

Supplement Review

Endothelial activation: intracellular signaling pathways

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Chapter summary

Tumor necrosis factor (TNF) is the prototypic proinflammatory cytokine and endothelial cells are the principal cellular targets of its actions. Here I review the responses of endothelial cells to TNF, with emphasis on the induction of endothelial leukocyte adhesion molecules. I focus on the biochemistry and cell biology of signal transduction in TNF-treated endothelial cells that lead to the expression of adhesion molecules.

Keywords: adhesion molecules, cytokines, inflammation, leukocytes, tumor necrosis factor

Introduction

Inflammation, defined as the local recruitment and activation of leukocytes, is an essential component of the innate immune response to pathogens and damaged cells. Consequently, mouse and human genetic defects in leukocyte recruitment manifest themselves as an increased frequency and susceptibility to infection and/or as a failure to remove degenerating tissues, such as the stump of a neonatal umbilical cord [1]. The innate inflammatory response has only a limited ability to distinguish normal from infected or damaged cells. Consequently, injury to healthy bystander cells at a site of inflammation is common. Moreover, unresolved inflammation can itself become a disease process, a clear example being rheumatoid arthritis. Inhibition of inflammation in such settings has become a primary goal of therapy irrespective of the underlying cause of the disease. For this reason, it is important to understand how inflammation develops and how it is regulated. In this chapter, I describe how tumor necrosis factor (TNF), an important

mediator of innate inflammation, acts on vascular endothelial cells (ECs) to promote the inflammatory response.

Historical background

A description of the inflammatory response to injured tissues at the level of light microscopy was first made over 100 years ago [2]. In those pioneering studies, Cohnheim noted that margination of leukocytes along the luminal surface of the postcapillary venule is the prelude to extravasation. Before the 1980s, these events were generally interpreted as a response of circulating leukocytes to chemoattractant substances elaborated within the tissue, either by infectious microbes (e.g. *N*-formyl peptides) or by the innate response (e.g. complement fragment C5a) [3]. Margination was explained by the observation that such substances not only induced chemotaxis but also triggered adhesion to endothelium, although the increase in adhesion was usually quite small (not more than twofold) [4]. This model did not explain why superfusion of chemotactic

substances does not cause circulating leukocytes to adhere to endothelium until they reach the venules [5].

Endothelial-cell-based model of inflammation

A re-evaluation of this paradigm began in the mid 1980s with the finding that exposure of ECs to cytokines, such as IL-1 or TNF, caused the ECs to bind 20 to 40 times as many leukocytes as untreated ECs, dwarfing the effects of chemotaxins [6]. The change in EC adhesivity arose from the induction of new surface proteins, collectively designated as endothelial leukocyte adhesion molecules (ELAMs), that bind counter-receptor proteins expressed on leukocytes [7]. Cytokine-treated ECs are also a source of chemoattractant cytokines (chemokines) that contribute to adhesion by activating the affinity of leukocyte counter-receptors for ELAMs [7]. These observations, combined with new experimental models such as parallel plate flow chambers and *ex vivo* videomicroscopy, led to the current multistep model of leukocyte recruitment centered on the responses of the vascular ECs lining postcapillary venules rather than on responses of leukocytes [8,9]. In brief, resting ECs are now viewed as noninteractive with leukocytes, so that random encounters with circulating white cells are short-lived, leaving both cells unaltered. Microbes and other inflammatory stimuli induce resident macrophages to release cytokines such as TNF or IL-1, which induce venular ECs to synthesize and express new proteins on their luminal cell surface. Critically, cytokine-treated ECs express several new ELAMs, namely E-selectin and integrin-ligands such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), that can interact with blood leukocytes. (Mouse ECs, but not human ones, also upregulate P-selectin in response to TNF or IL-1.) Cytokine-activated ECs also synthesize, secrete, and display (in association with cell-surface proteoglycans) chemokines on their luminal surface. Circulating leukocytes that bump into cytokine-activated ECs rapidly form low-affinity interactions mediated by binding to E-selectin and/or VCAM-1. Shear force, imparted by flowing blood, causes these interactions to be rapidly broken, only to reform rapidly as the leukocyte is displaced. Multiple iterations of these processes results in rolling of the leukocyte on the EC surface. Rolling, but not free-flowing, leukocytes encounter and respond to the surface-displayed chemokines, causing cell spreading and clustering of surface integrins (such as LFA-1 and VLA-4) at the contact area with the ECs. These leukocyte integrins are the counter-receptors for ICAM-1 and VCAM-1 on ECs, and enhanced interactions by clustered integrins produce firm attachment to the ECs. Bound chemokines also stimulate leukocyte chemokinesis, resulting in crawling on the EC surface. As crawling leukocytes reach the junction between ECs, they extravasate through the junction into the tissue space, resulting in inflammation.

