Inflammatory Mediator Expression

in Human Ocular Microvascular Endothelial Cells

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Next to leukocytes, the vessels and their endothelial lining play the most important role in inflammation"—Elie Metchnikoff, 1883.

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Abstract

Ocular inflammatory diseases are leading causes of blindness. Although our understanding of their underlying molecular mechanisms has exponentially increased in recent years, it nonetheless remains incomplete, with associated morbidity remaining high. Inflammation in the eye shares many of the same mechanistic features with inflammatory scenarios occurring elsewhere within the body. Key amongst these is the pivotal involvement of the endothelial cell (EC) monolayer, a specialized simple squamous epithelial lining of the entire luminal surface of the circulatory system, in regulating leukocyte traffic out of the bloodstream and into affected tissues.

For a long time, it was thought that the vascular EC lining of the body's circulatory system was essentially a biologically homogenous entity. A contradictory paradigm has emerged over the last 15 years or so, in which accumulating evidence suggests that EC from different regions of the vasculature are not necessarily identical to one another, but instead exhibit clear tissue-specific diversity in their structural and functional characteristics. Whether this EC heterogeneity is due to inherent genetic differences amongst the EC residing in different vascular beds, or strictly to their responses to the highly variable microenvironments that exist from tissue to tissue, still remains unclear.

The primary focus of this dissertation work was to test the hypothesis that inherent biological diversity exists amongst EC derived from the microvasculatures of different, closely situated, ocular tissues (*i.e.*, the iris and the retina). In order to test this premise, we first developed reliable methods for purifying and propagating donor-matched microvascular EC from human retinas and, for the first time reported, from irises. Using these cultured EC from the eye, coupled with a variety of gene, protein, and functional analytical methods, we extended our knowledge of the expression profiles of various biological mediators of inflammation (*e.g.*, adhesion molecules, chemokines, and cytokines), several of which were previously unelucidated

in the eye. More importantly, these approaches revealed the existence of distinct inherent biological differences amongst iris versus retinal microvascular EC, with respect to their expression of molecules that are essential in leukocyte recruitment during inflammation. This tissue-specific heterogeneity might be reflected in differing propensities of diverse ocular tissues to becoming inflamed.

Chapter 1. Introduction

1A. The Microvasculature in Inflammation

For more than a century, medical science has recognized the essential role of the microvasculature in mediating inflammation.[1] Inflammatory processes occur in the body's tissues in response to a broad range of harmful stimuli, in an attempt to neutralize the damaging agent and return compromised tissue to useful function. A hallmark trait of inflamed tissues is the accumulation within them of a fluid exudate containing large quantities of plasma proteins and leukocytes, both of which play important defensive roles against infective organisms that may be present.[2] This extravasation of blood components from the microcirculation into the tissue interstitium involves several interrelated biological phenomena, *i.e.*, vascular dilation and leakage, and the coordinate activation of the endothelium that lines blood vessels and of leukocytes that arrive in the bloodstream.

Vascular dilation results from relaxation of the smooth muscle cells and pericytes that encircle blood vessels, in response to locally produced vasoactive factors such as nitric oxide.[3, 4] Other inflammatory mediators, (*e.g.*, histamine, serotonin, and bradykinin), act in large part directly on the monolayer of endothelial cells (EC) that form the luminal lining of blood vessels. These agents stimulate reorganization of EC physical contacts with one another and with underlying cells, and thereby alter the barrier properties of the EC monolayer. This reorganization facilitates the leakage of plasma and plasma proteins out of the blood vessels and into surrounding tissues.[5, 6] As a result of exposure to these and other locally generated proinflammatory molecules (*e.g.*, tumor necrosis factor alpha, TNFα, interleukin-1, IL-1) and other activating agents that may be in the vicinity (e.g., bacterial lipopolysaccharide, LPS), the endothelium becomes "activated", and the cell's phenotypic profile is dramatically, though reversibly, altered, such that EC upregulate their expression of multiple prothrombotic,

chemoattractant, and adhesive molecules.[7, 8] By generating localized microthrombi, blood flow is impeded, thereby aiding the formation of gradients of soluble chemoattractant molecules, or chemokines, that function to draw in leukocytes towards the activated vessel wall.[7]

After leukocytes migrate to the bloodstream periphery, adhesion molecules on both EC and on leukocytes mediate attachment of these two cell types to one another, a prelude to leukocyte extravasation into surrounding tissues.[8-10] Many of the EC-expressed chemokines also activate the very leukocytes that are attracted to the vessel wall, resulting in upregulated leukocyte expression of adhesion molecules and additional chemokines. Attached leukocytes maintain and enhance the EC activation state, by virtue of signaling through their adhesive bonds which induces the release of their own cytokines and chemokines. Thus a complex reciprocal positive feedback system exists between these two cell types in inflamed scenarios, and this ensures a rapid and robust response to the initial aggravating stimulus.

1B. Cell Adhesion Molecules

The process of intercellular adhesion via specific molecular interactions plays pivotal roles in a wide variety of biological activities including embryo and organ development, hemostasis, the immune response, wound healing, and inflammation. Over the last two decades, there has been an exponential increase in our understanding of the molecular mechanisms that govern cell-cell adhesion and of adhesive interactions of cells with extracellular matrix proteins. In contrast to the comparatively slow intercellular adhesive processes at work during embryogenesis and tissue remodeling, the leukocyte/EC interactions that function during an inflammatory response are necessarily very rapid. In order for a leukocyte to leave the circulation, it must overcome significant physical forces imposed by the flowing blood. In the vasculature, the average speed of blood flow varies from upwards of 50 cm/s in the aorta to around 1 to 3 mm/s in

the capillaries and postcapillary venules.[11] Thus, efficient means must exist in order to effectively brake a circulating leukocyte if it is to exit the bloodstream.

In inflammation, leukocytes that exit the circulation into surrounding tissues are first captured from the flowing blood and temporarily immobilized on the EC luminal lining of the blood vessel. This attachment is accomplished via the interactions between a diverse array of cell adhesion molecules (CAMs) that are expressed on the surfaces of these two cell types (Fig. 1).[8] Structural characterization of these CAMs has allowed their convenient categorization into discrete molecular superfamilies that include, foremost, the selectins, the immunoglobulin superfamily, and the integrins. Besides mediating leukocyte/EC interactions, some of these CAMs also mediate the homotypic binding of adjacent EC to one another, thereby modulating the permeability characteristics of the endothelial monolayer to leukocytes and to soluble plasma constituents.[12] In addition to our understanding that cells can bind to one another in either heterotypic or homotypic fashions, similar heterotypic and homotypic adhesion events occur at the molecular level, as well (*e.g.*, ICAM-1 binding integrins vs E-cadherin binding E-cadherin).

Selectins

Leukocyte extravasation from the flowing blood is a complex process that can be divided into 4 distinct yet overlapping events: initial capture, rolling, firm adhesion, and transmigration through the endothelium (Fig 1). The selectins are a group of three structurally related membrane glycoproteins (i.e., E-, P-, and L-selectin) that mediate the first two events, i.e. leukocyte capture and rolling along the vascular luminal surface, by binding to cognate ligands on opposing cells.[13]

L-selectin (CD62L, LECAM-1, peripheral lymph node homing receptor), the first leukocyte adhesion molecule to be thoroughly characterized, is constitutively expressed on neutrophils, monocytes, T and B lymphocytes, and eosinophils,[14] in line with its demonstrated role in mediating the continual recirculation of patrolling leukocytes between the vascular and

interstitial compartments.[15] L-selectin is proteolytically shed upon cell activation, following a transient increase in binding activity.[16] E-selectin (CD62E, ELAM-1), which is present *in vivo* only in inflamed tissues, is synthesized *de novo* and transiently expressed on EC surfaces after cell stimulation with proinflammatory stimuli (*e.g.*, LPS, TNFα, IL-1).[17] By contrast, P-selectin (CD62P, PADGEM, GMP-140), is stored preformed in the Weibel-Palade bodies of EC and the α-granules of platelets, and is rapidly (within minutes in EC, seconds in platelets) mobilized to the cell surface in response to agonists such as histamine, bradykinin, leukotrienes, and thrombin.[18-20]

All three selectin family members possess a similar NH_2-terminal motif that is homologous to the Ca^{++}-dependent (type C) lectins, followed by an epidermal growth factor (EGF)-like domain, a chain of repeated domains similar to those found in complement regulatory proteins, a transmembrane domain, and a short cytoplasmic tail (Figure 2).[13]. The lectin domain is the ligand binding site, and, as is the case with other lectins, the selectin ligands are carbohydrate moieties that have a fucose-containing sialyl-Lewis^X-type structure also present in blood group antigens.[21] Several ligands have been identified, including CD34, PSGL-1, GlyCAM-1, and MAdCAM-1, which exhibit variable promiscuity in binding to the selectins.[22]

The selectins are structurally well-suited for mediating the initial capture and rolling of leukocytes on the endothelium. Their long single chain structure, rapid ligand binding and release rates, and their ability to withstand high bond tensions before disengaging allow selectins to transiently but tightly adhere leukocytes that approach the vessel wall.[13] Unlike other classes of adhesion molecules that require extremely close approximation of two cells in order to initiate ligation (e.g., integrin-mediated adhesion), the selectins can form relatively long "tethers" to their ligands, with predicted selectin-ligand bond lengths of 25, 40, and 50 μm for L-, E-, and P-selectin, respectively.[23] Such long binding distances, particularly in the cases of E- and P-selectin, avoid the energetically unfavorable scenario of glycocalyx interdigitation that occurs

when two cells are juxtaposed closely together, and present the additional benefit of steric flexibility along the bond.[24] Their rapid on/off rates result in transient ligations, wherein a dynamic cycle of release/readherance facilitates the controlled rolling of leukocytes across the EC monolayer. The high strains tolerated by selectin bonds likewise aid capture of leukocytes in the face of substantial physical shear stresses that are imposed by the flowing blood. Another characteristic of selectin-mediated binding is that such ligation typically does not result in overt activation of the bound leukocyte.[24] Although leukocyte tethering and rolling is a first step in the extravasation in pathological inflammatory processes, rolling does not necessarily commit a leukocyte to undergo extravasation, and occurs frequently under physiological conditions. In the absence of inflammation, while red blood cells traverse the postcapillary venules at 1-3 mm/s, the usual speed for leukocytes here is 5-300 μm/s with the majority traveling between 20-60 μm/s, with around 40% (20-70%) of these cells exhibiting rolling behavior at one time.[25] Thus, even in the absence of inflammation, selectin-mediated tethering of leukocytes assists normal physiological extravasation such as that which occurs when lymphocytes routinely patrol between the vasculature and secondary lymphoid organs, and also maintains other leukocytes in close proximity to the vascular wall, on high alert and ready to quickly respond at the first detection of an inflammatory stimulus.

