoding RNAs, including long intervening noncoding or lincRNAs (**5a**) and microRNAs (**5b**) can affect gene expression at the transcriptional as well as the posttranscriptional level. **6.** Via ubiquitinylation and subsequent proteasomal degradation, proteins can be eliminated from the cell (before or after they have exerted their function). **7.** When fully matured, proteins can be shuttled to their target compartment in the cell, or in the cell membrane, or to the outside of the cell. For clarity, the intracellular transport systems are not depicted in the scheme.

proteins to facilitate an additional wave of activation, as described for TNFα and soluble VCAM-1.[116]

The signal transduction section of this review has only paid attention to the class of proteins that contain substrate phosphorylation activity, the kinases, although nowadays the role of phosphatases as key coenzymes that fine-tune signaling cascades and cellular responses is also appreciated.[117] Protein tyrosine phosphatase 1B, or PTP1B, for example, negatively regulated VEGFR2 autophosphorylation and at the same time inhibited VE-cadherin phosphorylation in HUVEC,[118] implying a role in stabilizing cell-cell contact in the vasculature. Similarly, in HUVEC, the dual-specificity phosphatase MKP-1, rapidly induced upon TNFα or IL-1β activation, was shown to be involved in limiting cytokine-induced p38 MAPK and JNK signaling, and as such may play a role in limiting inflammatory effects.[54] Other negative feedback mechanisms to silence the endothelial inflammatory signaling response such as the rapid expression of family members of the ubiquitin editing enzyme A20[119] have also only been briefly mentioned, yet represent an additional level of control of endothelial activation that should be taken into account when trying to decipher the molecular mechanisms of heterogeneity in response to inflammatory stimuli.

A final remark on neglected subjects in this review deals with interindividual differences in endothelial

responsiveness. This represents a more technical issue that is of importance when working with primary (human) endothelial cell isolates to compare cellular and molecular reactions to cytokines between endothelial cell subtypes. Using 30 different endothelial isolates from human umbilical cord veins, and subjecting them for 24 hours to LPS stimulation, Beck and colleagues showed that these could be divided into low and high responders based on IL-8 production levels. Similar to reactivity to LPS, low responders also responded less extensively to TNFα and were found to require a higher concentration of activator to achieve maximal NFκB activity.[120]

PHARMACOLOGICAL CONSEQUENCES OF ENDOTHELIAL HETEROGENEITY

Kinases are a class of intracellular proteins that are critical for a variety of cellular responses in pathological conditions, and they are considered important targets for therapeutic intervention. Different classes of kinase inhibitors have been used as research tools, as described in Table 1, and many of these, or their derivatives, have been further developed for clinical application, with NFκB and p38 MAPK inhibitors being the most extensively studied.[121–125] One important pharmacological consequence of the existence of a complex network of different proteins interacting in concert to mount the desired reaction is that inhibition of only one pathway may lead to a more extensive activation of others. One example of such an adaptive response in endothelial cells is the effect of proteasome inhibitor MG-132 on TNFα-mediated cytokine production in human iliac and renal artery endothelial cells. Whereas MG-132 completely blocked TNFα-induced NFκB activation and concurrent MCP-1 expression, IL-8 expression was increased severalfold due to enhanced AP-1 transcription factor activity.[38] A combination of inhibitors is likely to be more effective in shutting down cell activation, as we showed for p38 MAPK/thioredoxin-NFκB inhibition in HUVEC challenged by TNFα or IL-1β.[59]

The effects of these kinase inhibitors and other anti-inflammatory drugs on endothelial cells in culture systems are undisputed, yet only limited data are available on how they affect microvascular endothelial cells in vivo. From the examples of similarities and differences in endothelial responsiveness it can be concluded that vascular segments respond to an inflammatory stimulus in a microenvironment-controlled manner. Although the outcome may be in part a vascular segment overlapping gene expression repertoire, different intracellular modes of relaying the signals to achieve this repertoire likely prevail. This implies that inhibitors of signal transduction such as the kinase inhibitors may be able to affect endothelial cells pharmacologically in one segment while being ineffective in others. This concept challenges the one-drug-fits-all model in which an inhibitor of NFκB or p38 MAPK could be used as a treatment of any inflammatory disease in the body to counteract microvascular endothelial cell activation irrespective of the location of the endothelial cells.