Several refinements of this EC-based model of inflammation are worth noting. One point involves the specialized nature of venular ECs. Although ICAM-1 is upregulated by TNF or IL-1 on essentially all ECs lining the microvasculature, E-selectin and VCAM-1 are normally confined to ECs of the postcapillary venule, the site of leukocyte rolling and margination [10]. However, in certain disease states (e.g. psoriasis), capillaries also may express these molecules, and the pattern of leukocyte extravasation changes to match that of expression of adhesion molecules [11]. In addition, the patterns of adhesion molecule expression (and chemokine expression) are dynamic. For example, expression of E-selectin, associated with neutrophil extravasation, peaks early (at 2–4 hours) after TNF addition, corresponding to the onset of neutrophil recruitment. VCAM-1, which is more closely associated with binding of mononuclear leukocytes, typically peaks at later times (12–24 hours) after TNF addition, corresponding to the onset of T-cell recruitment [12]. Finally, the EC response to TNF can be modified by T-cell-derived cytokines. For example, IFN- γ prolongs the expression of E-selectin [13], whereas IL-4 suppresses the expression of E-selectin while promoting that of VCAM-1 [14]. These alterations in the EC surface produce corresponding changes in the nature of the inflammatory leukocyte populations that are recruited. Specifically, IFN- γ favors recruitment of leukocytes associated with inflammation of the T-helper-1 type (dependent on E-selectin), whereas IL-4 favors inflammation of the T-helper-2 type (independent of E-selectin) [15]. In other words, the spatial, temporal, and qualitative patterns of EC adhesion molecule expression govern the location, evolution, and nature of the inflammatory response.

The biochemistry of TNF signaling in endothelial cells

A central role of TNF in inflammation has been established by observations that many inflammatory reactions are impaired in TNF or TNF-receptor (TNFR) knockout mice [16] and that, in humans, TNF inhibitors (soluble receptors or neutralizing antibodies) are effective anti-inflammatory therapeutics [17]. As I have noted, proinflammatory actions of TNF on ECs generally involve new protein synthesis. In general, these changes are initiated by new gene transcription [7]. Two specific transactivating (transcription) factors, NF- κ B and activator protein-1 (AP-1), are essential (although probably not sufficient) for TNF induction of ELAMs [18]. The evidence for this conclusion is that the E-selectin, ICAM-1, and VCAM-1 genes each contain DNA sequences in their 5' flanking regions that bind various forms of NF- κ B and AP-1 in electrophoretic-mobility-shift assays and that mutations of these sequences reduce TNF responses of transfected promoter-reporter genes. In addition, transfection experiments of wild-type and mutant forms of NF- κ B and AP-1 subunits into cultured ECs can regulate in the expression of E-selectin, ICAM-1, and VCAM-1.