The specific roles of individual selectins in mediating leukocyte trafficking have also been discerned in numerous animal studies.[26, 27] In selectin gene-knockout mice, L-selectin and P-selectin were shown to respectively mediate lymphocyte homing and inflammation.[28, 29] E-selectin-deficient mice did not show overt abnormalities of the inflammatory response, but closer scrutiny revealed the disappearance of the slow-rolling population of neutrophils in these mice.[30] Mice made doubly deficient in E- and P- selectin by dual gene knockout methods displayed a strong susceptibility to bacterial infections, thereby suggesting an overlapping function of these two selectins in mediating leukocyte response to infection.[31, 32] Recently, mice have been

generated that are deficient in all three selectins (E/L/P/-/-).[33] As expected, these mice display almost complete inhibition of leukocyte rolling and extravasation both basally and during inflammation.

In humans, the physiological relevance of the selectins is exemplified in leukocyte adhesion deficiency type 2 (LAD-2), a syndrome in which compromised immune responses and developmental defects in these patients are attributable to defects in selectin ligand structure.[34] Affected individuals have a markedly reduced ability of neutrophils to adhere to EC, and suffer recurrent bacterial infections. Neutrophils isolated from these individuals exhibit reduced binding to EC *in vitro*, and do not roll on intact venules under typical microvascular shear conditions.[35]

Immunoglobulin Superfamily Adhesion Molecules

Leukocyte capture from the circulation and rolling along the endothelium are both reversible phenomena. In the absence of additional braking mechanisms, rolling leukocytes will continue rolling downstream or may detach entirely from the vessel wall. The immunoglobulin (Ig) superfamily of adhesion molecules comprises one group of related molecules that function, among other things, to firmly adhere tethered leukocytes to the vascular lumen (reviewed in references 36 & 37). Structurally, these molecules contain one or more common 70-110 amino acid Ig-like domains (Figure 2). These CAMs are typically expressed on the extracellular surface of cells, although cleaved soluble forms of several of these molecules have been identified in the circulation, as well.[36] Typically, members of this family have a transmembrane domain and a short intracellular tail. In regulating inflammation, four of these Ig-like molecules have been shown to play important roles in leukocyte adhesion to EC: Intercellular adhesion molecules-1 and –2 (ICAM-1 or CD54, and ICAM-2 or CD102), vascular cell adhesion molecule-1 (VCAM-1 or CD106), and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) are EC-expressed molecules that bind to receptors present on leukocytes. A fifth Ig-family member, platelet

endothelial cell adhesion molecule-1 (PECAM-1 or CD31), has been primarily studied in the context of its mediating homotypic binding of adjacent EC, and thus of indirectly regulating leukocyte extravasation by modulating EC monolayer integrity.[37] More recent work, however, suggests that this molecule can also directly regulate leukocyte/EC interactions.[38]

The expression profiles of most of these Ig-like CAMs are tightly regulated to reflect their unique functions during inflammation. ICAM-1, for example, the first member of this group to be identified, is constitutively expressed by only a few cell types, but is induced in a variety of cell types during inflammation.[39] In EC, for example, ICAM-1 is basally expressed at low levels, but proinflammatory agents like LPS, TNFα, and thrombin strongly upregulate ICAM-1 expression to maximal levels within 8-12 hours.[40] VCAM-1 is barely detectable on quiescent EC, although this molecule, too, is potently induced by a variety of proinflammatory signals, albeit with different kinetics than ICAM-1.[41] MAdCAM-1 is specifically expressed on the high endothelial venules of lymph nodes and mucosal lymphoid tissues, and plays important roles in lymphocyte homing to these tissues. MAdCAM-1, too, is upregulated by cytokines such as TNFα.[42] Other adhesion molecules such as ICAM-2 and PECAM-1 are constitutively present on unstimulated EC, although they are not upregulated by the same inflammatory stimuli that induce ICAM-1.[43, 44]

The same experimental approaches that were used to discern selectin activities have also been used to evaluate the individual roles of the Ig superfamily CAMs in mediating tight adhesion of leukocytes to EC. ICAM-1-deficient mice by most appearances develop normally, but display impaired inflammatory responses.[45, 46] Monocyte adhesion to aortic EC cultured from ICAM-1-deficient mice occurred with only ≈15% the frequency of binding events to wild type EC.[47] ICAM-2-deficient mice also display a remarkably normal physiology, although eosinophil migration was slightly delayed in initial reports using a model of airway inflammation.[48] Other *in vivo* studies with ICAM-2 knockout mice and experiments using EC cultured from these animals

together demonstrate the importance of this molecule in T-cell extravasation during inflammation.[49] By contrast to ICAM-1 and –2 knockouts, VCAM-1 knockouts generated by classical methods are embryonic lethal.[50] Recently, conditional knockout mice have been developed that allow selective deficiency of VCAM-1 on EC and hematopoietic cells, while avoiding the problem of *in utero* death.[51] In these mice, lymphocyte homing to the bone marrow is markedly inhibited. Additional investigations into VCAM-1 involvement in inflammation using this model should be forthcoming. In addition to gene knockout studies, the involvement of all three of these Ig-family CAMs has been well characterized in a variety of inflammation models using neutralizing antibodies and antisense approaches.[49, 52, 53] Data from these studies suggest significant functional redundancy amongst these CAMs. Nonetheless, temporal and tissue-specific differences in CAM expression, together with the selective expression of their receptors on leukocyte subsets, provide an appreciable measure of selectivity in leukocyte recruitment during inflammation.

Integrins

The integrins (Figure 2) comprise a large family of noncovalently linked cell surface glycoproteins heterodimers that act as receptors for Ig-family CAMs and for extracellular matrix (ECM) proteins (e.g., fibronectin, vitronectin, laminin, etc.), and have diverse biological roles (reviewed in references 54 & 55). The name integrin reflects their important roles in integrating the cytoskeleton of their intracellular environment with the extracellular environments by binding to ECM components and to cell surface receptors. Combinations of the 18 known α-subunits (120-180 kDa) with the 9 known β-subunits (90-110 kDa), produces at least 24 receptors. Of these, 16 have been identified as important mediators of vascular biology, and 7 have been localized to EC.[54]

1E. Ocular Inflammation

In ophthalmology, a number of inflammatory pathologies preferentially affect one region of the eye, (*e.g.*, the iris or retina; Figure 5), while other nearby ocular tissues typically remain uninvolved. Anterior uveitis (AU) refers to a group of inflammatory disorders of disparate etiology that have in common the selective targeting of the iris/ciliary body (Figure 6).[73] Similarly, other ocular diseases are characterized by the selective inflammation of the choroid and/or retina.[74] One common feature of these diseases is the invariable and pivotal involvement of EC in mediating these inflammatory processes. To date, however, the essential roles of the iris and retinal microvascular EC, the "gatekeepers" in these ocular inflammatory processes, has not been thoroughly investigated.

Given the ethical and technical barriers to conducting mechanistic studies of AU and other ocular inflammatory diseases in humans, a variety of investigative models have been developed to address these questions, including numerous animal models, as well as cell and organ culture approaches.[73, 75] These systems have provided valuable insight into the biological underpinnings of ocular inflammation, and have revealed the importance of many cytokines, chemokines, and adhesion molecules in mediating ocular inflammation. However, these models

The integrin family is subdivided into categories based on shared biological functions.[54] The β_1 (CD29) integrins have a broad cell distribution and are constitutively localized to EC, epithelial cells, and fibroblasts, and some leukocytes. These β_1 integrins were originally called very late activation (VLA) antigens since the first that were identified appeared on lymphocytes only several weeks after exposure to activating antigen.[55] Although most β_1 integrins appear to bind to ECM proteins and play roles unrelated to leukocyte transmigration, VLA-4 ($\alpha_4\beta_1$) is an exception in that it expressed on a variety of leukocytes (neutrophils not included), and is a well-characterized ligand of endothelial VCAM-1.[56] Interestingly, another α_4 integrin ($\alpha_4\beta_7$), is expressed on subsets of B and T lymphocytes, and has been demonstrated to be important in recirculation of these cells through lymphoid tissues.[57] Thus in these two last cases, it is the α_4 and not the β subunit that dictates a role for these integrins in leukocyte trafficking.

In inflammatory processes, it is the β_2 (CD18) integrins that have dominated the investigative spotlight. These adhesive receptors have to date been localized exclusively to leukocytes, and have been demonstrated to play essential roles in meditating the leukocyte/EC interactions that are characteristic of inflammation, via binding to EC-expressed CAMs of the Ig superfamily. Indeed, the interactions between le13kocyte LFA-1 ($\alpha_L\beta_2$, CD11a/CD18) and endothelial ICAM-1 and –2, or between Mac-1 ($\alpha_M\beta_2$, CD11b/CD18) and endothelial ICAM-1, are well documented.[54] Thus, of the integrins, the β_2- and likely the α_4-containing subfamilies are the most important in regulating firm attachment of leukocytes to Ig-family CAMs on EC, and the subsequent efflux of these blood cells into the surrounding inflamed tissues.

1C. Chemokines

The chemokines comprise an expanding group of typically small polypeptide molecules (Figure 3) that mediate a variety of biological processes (Figure 4). Since the first chemokine (IL-

8) was characterized over 20 years ago,[58] a total of approximately 50 chemokines and 20 G-protein coupled receptors have been so far identified.in mice and humans.[7, 59] The small size and well-conserved structures of the chemokines makes them very suitable for identification from EST databases, and most of the recently identified members were discovered using this approach.[60] Structurally, the chemokines are divided into four subfamilies, based on their arrangement of highly conserved cysteine residues (Figure 3) Most chemokines have four such cysteines (lymphotactin, with two cysteines, is the exception), and are categorized based on the organization of their first two cysteines into alpha (CXC), beta (CC), gamma (C), or delta (CX_3C) classes. The β-class is further subdivided into those with an ELR (Glu-Leu-Arg) motif preceding the conserved cysteines, which confers angiogenic activity, and those without.[61] The receptors are also divided into four subfamilies, i.e., CR, CCR, CXCR, and CX_3CR, that interact with ligands from the respective group.