Microvascular segment specific control of engagement in disease can also provide an opportunity for endothelial selective intervention. Once we know the molecular basis for the control for each vascular bed in its pathophysiological context, drugs can be chosen accordingly. Selectivity can furthermore be achieved by targeted drug delivery, using antibodies or peptides specific for adhesion molecules,[126,127] integrins,[128,129] or growth factor receptors[130] as homing ligands for carrier systems that contain the drug cargo. For example, in our laboratory we designed high payload liposomal carriers harnessed with anti-E-selectin antibody as a homing ligand to selectively deliver drugs into endothelial cells engaging in inflammatory reactions. By this means we were able to interfere pharmacologically with microvascular endothelial cells in the glomerular compartment of kidneys of mice suffering from glomerulonephritis while upstream arteriolar and downstream postcapillary venule endothelial cells were not affected (Fig. 4).[131,132]

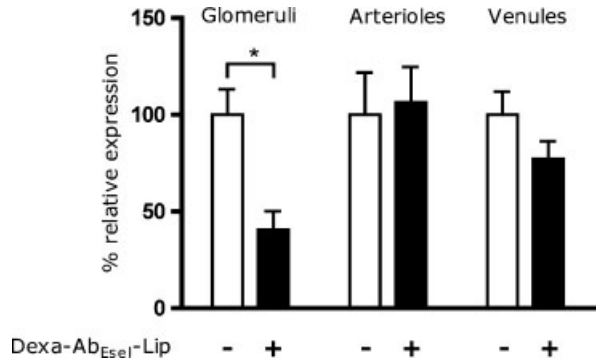

Figure 4 Endothelial heterogeneity in response to inflammatory stimuli allows for vascular segment specific therapeutic intervention by targeted drug delivery strategies. In the antiglomerular basement membrane mouse model of glomerulonephritis, endothelial cells in the affected glomeruli in the kidney express significant levels of E-selectin at an early stage of disease. Systemic administration of anti-E-selectin antibody modified liposomes containing the anti-inflammatory corticosteroid dexamethasone (Dexa-Ab$_{Esel}$-Lip) resulted in accumulation of the drug delivery preparation in the glomeruli (not shown), and in local inhibition of VCAM-1 expression in the glomerular compartment. Neither VCAM-1 expression in the arteriolar nor in the postcapillary venule compartments of the kidney were affected by the treatment. Compartmentalization of drug effects was determined by laser microdissection of the vascular segments prior to analysis of gene expression by real time RT-PCR.[131,132]

ADDRESSING ENDOTHELIAL HETEROGENEITY IN VIVO REQUIRES NEW TECHNOLOGIES

In an elegant study, Liu and colleagues showed that endothelial cells taken from their in vivo context rapidly lose their specific reactivity to an inflammatory stimulus.[30] Absence of VWF in glomerular endothelial cells in vivo[133] versus extensive VWF expression in isolated glomerular endothelial cells in vitro[134] is just another example of the molecular differences between endothelial cells in their in vivo context and in vitro culture conditions. Although in vitro studies are invaluable for generating new knowledge on endothelial behavior, the challenge for the coming decade will be to unravel the molecular basis of endothelial behavior in health, disease, and in response to drug treatment in their (patho)physiological context in vivo. To this end, one can isolate the endothelial cell subsets from an organ and directly map their molecular makeup using widely available gene and protein expression array platforms. Fluorescence-activated or magnetic bead–based cell sorting, for example, is a feasible technology to isolate peritubular endothelial cells from human kidneys by virtue of the fact that this endothelial subset selectively expresses MHC class II.[135] This is, however, a rare example of a molecule that is expressed by one subset of endothelium only.