An active area of research in the 1990s was the elucidation of signaling pathways through which TNF could activate NF- κ B and AP-1. Human ECs, like most other cell types, express two different TNFRs, designated TNFR1 (CD120a) and TNFR2 (CD120b) [19]. Signaling is initiated when ligand-occupied receptors recruit the binding of intracellular adaptor proteins to the intracellular portions of the receptor molecules (reviewed in [20,21]). Initially, ligand-occupied TNFR1 binds TNF-receptor-associated death-domain protein (TRADD) through interactions of homologous regions, called 'death domains' (DDs), expressed in both proteins [22]. The original DD was so named because it was found in the death-inducing receptor Fas as well as in an adaptor protein recruited to ligand-occupied Fas, called Fas-associated DD protein (FADD). The three-dimensional structure of the TNFR1 DD has recently been solved by two separate groups of researchers, and two interactive TRADD binding sites have been defined [23,24]. Unoccupied TNFR1 associates with a DD-containing protein, called silencer of DDs (SODD), which is displaced by TRADD upon TNF binding [25]. Receptor-bound TRADD can recruit FADD through DD interactions and thereby mimic the death-activation responses of Fas [26].

TRADD recruitment also initiates the recruitment of two other adaptor proteins that have been linked to activation of NF- κ B and for AP-1, namely receptor interacting protein (RIP) and TNF-receptor-associated factor 2 (TRAF2). RIP is a serine/threonine kinase that contains a DD that mediates binding to TRADD [27]. The mechanism of action of RIP is unclear; kinase-inactive RIP can function when overexpressed, but this may involve recruitment of endogenous RIP molecules with an intact kinase activity. Thymocytes from knockout mice lacking RIP cannot activate NF- κ B in response to TNF but can still respond to TNF by activating AP-1 [28]. TRAF2 binds to the N-terminal domain of TRADD, i.e. outside the DD [29]. TRAF2 contains an N-terminal RING domain, which is essential for its signaling, a series of zinc fingers, and C-terminal TRAF domains shared with other members of the TRAF family. The TRAF domains mediate both self-association and binding to adaptor proteins (e.g. TRADD). Unlike RIP, TRAF2 has no known enzymatic activities (although RING domains may act as E3 ubiquitin ligases) [30]. Embryonic fibroblasts lacking TRAF2 show a partially impaired ability of TNF to activate NF- κ B and show complete loss of TNF-induced activation of AP-1 [31]. (The incomplete effect of TRAF2 deficiency may arise because of redundancy with TRAF5 [32].) TRAF2 may indirectly contribute to NF- κ B activation by recruiting and/or stabilizing the interactions of RIP with its downstream targets [33]. Overexpression of either RIP or TRAF2 in wild-type cells can initiate both NF- κ B and AP-1 signaling independent of TNF, TNFR1, or TRADD, possibly by driving association of adaptor proteins in a nonphysiological manner.

The physiological downstream targets of RIP or TRAF2 remain uncertain. Both the NF- κ B and the AP-1 pathways are activated by members of the mitogen-activated-protein kinase (MAPK) kinase kinase (MAP3K, also known as MEKK) family. Experiments in gene-knockout embryonic fibroblasts implicate MEKK-1 in the AP-1 pathway [34] and MEKK3 in the NF- κ B pathway [35] activated by TNF. Several different MEKKs can phosphorylate and activate a cytosolic enzymatic complex called I κ B kinase (IKK) [36]. This multiprotein complex contains at least two active kinases (IKK- α and IKK- β), as well as a regulatory protein that lacks kinase activity, called IKK- γ , or NEMO. Studies in gene-knockout animals point to IKK- β as the crucial component that mediates TNF-induced phosphorylation of cytosolic inhibitor of κ B (I κ B) proteins [37,38] and show that IKK- γ (NEMO) is required for this response [39].