In addition to their role as leukocyte chemoattractant molecules, chemokines are now known to mediate various other biological processes including tumor metastasis, angiogenesis, stem cell homing, immune cell differentiation and function, and viral infectivity (Figure 4).[59] In mediating leukocyte efflux in inflammation, chemokines play two primary roles, i.e., activation of leukocytes and guiding their migration. First, after leukocytes are captured from the flowing blood by adhesion molecules on the vascular wall, chemokines expressed on the endothelial surface bind leukocyte receptors. Chemokine/receptor ligation results in activation of leukocytes, with cell surface integrins undergoing structural reorganization to a high avidity state, thus promoting firm adhesion to the vascular lumen.[62] Additionally, chemokine stimulation of attached leukocytes also induces them to release a variety of biological effectors, including additional chemokines, cytokines, reactive oxygen species, and proteases.[7] These molecules promote the recruitment and activation of additional leukocytes, and alters the EC monolayer integrity and the underlying ECM architecture so that leukocyte diapedesis is facilitated. Second,

when situated in gradients of soluble chemokines, leukocytes bearing the appropriate receptors polarize their morphology and migrate toward the source of these chemical gradients.[63] These morphological changes have important implications for the migrating leukocyte. When initially attaching to the vascular wall, chemokine-induced shape changes include flattening of the leukocyte to reduce the size of its profile presented to the flowing blood, thus minimizing the likelihood of detachment. Once a leukocyte is arrested from the bloodstream, this cell utilizes stromal-derived chemokines as directional cues while migrating between adjacent EC and into the surrounding tissues.

The existence of a large number of chemokines and their receptors suggests a large measure of functional redundancy among these molecules. There also seems to exist a high degree of tissue-specificity in chemokine-mediated leukocyte trafficking. The first chemokines to be identified displayed the widest tissue distribution. More recently, several chemokines possessing much more restricted expression profiles have been identified using expanding genome/EST databases.[7] Conversely, leukocytes of different derivation appear to possess widely divergent chemokine receptor profiles, suggesting that these cells respond uniquely to the chemokine cocktail present in a given tissue environment.[64] Leukocyte chemokine receptor expression profiles appear to be determined by not only lineage, but also by the cell's differentiation status. In the thymus, for example, segregation of maturing thymocyte subsets to the cortex versus medulla appears to result from differential responses to the local chemokine milieu.[65] Importantly, chemokine binding can elicit distinct migrational activities in a target cell, depending on the ligand/receptor combination employed. One example of these diverse actions of chemokines is the regulation of initial firm attachment of emigrating leukocytes by GROα and fractalkine (FKN), while MCP-1 mediates subsequent leukocyte spreading and diapedesis.[66] Thus, the sensing and signaling employed by chemokine family members is very precisely tuned to appropriately direct leukocyte traffic in a variety of biological scenarios. Moreover, although

some chemokines and receptors are constitutively expressed, many of these molecules are inducible by a variety of inflammatory mediators.[59] Thus, multiple levels of regulation govern chemokine activity, and coordinate these molecules' function in a variety of both overlapping and distinct biological processes.

Chemokines appear to play key roles in the pathogenesis of a variety of human inflammatory disorders, including rheumatoid arthritis (RA), multiple sclerosis, Grave's disease, and systemic lupus erythematosus.[7] In RA, for example, inappropriate recruitment and reduced apoptosis of infiltrating leukocytes results in their accumulation in diarthroidal joints. In arthritis, these cells mediate the chronic synovitis and eventual cartilage and bone destruction that are hallmarks of the disease. Elevated levels of several chemokines (*e.g.*, MCP-1, MIP-1α, IL-8, MIG, SDF-1, etc.) have recently been reported in specimens from RA patients, suggesting a role for these molecules in the inordinate accumulation of leukocytes in affected joints.[67]

1D. Tissue Specificity in Inflammation

Although inflammatory processes throughout the body share the same basic mechanistic paradigms, the existence of more subtle tissue-specific differences in regulating these processes is becoming increasingly evident. During sepsis, for example, fluid loss from the vascular compartment is not uniform throughout the body but instead preferentially occurs in the vascular beds of the pulmonary, hepatic, and abdominal regions.[68] Tissue specificity is also supported by the observed etiologies of inflammatory diseases such as RA, wherein inflammation occurs selectively in some tissues while other nearby tissues are spared.[67] In order for leukocytes to accumulate in inflamed tissues, these cells must first interact with the microvascular endothelium in that tissue. While selective interaction of leukocytes with specific microvascular beds clearly occurs, the underlying mechanisms of this selective

engagement remain controversial. Site-specific differences in vascular involvement during inflammation are likely attributable, in part, to variations in local environmental factors such as hemodynamics and perivascular cellular and extracellular matrix compositions. Other work, however, suggests that there exist inherent genetic differences amongst EC from different microvascular beds, and that these differences might be important determinants of a tissue's susceptibility to involvement in inflammatory processes.[69-72] Relevant differences reported to date include morphology, adhesion molecule expression, and susceptibility to neutrophil-mediated damage.

do have certain shortcomings. For example, observations from gene-deficient animals or neutralizing antibody studies can sometimes prove difficult to interpret when these approaches are used to investigate systems with high degrees of redundancy (*e.g.* adhesion, chemoattractant). Also, species differences can make it difficult to extend the findings from animal studies over to the human condition.

In several scenarios, inflammation preferentially occurs in one ocular tissue, while other nearby tissues are spared.[73] It remains unclear whether inherent biological diversity exists amongst EC derived from different ocular tissues, and if this diversity might explain tissue-specific inflammation. This gap in knowledge provided the primary impetus for the dissertation studies reported hereafter, which focus on testing the hypotheses that intrinsic biological differences exist between human iris and retinal EC. The obvious corollary, although beyond the scope of the current dissertation, is that heterogenenous inflammatory mediator expression between the iris and retinal microvasculature might render one ocular tissue more prone to becoming inflamed.

In this work, I detail the development and characterization of a donor-matched human iris and retinal EC culture model, for use in testing the hypothesis that inherent tissue-specific biological differences exist among the vascular beds of iris and retina. Using this model, in conjunction with various gene and protein expression assays, I have undertaken an in depth characterization of the molecular profiles of these tissue-specific ocular EC. By identifying molecular expression profiles of these cells in constitutive and cytokine-activated (*i.e.*, simulated inflammation) scenarios, I have begun to unravel the extraordinary molecular complexity that underlies ocular inflammation. Ultimately, these approaches, in conjunction with additional laboratory and clinical investigative efforts, might together present us with novel molecular targets at which highly specific anti-inflammatory therapies can be aimed.

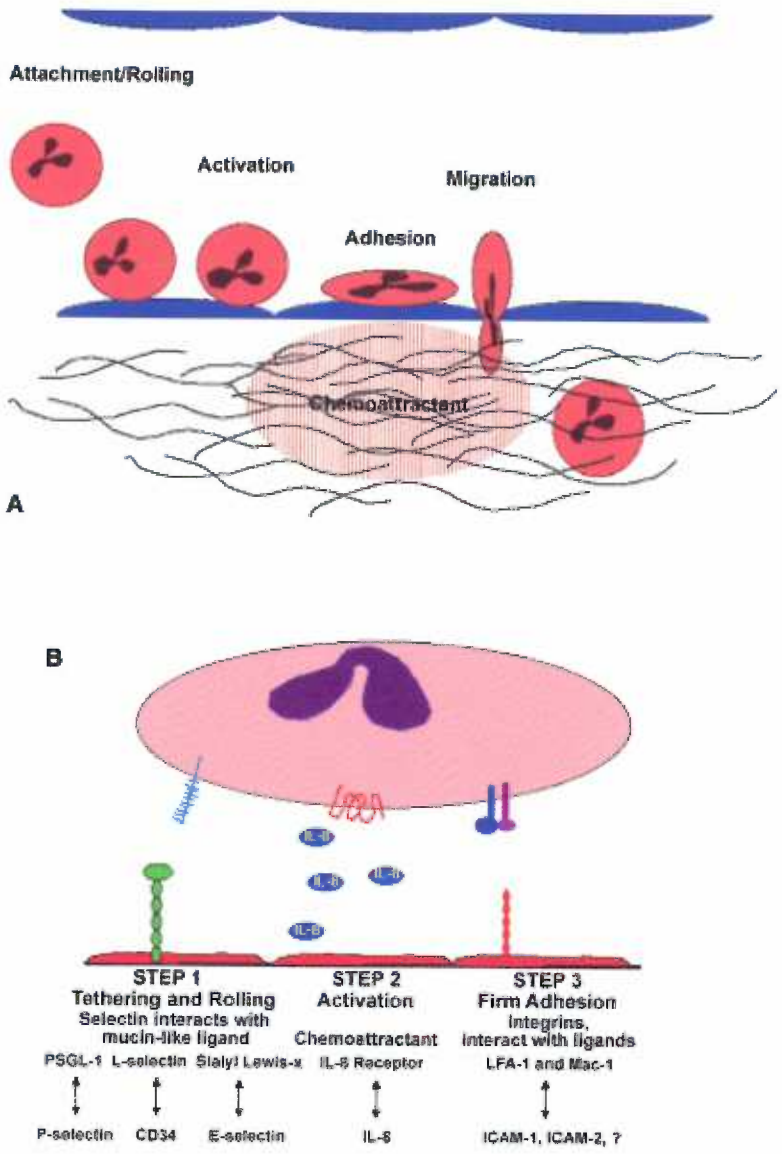

Figure 1. The recruitment of leukocytes from the circulation. As seen in (**A**), recruitment of leukocytes from the circulation to the site of inflammation is a complex and well-orchestrated event that can be divided into four steps, attachment and rolling, activation, adhesion, and migration. Specific molecular events play a role at each step, as depicted in (**B**). Although selectins play a central role in tethering and rolling, chemokines are secreted and activate the neutrophil, and integrins are involved with firm adhesion, the exact molecular details of the regulation of these events remain under intense scrutiny. Adopted from Petruzzelli, *et. al.,* 1999.