Transgene mouse models that overexpress, or lack genes of interest, or express reporter genes in the endothelial cell compartment are instrumental in our endeavor to understand endothelial behavior in vivo.[95,136–138] The use of such models recently revealed the role of DSCR-1s (Down syndrome critical region gene 1, short variant) in heterogenic endothelial responsiveness to an inflammatory stimulus.[139] DSCR-1s promoter-lacZ mice showed enhanced promoter activity after LPS challenge in major organs. Upon LPS challenge of *Dscr-1* −/− mice, superinduction of E-selectin and ICAM-1 was observed in heart, while enhanced expression of VCAM-1 was observed in lung vasculature only. These data elegantly revealed a role for DSCR-1s in a negative feedback loop of endothelial cell gene expression control that is differentially regulated throughout the vascular system.

Laser microdissection (LMD) of endothelial cells from microvascular segments in tissues does not depend on advanced transgene animal models. It can be applied to both animal and human tissues, it does not affect RNA, protein and DNA integrity, and it allows for enrichment of endothelial cells from predestined (micro)vascular segments that can be prior assessed for disease activity. As such, it is an important technology for studying in vivo endothelial heterogeneity issues. Using this technique, basal and inflammation-related renal vascular compartmentalization of gene expression and local consequences of drug treatment on vascular segments could be revealed by us and others.[131,140,141] Also, epigenetic marks in specific vascular compartments can be interrogated using LMD-based vascular segment enrichment prior to sample analysis.[142] Combining targeted drug delivery systems that carry siRNA as a cargo to selectively knock out genes in restricted microvascular segments[143] with LMD-based validation of knockout and analysis of downstream molecular consequences in the target endothelial compartment furthermore represents a new, powerful multidisciplinary approach for in vivo endothelial cell biology studies.

A vast array of kinase inhibitors has been developed as potential drugs for the treatment of inflammatory diseases. For the design of effective drug regimen, knowledge about the kinase activity status in the tissues, and more specifically in the endothelial cells in the different microvascular segments, is of crucial importance. For this, availability of antibodies to detect phosphoproteins on tissue sections with intact morphology is a prerequisite.[144] Considering the fact that endothelial cells are numerically underrepresented in almost all tissues and are furthermore often difficult to discriminate morphologically from other cells, these phosphokinase antibodies should ideally be suitable for use in immuno(fluorescence) double staining protocols and in tissue quantitation techniques. A limited number of antibodies are at present available for this purpose, and some of them have been instrumental in detecting activated p38 MAPK in endothelial cells in human glomerulonephritis[145] and in determining the consequences of drug treatment on kinase activity in tumor endothelium.[146] Novel, superior performance phosphoprotein-specific antibodies are needed to expand the number of kinases and transcription factors to be analyzed. Combining these techniques will provide a wealth of new information on endothelial involvement in human and mouse pathology, and when including careful inspection of endothelial cells in microvascular segments, will allow us to create a view of in vivo endothelial heterogeneity to its full extent.

CONCLUDING REMARKS

Endothelial cells in the body microvasculature actively engage in inflammatory reactions. In quiescent conditions, endothelial cells exert a heterogenic phenotype while the existence of endothelial subset specific responsiveness to proinflammatory cytokines is now also gradually revealed. The nature of responsiveness is clearly vascular segment dependent, and it represents an intriguing and, from a molecular point of view, a mysterious feature of an organ that is situated within all organs of the body and that was known to exist since the 17th century. Unraveling the molecular control underlying endothelial heterogeneity will be essential for a better understanding of the role of endothelial cells in pathophysiological processes and for the design of

effective pharmacological intervention of inflammatory diseases.

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