In unstimulated cells, I κ B proteins normally sequester dimeric NF- κ B complexes in the cytosol, preventing their entry into the nucleus where gene transcription occurs [40]. In response to TNF, I κ B proteins are phosphorylated by IKK upon critical serine residues, and, once phosphorylated, are rapidly ubiquitinated and then degraded by the cytosolic proteasome. This process occurs within 15 minutes of treating human umbilical-vein-derived endothelial cells (HUVECs) with TNF. In HUVECs, TNF causes degradation of I κ B- α , - β , and - ϵ [41,42]. Once an I κ B protein is degraded, the associated NF- κ B is free to move from the cytosol to the nucleus and activate transcription by binding to specific DNA sequences in the enhancers of target genes. In HUVECs, TNF-activated NF- κ B is formed of homodimers or heterodimers involving three different members of the Rel family, namely p50 (also called NF- κ B1), p65 (also called Rel A), and c-Rel. Homodimers of p50 appear to be constitutively present in the nucleus and are not regulated by I κ B degradation. A recent report has suggested that I κ B- α and I κ B- β associate primarily with p50/p65 or p50/c-Rel heterodimers, whereas I κ B- ϵ associates primarily with p50/c-Rel or c-Rel homodimers [42]. Minor variations in a κ B-binding DNA sequence may favor binding of one form of NF- κ B over another. In experiments using electrophoretic-mobility-shift assay, the three E-selectin elements preferentially bind p50/p65 heterodimers [43–45]. The VCAM-1 promoter contains two tandem κ B-binding sites that also appear to preferentially bind p50/p65 [46,47]. In contrast, the ICAM-1 promoter contains one κ B element that may preferentially bind p50/c-Rel heterodimers or c-Rel heterodimers [42,48].

AP-1 activation occurs when an MAP3K, probably MEKK-1, phosphorylates and activates several MAP2Ks (also known as MEKs), which, in turn, phosphorylate and activate several MAPKs (also known as stress-activated protein kinases, or SAPKs) such as c-Jun N-terminal kinase (JNK)-1 and -2 and p38 MAPK [49]. JNK-1 and -2

phosphorylate the transactivating domain of c-Jun, a component of AP-1, and thereby enable AP-1 to activate gene transcription. Normally, c-Jun forms heterodimers with members of the Fos family, such as c-Fos or FosB, but c-Jun also can heterodimerize with activating transcription factor 2 (ATF2) to form a variant form of AP-1. TNF increases the binding of c-Jun/ATF2 to a DNA sequence in the E-selectin promoter [50]. Transfected E-selectin promoter-reporter genes lacking the c-Jun/ATF2-binding site are much less active than wild type [51], and E-selectin transcription does not occur in mice lacking ATF2 [52]. The transcriptional potential of ATF2, like that of c-Jun, can be increased by phosphorylation of its transactivating domain, catalyzed by p38 MAP kinase. However, while overexpression of a c-Jun mutant that cannot be phosphorylated (or of dominant negative JNK isoforms) will inhibit E-selectin transcription, a mutant form of ATF2 that cannot be phosphorylated is not inhibitory [51]. This suggests that c-Jun/ATF2 is necessary for E-selectin transcription but that only the c-Jun subunit needs to be phosphorylated for efficient gene transactivation. Both ICAM-1 and VCAM-1 also contain AP-1 binding sites (commonly called tetrahydrophorbol response elements, or TREs) that bind AP-1 in TNF-treated ECs [53,54]. In this case, AP-1 consists of c-Jun/cFos heterodimers. The significance of the canonical TRE in the VCAM-1 promoter has been questioned because this element is lacking in mice [54]. However, human and mouse VCAM-1 may not be regulated in the same manner, and a role of AP-1 in VCAM-1 transcription has been demonstrated using promoter-reporter genes in human ECs, although the site where AP-1 appears to bind in the model is distinct from the consensus TRE and is instead located between the two κ B-binding elements [55].

Although the activation of NF- κ B and AP-1 may involve divergent pathways, these factors interact in the nucleus through concomitant binding of gene coactivators, such as histone acetyl transferases like CREB-binding protein (CBP), or p300 [56]. The steric positioning of individual transcription factors bound to DNA, which permits coordinate interactions with coactivators, may depend upon DNA bending, controlled by proteins such as the high-mobility-group protein HMG-Y1 [43–45]. The basic unit of such coordinated complexes has been called an 'enhanceosome'. The three NF- κ B binding sites and the AP-1 binding site in the E-selectin promoter appear to fit this definition.