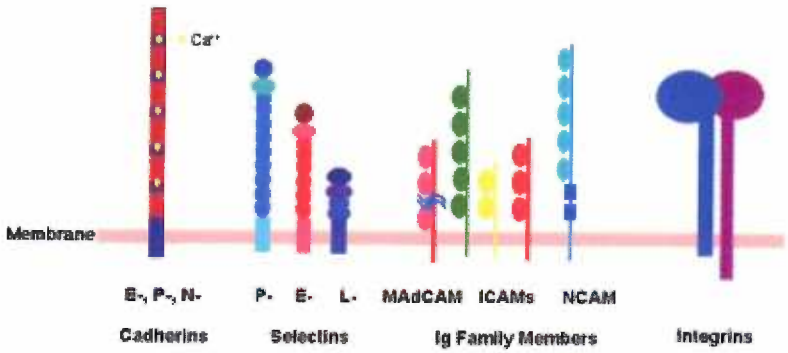

Figure 2. Schematic representation of four classes of adhesion molecules, the cadherins, selectins, Ig family members, and integrins. The cadherins are transmembrane proteins that contain, in the extracellular domain, five motifs that are bridged by Ca^{++}. The selectin family members are transmembrane proteins that contain a N-terminal lectin domain, an EGF-type domain, and two to nine complement regulatory repeats. The Ig family members are structurally most diverse, but each have two to five Ig repeats in their extracellular domain and most, but not all, are transmembrane proteins. The integrins are noncovalently linked heterodimers composed of α and β subunits. Adopted from Petruzzelli, *et. al.*, 1999.

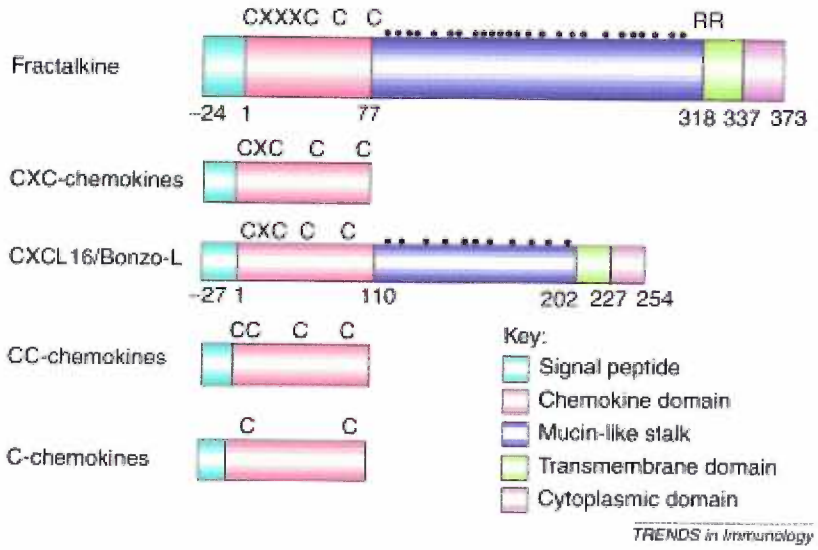

Fig. 3. Schematic structure of chemokines. Fractalkine is a large protein of 373 amino acids containing multiple domains and is structurally distinct from the CXC-, CC- and C-chemokines. Beginning with the predicted signal peptide, fractalkine comprises an N-terminal chemokine domain (residues 1-76) with a unique three-residue insertion (CX_3C), a mucin-like stalk (residues 77-317) with predicted *O*-glycosylated serine and threonine residues (●), a transmembrane domain (residues 318-336) and an intracellular domain (residues 337-373). RR is a membrane-proximal dibasic motif similar to a dibasic cleavage site in syndecans. The CXCL16 chemokine has several features in common with fractalkine and is predicted to be membrane bound. Adopted from Umehara, *et. al.*, 2001.

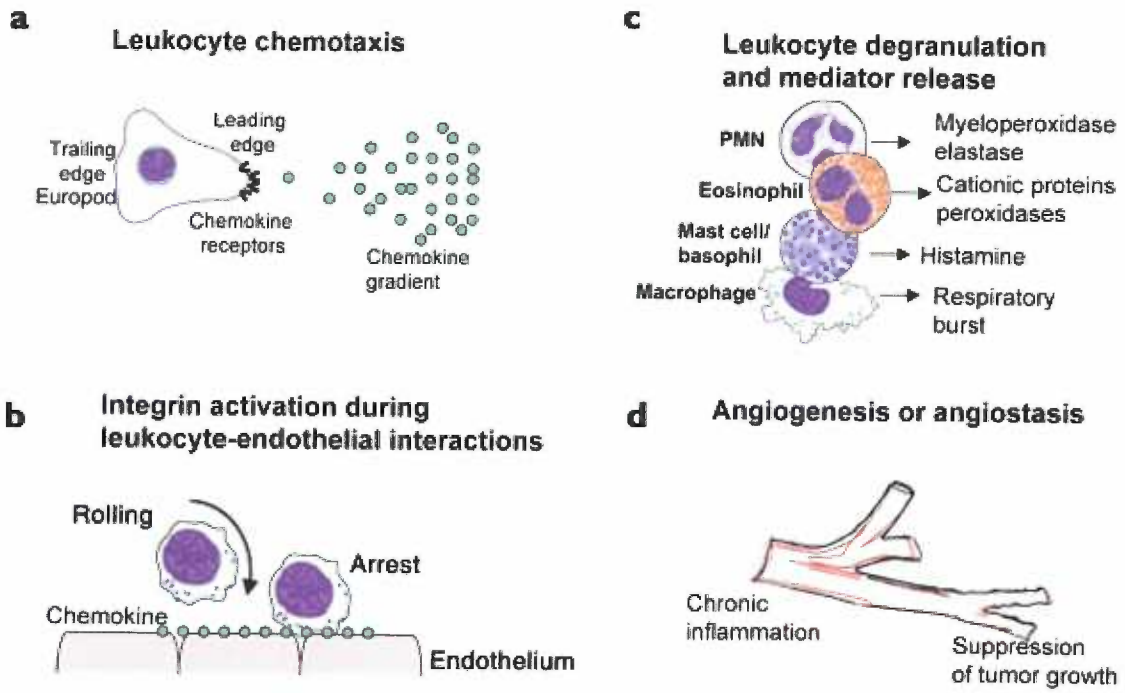

Figure 4. Biological functions of chemokines. (**a**) Chemoattractants provide directional cues for leukocyte motility through the formation of gradients that migrating cells detect. (**b**) Chemokines activate leukocyte integrins during rolling and attachment. (**c**) Chemokines stimulate leukocyte degranulation or release of inflammatory mediators, and the respiratory burst, which results in production of reactive oxygen intermediates. (**d**) Chemokines can also stimulate angiogenesis or angiostasis. The biological relevance of angiogenic or angiostatic properties of chemokines could relate to tumor suppression or to inflammatory responses where angiogenesis is an important requirement for disease progression. These four different properties of chemokines are used in combination for various biological responses. For example, tumor rejection involves recruitment of leukocytes from blood, chemotaxis to the tumor and angiostasis; allergic inflammation involves leukocyte recruitment and release of inflammatory mediators. Adopted from Mackay, *et. al.*, 2001.

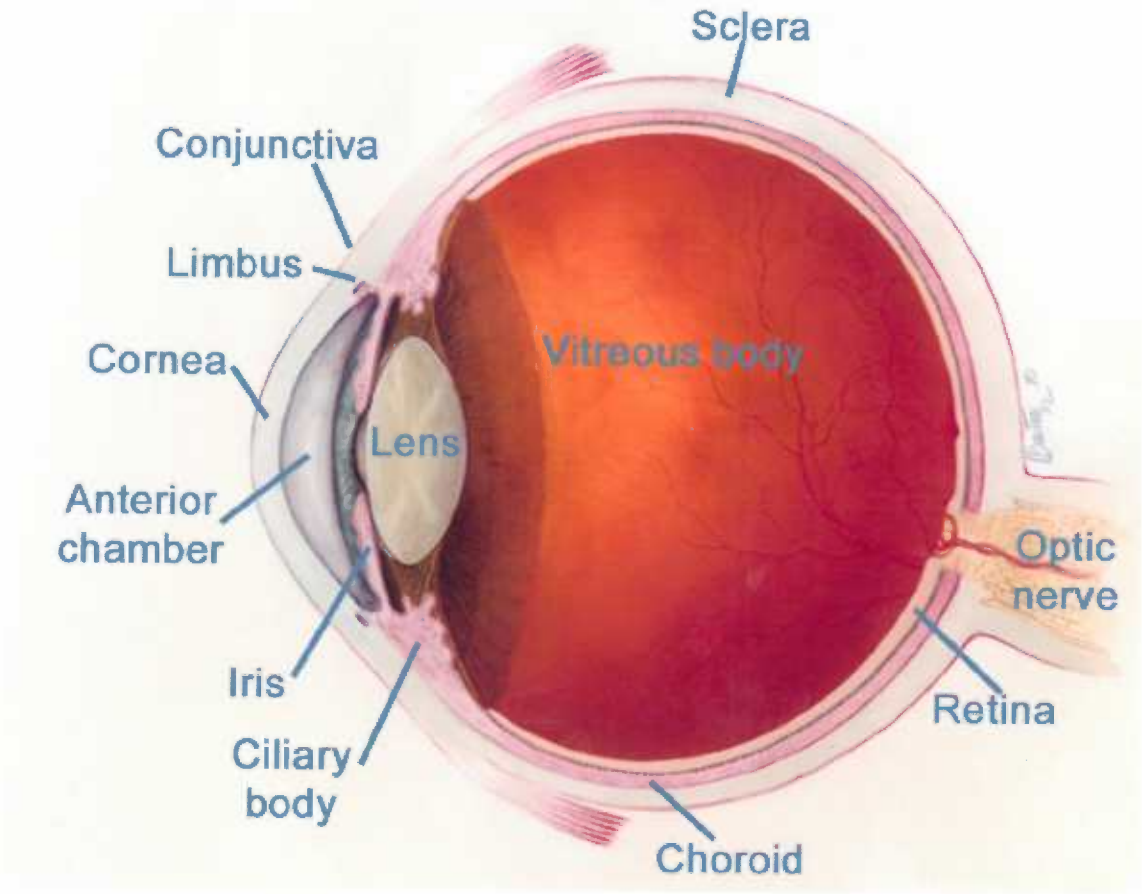

Figure 5. Schematic of the eye. The uvea, derived from the Latin term for "grape", includes the iris, ciliary body, and choroids. Anterior uveitis is manifested in inflammation of the iris (iritis) and in some cases the ciliary body (iridocyclitis). Posterior uveitis involves the choroid and sometimes encompasses the retina, as well (retinochoroiditis). Retinitis may also occur in the absence of other tissues' involvement. Adapted from the NEI website, with modifications made by Dr. Stephen Planck.

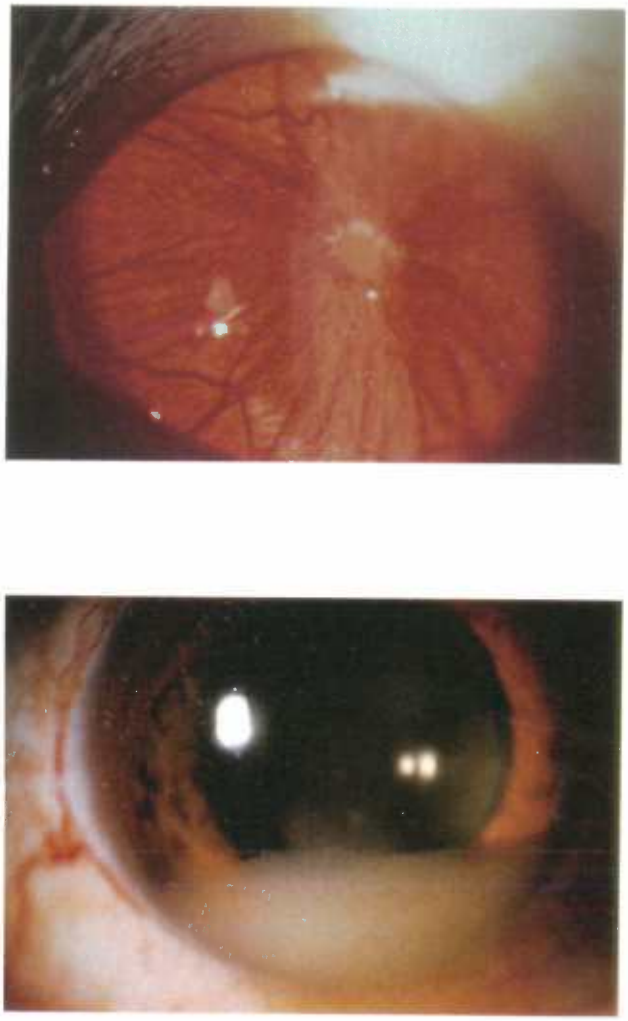

Figure 6. Clinical manifestations of acute anterior uveitis. Top panel depicts a rat eye that was injected intraocularly 16 hours prior with bacterial lipopolysaccharide (LPS), resulting in intense iridial hyperemia and a fibrinous exudates in the pupil area. **Bottom** panel : Gravity sedimentation of large numbers of infiltrating leukocytes within the anterior chamber (hypopyon) in a patient with acute anterior uveitis. Adopted from BenEzra, 1999.

In satellite studies to evaluate protein expression in mouse eyes, normal mice (F1 hybrids of C57BL/6J and 129/J; Jackson Laboratories, Bar Harbor, ME), received food and water *ad libitum*, and were treated in compliance with the guidelines of the ARVO Resolution on the Use of Animals in Research. Upon sacrifice, mouse eyes were enucleated, dissected, and processed for total RNA isolation as described below for human tissues.

Iris and Retinal Explants

For iris dissection, corneas, if present, were excised, and irises were gently disengaged from surrounding tissues using a forceps. Irises were aseptically transferred to 60 mm^2 sterile petri dishes containing Dulbecco's phosphate buffered saline (PBS), and any attached ciliary body tissue was removed with a scissors. For iris cell culture establishment, pigment epithelial cells were manually removed using a sterile cotton swab in conjunction with several washes in fresh PBS prior to cell isolation (detailed below). Retinas were manually separated using forceps from the underlying choroid of the posterior two-thirds of the poles, after elutriation of the vitreous humor. For retinal cell culture establishment, pigment epithelial cells were manually removed as described for iris tissues above. For immunohistochemical studies, iris and retinal specimens were fixed in 4% paraformaldehyde (4°C, overnight), paraffin embedded, and sectioned into 5-μm thin sections.

Human Iris Endothelial Cell (HIEC) Cultures

Depigmented irises were digested in 0.2% type II collagenase (Sigma) in medium MCDB-131, for 20-30 min at 37°C. Following digestion, EC were purified from iris stromal cells using monoclonal anti-human platelet-endothelial cell adhesion molecule-1 (PECAM-1) antibody-coated magnetic beads (Dynal, Inc.), and were cultured in MCDB-131 supplemented with 10% fetal bovine serum (FBS, Life Technologies), endothelial cell growth factors (EGM-

MV2 BulletKit™, with hydrocortisone omitted; Clonetics/Biowhittaker; complete medium), gentamicin (10 μg/ml), and amphoteracin-B (Fungizone®, 250 ng/ml, Gibco). Cultures were trypsin passaged at a 1:3 split ratio, and used in subsequent experiments between passages 3 and 6. EC-depleted iris stromal cell cultures were propogated in the above medium, without supplemental growth factors, and were used between passages 3-4. In a limited number of experiments, human choroid EC (HCEC) cultures were generated using identical methods employed with iris tissues.

Human Retinal Endothelial Cell (HREC) Cultures

Retinas collected as described above were digested overnight at 37°C, in complete medium containing 0.3 mg/mL dispase (Sigma), with gentle rocking. Following gentle trituration the next morning, intact microvessels were recovered from the surrounding stromal tissue that had been digested away. These vessels were further digested, with 0.2% collagenase type II in MCDB-131, (37°C, up to 20 minutes with gentle mixing), to yield single cell suspensions from which EC were immunomagnetically isolated and propogated, and EC-depleted stromal cells cultured, as described above for iris cultures. In some preliminary experiments, we tested commercially available human retinal EC (Applied Cell Biology Research Institute, Kirkland, WA).

Confirmation of EC Nature of Cultured Cells

Following one or two rounds of magnetic separation, EC cultures were ≥99.5% pure on the basis of PECAM-1 and von Willebrand factor (vWF) expression,[71, 76] and uptake of dil-acetylated-LDL.[77] In specialized culture conditions with a provisional extracellular matrix, monolayers of EC are capable of reorganizing into capillary-like networks.[78] Seventy-five thousand EC were plated on polymerized Matrigel® (Beckton-Dickinson, Inc., 14.1 mg/ml, 200

μl/well) within 24-well tissue culture plates, and were allowed to attach overnight. Cells were then refed, with some wells receiving 10 ng/ml phorbol myristate acetate (PMA; Sigma) to induce tube formation.[79]

2C. mRNA Expression Analysis

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Expression of mRNA for multiple cytokines, adhesion molecules, and chemokines were evaluated in ocular explants, EC, and/or stromal cell cultures; specific primer pair sequences employed are tabulated below. For mRNA studies, ocular explants or confluent EC or stromal cells grown in 6- or 12-well tissue culture plates were stimulated with various combinations of cytokines for up to 24 hours. At experimental termination, tissues or cell monolayers were washed in PBS, and total RNA was isolated using a commercial kit (RNApure, GenHunter Corp, Nashville, TN; or RNEasy™, Quiagen, Inc., Valencia, CA). RNA samples were stored in nuclease-free water at -80°C until used in RT-PCR reactions.

Touchdown RT-PCR detection of gene expression was done as previously described in detail.[80] First strand cDNA synthesis was accomplished with oligo (dT)-primed MMLV reverse transcriptase (BRL Life Technologies, Rockville, MD). Gene-specific cDNA was amplified by a hot start/touchdown PCR procedure, using Taq polymerase (Applied Biosystems, Foster City, CA) and specific primer pairs. Twenty touchdown cycles were run, with a stepwise decrease in annealing temperature (1°C every two cycles) from 69°C to 60°C. Twelve to twenty additional cycles were then run at a constant 55°C annealing temperature, followed by a final 7-minute elongation step at 72°C. Positive-control total RNA was obtained from synovial samples of rheumatoid arthritis patients. Nuclease-free water (Ambion, Inc.) was used as a negative-control. A primer pair for either constitutively expressed glyceraldehyde 3'-phosphate dehydrogenase (GAPDH) or cyclophilin was included in each assay as an internal control. PCR reaction products

were electrophoresed in 3% agarose gels in Tris-acetate buffer containing ethidium bromide, and UV-induced fluorescent bands were photographed.

RT-PCR Primers

The following human genes were evaluated using the following indicated primer sets, which were designed by SR Planck, PhD, and were synthesized by either Operon Technologies, Inc., or by Integrated DNA Technologies, Inc:

ICAM-1	Sense, 5'–CCGGAAGGTGTATGAACTG–3' Antisense, 5'–TCCATGGTGATCTCTCCTC–3'
ICAM-2	Sense, 5'-CCGTGGCAATGAGACTCTGCACTA-3' Antisense, 5'-ATGGTTGCTATGGCCGGAAGG-3'
VCAM-1	Sense, 5'-CTCCGTCTCATTGACTTGC-3' Antisense, 5'GAACAGGTCATGGTCACAG-3'
IL-1α	Sense, 5'-GTCTCTGAATCAGAAATCCTTCTATC-3' Antisense, 5'-CATGTCAAATTTCACTGCTTCATCC -3'
IL-1β	Sense, 5'-AAACAGATGAAGTGCTCCTTCCAGG-3' Antisense, 5'-TGGAGAACACCACTTGTTGCTCCA-3'
IL-6	Sense, 5'-CCTTCTCCACAAGCGCCTTC-3' Antisense, 5'-GGCAAGTCTCCTCATTGAATC-3'
FKN	Sense; 5'-CAGAGGAGAATGCTCCGTCTGAAG-3' Antisense; 5'-CAGAAGAGGAGGCCAAGGAAGG-3'
Cyclophilin	Sense, 5'–TGTTCTTCGACATTGCCGTCGAC–3' Antisense, 5'–GCATTTGCCATGGACAAGATGCCAGGA– 3'
GAPDH	Sense; 5'-AGCTGAACGGGAAGCTCACTGG-3' Antisense; 5'-GGAGTGGGTGTCGCTGTTGAAGTC-3'