E-selectin transcription and AP-1 activation are both transient in TNF-treated HUVECs [57], but NF- κ B activation is not [41]. A decrease in phospho-c-Jun/ATF2, resulting from shutting off of JNK activity, probably accounts for the termination of E-selectin transcription [57]. Once the gene turns off, E-selectin cannot be effectively reinduced by TNF without a rest period of 18–24 hours. During the

refractory period, it can be reinduced by IL-1 or CD40 ligand, and reinduction correlates with the reactivation of JNK. Since JNK activation is mediated by TRAF proteins and since TNF, IL-1, and CD40 ligand all use distinct TRAF proteins (although CD40 ligand does recruit TRAF2 and TRAF6 as well as TRAF3 and TRAF5), an attractive explanation for receptor-specific desensitization is that specific TRAFs are somehow selectively inactivated. TRAF proteins contain RING domains that can act as ubiquitin E3 ligases [30], and it is possible that ubiquitination is involved in TRAF inactivation and is responsible for the reduction of JNK and AP-1 activity. This speculation is supported by the observation that TRAF activation by CD30 in T cells is terminated by TRAF ubiquitination and degradation [58].

In some cells, TNF signaling may involve signaling pathways in addition to those described above, including the activation of phosphatidylinositol-3 kinase and protein kinase B (also known as Akt) [59]; activation of neutral or acidic sphingomyelinases to generate ceramide [60]; *de novo* sphingosine-1 phosphate (S-1P) synthesis [61]; and a Ras/Raf/ERK growth-control pathway [62]. My laboratory has shown that the Akt and ceramide pathways do not contribute to adhesion molecule expression in ECs [63,64]. It is possible that the S-1P and Ras pathways do participate in ELAM regulation, but, if so, the biochemical links to ELAM gene transcription are unknown.

The cell biology of TNF signaling in ECs

The biochemical view of TNF signaling described above is well supported by genetic and molecular data. However, my colleagues and I believe that it is an incomplete description, because it does not consider the capacity of cells to regulate interactions of receptors and adaptor proteins through control of their subcellular localization. Much of our recent work has focused upon this aspect of TNF signaling. HUVECs have very little TNFR1 on their surface; the predominant surface receptor is TNFR2 [65,66]. Although TNFR2 can directly bind TRAF2 (as well as TRAF1), it does not activate expression of adhesion molecules in these cells [19] (although it can do so, independently of ligand, when overexpressed) [67]. However, the presence of TNFR2 increases the cells' sensitivity to TNF [19], consistent with the hypothesis of ligand passing from a higher-affinity (TNFR2) to a lower-affinity (TNFR1) receptor [68].

When TNF binds to ECs, much of it is rapidly internalized. Most of the internalized ligand follows a coated-pit/coated-vesicle pathway, winding up in endosomal/lysosomal compartments [65]. TNFR2 shows a similar pattern of internalization, and it is likely that most TNF uptake is mediated via this nonsignaling receptor. Surprisingly, a significant fraction of internalized TNF molecules end up associated with mitochondria [69] and a third TNF-binding

protein (of approximately 60 kDa) has been identified in the inner membrane of this organelle [69]. The complete identity of this molecule, the pathway by which TNF is transported to the mitochondria, and the function, if any, of mitochondrial TNF are still unknown.

In cultured HUVECs, as in many other cell types, the majority of TNFR1 molecules are located within the Golgi apparatus, retained there through an undefined interaction involving the DD [65,66]. Until recently, the cellular and subcellular distributions of TNFR1 were known only from studies of cultured cells. Interestingly, in our initial analysis of a human tissue, namely the kidney, my colleagues and I found that TNFR1 was confined mainly to the Golgi apparatus of glomerular and peritubular capillary ECs [70], consistent with findings *in vitro* (although the absence of TNFR1 in cell types other than ECs was unexpected). The role of the Golgi population of receptors is unknown. Plasma-membrane receptors can be internalized to an ER-like compartment in response to MAPK-mediated phosphorylation [71]. My colleagues at Cambridge University and I have recently found that Golgi receptors can be mobilized to appear on the cell surface by stimulation of cultured ECs with histamine (Jun Wang *et al.*, unpublished observations).