2D. cDNA Array Analysis of EC Gene Expression Profiles

We used two different nylon-based cDNA array kits from Clontech, Inc.'s (Palo Alto, CA) Atlas Array™ series; the human cDNA array (catalog #7740-1) measures expression of 588 known genes of various functional classes, and a human hematology/immunology array (catalog #7737-1), which evaluates 406 known genes with a focus on immune/circulatory system activities (*e.g.*, a larger proportion of chemokines and adhesion molecules are targeted). Both kits use nylon membranes with target sequences spotted in duplicate for quality control. Five μg aliquots of DNAsed (MessageClean™ kit, Genhunter, Nashville, TN) total RNA aliquots were MMLV-reverse transcribed into α-^{32}P-ATP-labeled cDNA probes, using kit-specific primer collections, according to manufacturer's instructions. Radiolabeled probes (2-20 x 10^6 cpm) were hybridized overnight to DNA arrays, which were then washed and exposed for varying timepoints (2 hrs to 1 week, depending upon probe specific activity) to X-ray film (Kodak Biomax-MS), at -80° C. After developing, images were digitized and densitometrically analyzed using AtlasImage 2.0 software (Clontech). The linear range of densitometry data ranged from 1000 to 45000 arbitrary densitometric units, after background subtraction. Genes whose expression were not detectable were artificially assigned a value of 1000 for calculating fold changes in expression. Data are presented as average fold differences between control vs stimulated conditions within a cell type, or average fold differences between HIEC and HREC, after subtraction of non-specific background signals, and normalization to the median intensity of all gene signals on the array. Signal intensities between comparison groups were considered to be significantly different if they met the following two criteria: 1) they were at least two-fold increased, or decreased by 50%, relative to one another; and 2) the p value was less than 0.05 when the means of two groups were compared by ANOVA (SigmaStat, Jandel Corp).

2E. Protein Analysis

Enzyme-Linked Immunosorbent Assay (ELISA)

Soluble IL-6, IL-8, MCP-1, and E-Selectin were measured in EC-conditioned media using commercially available ELISA kits (R & D Systems), according to manufacturer's instructions. Measured values were normalized to cell number, as evaluated using a fluorimetric cell enumeration kit (CyQuant, Molecular Probes, Inc.), which evaluates total nucleic acids in lysed cell samples. Preliminary studies demonstrated that this method gives a reliable linear range of quantitation of between 50 and 30,000 EC when employed in a 96-well format, according to manufacturer's instructions.

Enzyme-Linked Immunocellular Assay (ELICA)

Cell surface protein expression of ICAM-1, ICAM-2, VCAM-1, E-selectin, and fractalkine (FKN) was evaluated on intact EC monolayers using a fluorescent enzyme-linked immunocellular assay (ELICA) in a 96-well microtiter plate format. Mouse monoclonal anti-human ICAM-1, VCAM-1, and E-selectin antibodies, rabbit polyclonal anti-human ICAM-2 antibody, and goat polyclonal anti-human FKN were used as primary detection antibodies (all diluted to 1 μg/ml in 1% bovine serum albumin/bicarbonate buffered saline). Affinity-purified, alkaline phosphatase-conjugated, goat anti-mouse or anti-rabbit, or sheep anti-goat polyclonal antibodies (1:1000, Sigma) were used for secondary detection. Fluorescent ELICAs were performed as previously described in detail.[81] Briefly, after a short glutaraldehyde fixation in situ, and blocking in 20% FBS/PBS, cell surface molecules were detected with the appropriate primary detection antibody, which were then decorated with an appropriate alkaline phosphatase-conjugated secondary antibody. Detection of bound antibody utilized the fluorogenic alkaline phosphatase substrate, methylumbelliferyl phosphate (Sigma; 100 μg/mL in bicarbonate-buffered saline). Fluorescence was measured using a fluorescence microplate

reader (Bio-Rad Laboratories, Hercules, CA) with 360/460 nm excitation/emission wavelengths. Background fluorescence from cells in which primary detection antibody incubation was omitted was subtracted from experimental values. All conditions were replicated in 3 to 6 wells/experiment, for the indicated number of experiments.

Western Blotting

In some cases, ELICA results were confirmed by western blotting in which HIEC monolayers were washed in PBS and then collected in immunolysis buffer (20mM Tris base, 137mM NaCl, 10% glycerol, 1% Triton-X100, and protease inhibitors). Lysates equivalent to 3 x 10^4 cells/lane were electrophoresed on 4-15% linear gradient SDS-PAGE gels (BioRad, Inc., Hercules, CA), followed by transfer to nitrocellulose membranes and protein detection with the same antibodies that were used in the ELICA. Bands were visualized using an NBT/BCIP detection kit (Vector Labs), and images were photographed.

2F. Immunohistochemistry

Five-micron sections of formalin-fixed, paraffin-embedded iris and retina explants were mounted on microscope slides, xylene-deparaffinized, ethanol-dehydrated, and sections were treated with proteinase K (20 μg/ml, 20°C, 5 min), to assist antigen retrieval. Sections were then washed in 0.1% Tween 20/Tris-buffered saline, pH 7.4 (TBST), and nonspecific binding sites were blocked with 2% bovine serum albumin/TBST. FKN detection employed mouse monoclonal anti-human FKN antibody (20 μg/ml, 20°C, 4 hrs; R & D Systems), followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG (1:25 dilution, 20°C, 2 hrs; Sigma cat # A-2179), both diluted in blocking solution. FKN was visualized using a Fast Red substrate kit (BioGenex, Laboratories, San Ramon, CA).

2G. Leukocyte Adhesion Assays

To ascertain the utility of the ocular EC cultures as an *in vitro* model of ocular inflammation, I tested the ability of activated HIEC cultures to adhere U937 monocytes (ATCC). HIEC were grown to confluence in 48-well tissue culture plates and were then stimulated with either LPS (10 μg/ml), TNFα (10 ng/ml), or VEGF (100 ng/ml, R&D Systems) for 12 hrs. In some cases, LPS-stimulated HIEC were incubated with either anti-ICAM-1 mAb (20 μg/ml), anti-ICAM-2 pAb (10 μg/ml), or anti-VCAM-1 mAb (all reportedly neutralizing at these concentrations; same sources as those used for protein detection studies), for the last 30 minutes prior to monocyte addition. All stimulations and antibody incubations were done in complete medium at 37°C. Following EC treatments, the wells were aspirated, quickly washed with MCDB131/10% FBS, and 500 μl of the same medium containing 2.5 x 10^5 monocytes was added to each well and incubated for 15 minutes at 37°C. After gently washing off unbound monocytes, the EC monolayers and adherent monocytes were briefly fixed with 0.5% glutaraldehyde, and attached monocytes were visually counted (four 1-mm^2 regions per well).

We employed similar adhesion assays to discern the relative involvement of cell-surface and soluble E-selectin in mediating leukocyte adhesion to ocular microvascular EC. In these experiments, human retinal EC were grown to confluence in 96-well culture plates, in complete tissue culture medium MCDB131. EC monolayers were dose-dependently stimulated with TNFα (0 to 1 ng/ml) for various timepoints (2-8 hrs) to upregulate cell surface E-selectin. Following three media washes (MCDB131 containing 10% FCS and antibiotics, growth factors omitted), of EC, 2 x 10^5 fluorescently tagged leukocytes (in this case U937 monocytes, Jurkat T-cells, or freshly isolated human polymorphonuclear cells), were added to each well in 200 μl wash medium, and allowed to sediment and

attach for 15 minutes. Leukocytes had been fluorescently labeled, after washing in PBS twice, by incubating 10 to 40 x 10^6 cells/ml in PBS containing 5 μM 5-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes Inc., Eugene, OR), for 15 minutes at 37°C, according to manufacturer's recommendations. Labeled leukocytes were then washed once in PBS and resuspended in MCDB131 containing 10% FCS in preparation for attachment assays. In some cases, neutralizing antibody against E-selectin (sheep anti-human E-selectin polyclonal antibody or recombinant human soluble E-selectin (both from R & D Systems, Inc.), were added to the leukocyte suspensions immediately prior to conducting the adhesion assays. After the attachment period, wells were washed 3x with 200 μl/well of serum-free media. Bound leukocytes were quantified by evaluating the fluorescence of each well on a microplate reader, with excitation and emission filters set at 495 nm and 530 nm, respectively. All experimental conditions were conducted in 4-8 wells per condition per experiment, and were repeated in 2-3 experiments. Background fluorescence from wells containing EC only was subtracted from fluorescence measured in wells in which leukocytes had been incubated with EC.

2H. Statistical Analysis

Data presented for ELISA/ELICA represent mean fluorescence/well or per unit number of cells, as indicated in the figure legend. Data presented for monocyte adhesion assays represent number of monocytes bound/mm^2 HIEC monolayer surface area, or mean fluorescence/well, for the indicated number of replicates. Data from gene arrays are presented as percentage or fold change versus the appropriate controls, as indicated. Data presented for Asterisk (*) in figures indicates a significant difference ($p < 0.05$) between the means of experimental and respective

control groups, by ANOVA (SigmaStat™ 2.0 software, Jandel Corp.), and all error bars indicate standard deviation (SD).

Chapter 3

Isolation, Culture, and Characterization of Human Ocular Microvascular Endothelial Cells

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The work that comprises this chapter appeared, in part, in published form in *Investigative Ophthalmology and Vision Science*, 42(12):2861-6 (2001). The experiments for Figure 1 were a joint effort of MDS, DOZ, and YP. The experiments for Figure 2 were a joint effort of MDS and DOZ. The experiments in Figures 3, 5, 6, and 8 were performed by MDS. The experiments in Figures 4 and 7 were performed by MDS and YP, with the technical assistance of Leslie O'Rourke and Xiaona Huang.

Summary

Considering the scientific community's increasing appreciation of EC heterogeneity as it might pertain to pathogenesis, we sought to establish pure cultures of human iris, retinal, and choroid microvascular EC (HIEC, HREC, and HCEC, respectively), to test as an *in vitro* model of vascular involvement in ocular inflammation. Here we detail the development of methods to reliably isolate pure human ocular microvascular EC, ascertain their maintenance of a differentiated EC phenotype, and explore their constitutive and inflammatory agent-modulated expression of ICAM-1 and –2, VCAM-1, and E-selectin, IL-1, IL-6, and IL-8. These results demonstrate the establishment and propagation of cultured ocular microvascular EC that maintain their expression of typical endothelial morphological, biochemical, and functional criteria. These cells respond to inflammatory agents such as TNFα and LPS, by increasing their functional expression of various adhesion molecules, chemokines, and cytokines. In addition to being the first report of culturing HIEC, this is also the first documentation of functional ICAM-2 expression, and its regulation by inflammatory cytokines, in any ocular cell type. Thus, in addition to elucidating the regulated expression in HIEC of molecules with known roles in mediating ocular inflammation, these data suggest a likely role for ICAM-2, as well, in mediating leukocyte efflux into the inflamed iris.