When HUVECs are exposed to noxious stimuli, TNFR1 is shed from the surface [72]. This response is mediated by a protease inhibited by the compound TAPI (TACE protease inhibitor), and thus likely to be identical to TNF- α -converting enzyme (TACE). Shedding of receptors both desensitizes HUVECs to TNF [72] and serves as a source for soluble receptor (sTNFR), a natural inhibitor of TNF function [73]. The Golgi pool of receptors could thus be a reservoir for replacing shed receptors on the surface or could serve as a precursor pool for increasing the number of shed receptors. Our recent observations indicate that the same signals that mobilize receptors from the Golgi apparatus also favor receptor shedding, supporting the latter hypothesis (Jun Wang *et al.*, unpublished observations). The importance of receptor shedding is highlighted by the finding that deficiencies in shedding receptors due to structural mutations in TNFR1 underlie TNFR-associated periodic syndrome (TRAPS), characterized by febrile episodes related to overreaction to TNF [74].

Even though only a minority of TNFR1 molecules are present on the plasma membrane, these molecules are critically important, because they are the only population that interacts with TRADD upon TNF treatment [75]. The cellular compartment(s) in which FADD, RIP, and TRAF2 are recruited to the TNFR1/TRADD complex is unknown. However, my colleagues and I and others have also observed that treatments that reduce membrane trafficking (e.g. endocytosis) also block TNF signaling [76,77], and such observations imply that TNFR1 signaling com-

plexes move from the plasma membrane in order to signal efficiently. My colleagues and I also have found that internalized receptors rapidly dissociate from TRADD, potentially limiting signaling [76].

A recent idea in receptor function is that activated receptors efficiently interact with adaptor proteins (and engage in crosstalk with other receptors) only when the relevant proteins are brought into proximity within cholesterol- and sphingomyelin-rich patches of the plasma membrane called lipid rafts [78,79]. In ECs and certain other cell types, lipid rafts bind cytoskeletal scaffolding proteins called caveolins [80]. Caveolin binding causes the rafts to invaginate, forming specialized organelles known as caveolae. Although caveolae were first identified for their role in initiating transcellular vesicular transport, they are now (additionally) thought to facilitate signaling and permit receptor crosstalk. It is thus noteworthy that in ECs, TRAF2 is normally associated with caveolin-1 [67]. This finding suggests that the TNFR1 signaling complex, through binding of TRAF2, can be recruited via caveolin-1 to caveolae, where interactions with other signaling pathways may occur.

Concluding remarks

TNF-mediated induction of ELAM gene expression in vascular ECs is a central event in inflammation. Over the past 10 years, a reasonably good biochemical model has been developed of how TNF can activate two families of transcription factors, namely NF- κ B and AP-1, whose activation is necessary for expression of ELAM genes. Our current focus of investigation is how this biochemical model operates within the living ECs, where various interactive components may either be segregated or brought together in response to ligand binding. The more complete picture of TNF signaling, which is being developed from these studies, may lead to new, more nuanced therapeutic approaches to regulating the inflammatory process.

Glossary of terms

ATF = activating transcription factor; DD = death domain; ELAM = endothelial leukocyte adhesion molecule; FADD = Fas-associated DD protein; HUVEC = human umbilical-vein-derived endothelial cell; IKK = I κ B kinase; JNK = c-Jun N-terminal kinase; MAPK = mitogen-activated protein kinase; MEK = MAPK kinase (also called MAP2K); MEKK = MEK kinase (also called MAP3K); RIP = receptor interacting protein; TRADD = TNF-receptor-associated death-domain protein; TRAF = TNF-receptor-associated factor; TRE = tetrahydrophorbol response element.

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