Introduction

The molecular mechanisms responsible for the tissue-specific inflammation in anterior uveitis (AU) are incompletely understood. AU is often associated with systemic illnesses such as ankylosing spondylitis, inflammatory bowel disease, Behcet's disease, juvenile rheumatoid arthritis, reactive arthritis or sarcoidosis.[73, 75] However, AU may also arise in an idiopathic fashion without an associated systemic disease, or it may be secondary to a localized infection such as herpes simplex virus.[82] Regardless of its etiology, the mechanism for triggering an inflammatory response specifically in the iris, while often sparing other ocular tissues, remains unknown. Recently, much emphasis has been placed on understanding how cells in the bloodstream preferentially penetrate specific tissues, e.g., in leukocyte and stem cell migration, and in cancer cell metastasis.[9] In AU, transmigration of leukocytes into the anterior chamber requires that endothelial cells (EC) and the leukocytes within the iris microcirculation become "activated", and then physically approximated. These leukocytes can then leave the bloodstream and migrate through the iris stroma, often into the anterior chamber. Although leukocytes are vital cells in the pathogenesis of inflammation, the essential role of the iris microvascular EC, the gatekeepers in AU inflammatory processes, has received little attention.

Given the limitations of investigating the molecular mechanisms of AU directly in human subjects, a variety of animal models of ocular inflammation have been developed, including endotoxin-induced uveitis (EIU), experimental-autoimmune uveoretinitis (EAU), experimental melanin-induced uveitis, and experimental-autoimmune encephalomyelitis.[73, 75] These models attempt to mimic human disease and have provided important insights into the etiology of AU. Nonetheless, species differences often make it difficult to translate the findings directly from these animal studies over to the human condition. Tissue culture systems provide another means to investigate the biology of ocular inflammation, and others have previously isolated and cultured human choroid and retinal EC for this purpose.[83, 84] Functional heterogeneity is known

to exist amongst cultured EC derived from the microvasculature of different, often proximally situated, tissues.[70, 71, 85] Thus, in order to evaluate microvascular involvement in AU in an *in vitro* system, we developed a methodology to selectively culture human iris endothelium (HIEC).

In this report we provide the initial characterization of cultured HIEC and describe the constitutive and inflammatory agent-regulated expression of representatives of the cell adhesion molecule, chemokine, and cytokine families in HIEC. These molecules are likely key mediators of the leukocyte infiltration observed in AU. Leukocyte adhesion assays suggest that in addition to intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), both of which have been previously implicated in ocular inflammation, ICAM-2 also has the potential to play an important role in leukocyte extravasation through the iris microvascular wall in uveitis.

Results

Ocular EC Culture Establishment

Initial digestion of iris tissue yielded a mixed cell population that included cells with morphologies characteristic of EC, fibroblasts, smooth muscle cells, pigment epithelial cells, and pericytes. After a single PECAM-1-based magnetic bead separation, we were able to significantly reduce the number of contaminating cells (Figure 1). We have consistently been able to generate, within two weeks, essentially pure experiment-ready HIEC cultures. To date, we have been successful with over 40 donors. The monolayers thus generated are ≥99.5% pure, as assessed by their cobblestone morphology (Figure 1), and by biochemical and functional criteria (detailed below). The average number of HIEC initially isolated from an iris pair is ≈50,000, and these can typically be expanded in culture to ≈3.0 x 10^6 cells (i.e., 6-7 population doublings), without any detectable changes in morphology or physiology (i.e., CAM expression and stimulability). Additionally, these HIEC cultures are amenable to trypsin passaging and display expected survival rates (≈70-80%) when recovered from cryopreservation.

Choroidal EC were also established using the same protocols detailed for HIEC isolation, with reliable success (not shown). Given the larger size of the choroid versus the iris tissue in the human eye, with comparable vascularity, we generally were able to obtain larger starting numbers of HCEC.

We originally isolated HREC from donated human eyes using the same collagenase-based protocol designed for HIEC/HCEC purification, and achieved reasonable cell yields. However, while testing different enzyme digestion protocols (in large measure conducted by Yuzhen Pan), we observed greater initial viable EC yields,

on par with those obtained using iris tissue, when we employed the dispase-based digestion protocol detailed above. Since we ultimately planned to compare gene expression profiles in donor-, population doubling-, passage-, and time in culture-matched HIEC and HREC, obtaining similar initial yields of both cell EC types was essential in maintaining control over these variables.

Physiological Characterization of Ocular EC

We characterized iris, retinal, and choroid PECAM-1^+ cells as EC by several biological criteria. The presence of functional LDL scavenger receptors on cell surfaces was demonstrated by the uptake of Di-I-Ac-LDL (Figure 2). After 4 hrs incubation with fluorescently-labeled Di-I-Ac-LDL, punctate staining was observed intracytoplasmically throughout the EC monolayers. vWF is considered to be a classical marker of EC since it has only been found in EC, megakaryocytes and platelets, the latter two not posing a culture contamination problem. Immunocytochemistry of ocular EC monolayers demonstrated punctate vWF expression within the cytoplasm (Figure 2). Although vWF immunostaining intensity varied from cell to cell, greater than 99% of all EC types stained positively. Lastly, *in vitro*, EC have the ability to form capillary-like networks when grown on three-dimensional matrices under specialized conditions.[78] This characteristic has been used to differentiate between cells that morphologically and biochemically appear to be EC.[86] HIEC isolated by our methods indeed form capillary-like structures *in vitro* when grown on a three-dimensional matrix of Matrigel® and stimulated with PMA. Confluent monolayers of HIEC atop Matrigel® quickly

reorganized themselves to form tube-like structures (Figure 2), with reorganization apparent as early as 4 hrs after PMA addition.

Adhesion molecule mRNA Expression in HIEC

Since cultured HCEC and HREC have been studied previously, we initially focused our EC characterization efforts on HIEC. Cultured HIEC were tested for their steady-state mRNA expression of various CAMs in response to the inflammatory mediators LPS and/or TNF-α. By RT-PCR (Figure 3), unstimulated HIEC constitutively express low but detectable levels of ICAM-1 message. Stimulation with 10 μg/ml LPS caused a time-dependent upregulation of ICAM-1 mRNA; an effect obvious at 1.5 hrs post-exposure, maximal around 3-5 hrs, and maintained through at least 21 hrs of continuous stimulation. Similar kinetics of ICAM-1 upregulation were observed when HIEC were stimulated with 10 ng/ml TNFα or with 10 ng/ml IL-1α (not shown). We also evaluated ICAM-2 mRNA expression in these cells by RT-PCR (Figure 3). We detected significant constitutively expressed ICAM-2 mRNA in HIEC, which was not measurably altered by inflammatory agent stimulation, using RT-PCR. Only barely detectable VCAM-1 signals (Figure 3) were seen in unstimulated HIEC. Following activation with LPS or TNFα, however, we saw a rapid upregulation of VCAM-1 message that was declining after 21 hrs of stimulation.

Adhesion Molecule Protein Expression in HIEC

In line with the mRNA data, using an ELICA we measured a low but detectable constitutive expression of ICAM-1 protein on HIEC cell surfaces, which was markedly

elevated by LPS and TNFα stimulation (Figure 4A). Upregulated ICAM-1 reached maximal levels after 8-12 hours of LPS or TNFα stimulation, and remained maximally elevated through at least 2 days of continued stimulation. ICAM-1 upregulation by LPS was confirmed by western blotting of HIEC lysates (Figure 4A, Inlay). We also detected significant constitutive expression of ICAM-2 protein in HIEC (Figure 4B), which, conversely, was decreased in TNFα- and LPS-stimulated HIEC. This trend appeared as early as 6 hrs post-stimulation, and by 24 hrs had culminated in a significant 50% decrease in immunodetectable ICAM-2 on HIEC surfaces. Unlike the clear constitutive expression of ICAM-1 and -2, only very low levels of VCAM-1 and no E-selectin protein was detected on unstimulated HIEC. In response to either LPS or TNFα stimulation, however, VCAM-1 protein was markedly elevated by 6 hrs (Figure 4C). After 24 hrs of stimulation, VCAM-1 in TNF α-stimulated EC had begun declining towards baseline, but was still significantly elevated versus unstimulated controls. After 24 hrs of stimulation with LPS, VCAM-1 protein had already decayed to control levels (Fig 5?C), in good temporal concordance with the declining VCAM mRNA signal observed in these cells. In the case of E-selectin, upregulation by LPS was rapid, with maximal protein levels detected on HIEC surfaces after only 4 hrs stimulation (Figure 4D). This was followed by a similarly rapid decline to control levels after only 12 hrs.

Leukocyte Adhesion to HIEC

We next investigated whether cultured HIEC monolayers could become more adherent for leukocytes when activated by inflammatory stimuli. When HIEC monolayers were stimulated with LPS (10 μg/ml, 12 hrs), subsequent monocyte adhesion

increased almost 10-fold (Figure 5). Similarly, HIEC stimulation with vascular endothelial growth factor (VEGF, 100 ng/ml, 12 hrs; R&D Systems) resulted in approximately 3-fold increases in monocyte adhesiveness. Pre-incubation of LPS-stimulated HIEC with blocking antibodies against the cell adhesion molecules ICAM-1, ICAM-2, and VCAM-1 resulted in respective 40%, 40%, and 20% decreases in monocyte binding, demonstrating the *in vitro* functioning of multiple adhesion molecules in these activated HIEC.

Cytokine Expression in HIEC

In addition to surveying adhesion molecule expression in HIEC, we also investigated their constitutive and LPS-induced expression of the inflammatory cytokines IL-1α, IL-1β, and IL-6, at the mRNA and/or protein levels (Figure 6). Both IL-1α and IL-1β mRNAs were constitutively expressed at low levels, with baseline IL-1α signals typically stronger than those observed for IL-1β using similar RT-PCR reaction conditions. IL-6 mRNA was also expressed in unstimulated HIEC. All three cytokines were markedly upregulated by LPS stimulation (10 μg/mL), to maximal levels by 3 hours. This maximal induction was maintained thorough at least 21 hours of stimulation. (Figure 6). Choroidal EC (HCEC) behaved similarly as HIEC in terms of upregulating the mRNAs for all three cytokines investigated (not shown).

Discussion

Microvascular EC play key regulatory roles in the initiation and progression of inflammation.[9]The pathological sequelae of AU are often limited to specific tissues (i.e., iris and ciliary body) while neighboring tissues (e.g., retina, choroid, and sclera) are spared, supporting the likely existence of functional heterogeneity amongst the vasculatures of these disparate tissues. That EC from diverse anatomical sites can differ markedly in form and function is becoming increasingly appreciated; such endothelial heterogeneity has been evidenced both by *in vivo* work and in studies employing cultured EC.[70, 71] In ocular pathologies, for example, cytomegalovirus preferentially infects the posterior eye causing a retinitis, while anterior structures are typically unaffected.[87] Additionally, intraocular lymphomas also appear to selectively target the retina and not anterior eye tissues.[88] In appreciation of the likely existence of tissue-specific gene expression amongst the microvasculature of different ocular tissues, we endeavored to establish pure HIEC cultures to investigate some of the molecular mechanisms employed in AU pathogenesis. Although other researchers have previously cultured and experimented with ocular EC from human retina and choroid,[83, 84] this is the first report testing pure microvascular EC isolated from human irises.

We evaluated in HIEC the expression of three adhesion molecules of the immunoglobulin superfamily (i.e., ICAM-1, -2, and VCAM-1), and one member of the selectin family (E-selectin), all of which are known to support leukocyte adhesion. Consistent with previous demonstrations in other EC types,[81] these cells display a low but measurable constitutive expression of ICAM-1 that is markedly upregulated at both the mRNA and the protein levels by stimulation with either LPS or, more potently, by

TNFα. By contrast, constitutively expressed ICAM-2 protein is markedly downregulated by the same inflammatory stimuli over the same time period. That we did not detect a coordinate decrease in ICAM-2 mRNA levels using a multiplex RT-PCR assay is likely due to the semi-quantitative nature of this method, since in preliminary experiments using gene arrays we saw an approximate 50% decline in ICAM-2 signal strength after 24 hrs of LPS stimulation (discussed in chapter 3). By contrast to the clear constitutive expression of ICAM-1 and –2, negligible VCAM-1 and no E-selectin expression is detectable in unstimulated HIEC, at both mRNA and protein levels. As with ICAM-1, both VCAM-1 and E-selectin are potently upregulated in iris EC upon activation with inflammatory stimuli, in line with their previously suspected roles in uveitis.[89-94] Unlike the persistent ICAM-1 elevation, VCAM-1 and E-selectin upregulation is transient.

The activated phenotype of HIEC is reflected at the functional level, whereby LPS-stimulated HIEC show a significantly increased adhesiveness for U937 monocytes. This effect is in part blocked by the pre-incubation HIEC with neutralizing antibodies against various adhesion molecules (i.e., ICAM-1 and -2, and VCAM-1). This is consistent with a demonstrated role for EC-expressed ICAM-1 in mediating ocular inflammation in both animal models and clinical investigations,[80, 89-92] and suggests that these cultured HIEC can qualitatively mimic the *in vivo* scenario. Previously, immunohistology performed on biopsied iris specimens from patients with either acute or chronic AU has revealed upregulated expression of ICAM-1 and VCAM-1 on human iris microvascular EC, implicating both adhesion molecules in the increased leukocyte flux through the iris in AU.[92, 94] Our current data support this likelihood.

To date, this is the first report investigating a role for ICAM-2 in ocular inflammation. Although ICAM-2 is appreciably downregulated in activated HIEC, as has been reported in TNFα- and IL-1β-stimulated umbilical vein EC,[95] we observed that it nonetheless remains present in sufficient amounts to significantly mediate the binding of leukocytes to these vascular cells. Endothelial ICAM-2 is known to support the adhesion a variety of leukocyte subsets to EC, by acting as a ligand for the leukocyte-specific β_2 integrins, LFA-1 and Mac-1,[49, 96] and appears to be essential for T-cell transendothelial migration. Interestingly, peptide fragments of ICAM-2 and soluble ICAM-2/Fc chimeras can bind to and rapidly enhance the affinity of these leukocyte integrins for both ICAM-1 and ICAM-2, resulting in a feed-forward enhancement of adhesion.[97]

Soluble ICAM-1 exists in normal human plasma in ng/ml quantities, and circulating levels are significantly increased in a variety of inflammatory disorders, including uveitis.[91, 98] Additionally, soluble adhesion molecules, including ICAM-1, are elevated in the aqueous humor of uveitis patients, [91] and in the vitreous of patients with proliferative diabetic retinopathy and proliferative vitreoretinopathy.[99] Very recently, soluble ICAM-2 has been detected in the bloodstream of normal humans and leukemia patients, which is significantly lowered after chemotherapeutic reduction of circulating leukemic cells.[100] Although currently speculative, if cleavage or shedding of membrane ICAM-2 accompanies its downregulation in activated HIEC, the resulting increased local levels of soluble ICAM-2 might perpetuate inflammation in the iris by activating integrins on nearby leukocytes.[97] Taken together with prior experimental observations, our current data suggest a potentially important role for ICAM-2 in mediating leukocyte infiltration in uveitis, and warrant further experimentation to this end.

We also observed low constitutive expression of the inflammatory cytokines IL-1α, IL-1β, and IL-6 in HIEC, which were all markedly upregulated by exposure to bacterial LPS. All three of these cytokines have been well-studied as pathogenic modulators in various models of anterior uveitis.[75] Their demonstration here of regulated expression in cultured HIEC further supports a role for them in mediating inflammation involving the iris, and additionally validates the use of these cultured cells as an *in vitro* model for studying mechanisms of iritis.

Endothelial expression of various chemokines, cytokines, and cell adhesion molecules are critical determinants in the development of the inflammation in AU. Understanding the EC expression profiles of these molecules and determining their relative importance in uveitis is difficult to test in the clinical scenario, since experimental data gathered from AU patients are limited to procuring small samples of aqueous humor or iris biopsy specimens from patients undergoing ocular surgery. Additionally, anti-inflammatory pharmacologic regimens are often underway in these patients, and can complicate interpretation of findings. In order to circumvent these limitations while still working in a relevant human model, cultured HIEC provide a versatile means to expand our understanding of the mechanistic origins of AU. Although cell culture systems are not without their own inherent experimental limitations, this approach complements the existing animal models and clinical investigations in together elucidating the molecular mechanisms of uveitis. Additionally, our ability to culture HIEC was an essential gateway for beginning the investigations into potential tissue-specific EC gene expression that are highlighted later in this dissertation.

Acknowledgements

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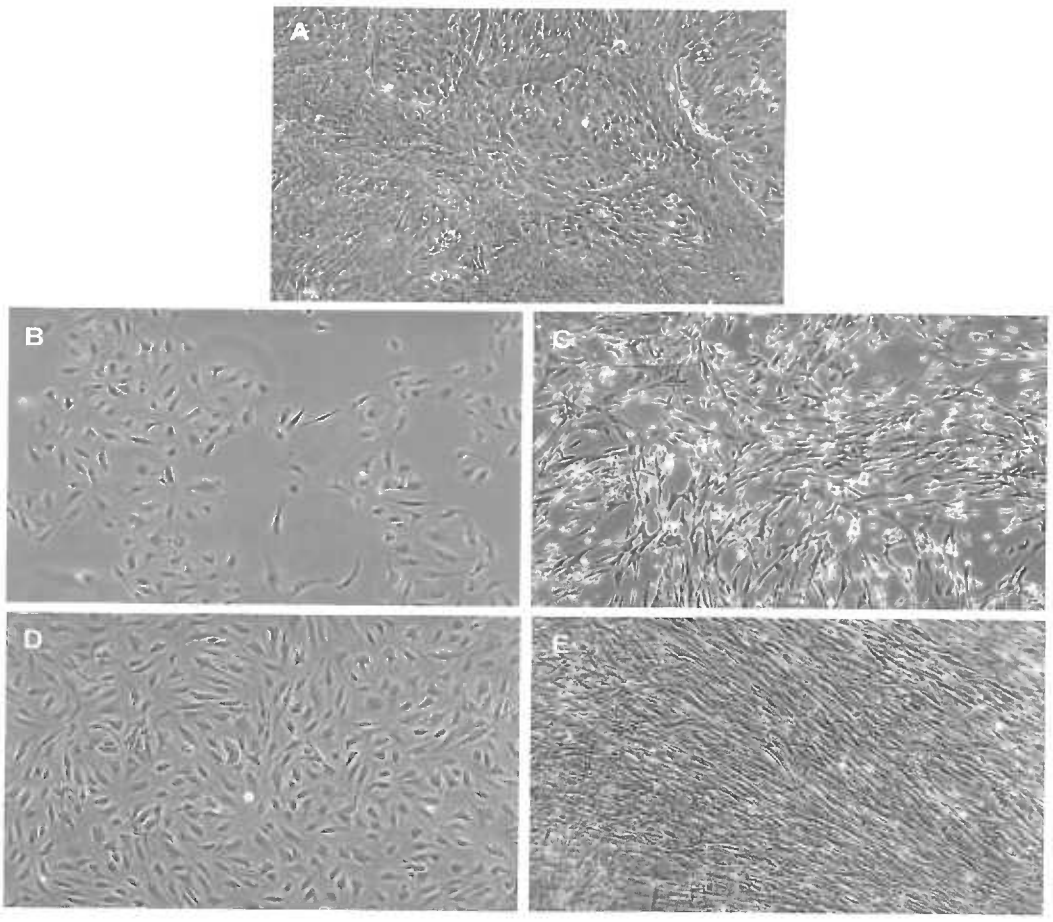

Figure 1. Isolation of human iris endothelial cells (HIEC). A) Simple collagenase-digests of iris tissue grow as a mixed culture containing EC and a variety of stromal cells. Separation of the PECAM-1^{+} fraction of these cultures using antibody-conjugated magnetic beads results in **B)** the positive selection of pure HIEC cultures, and **C)** the negatively selected mixed stromal cell cultures that remain. Panels **B** and **C** were photographed after 2 days in culture following separation. Panels **D** and **E** show the same cells after 5 days in culture

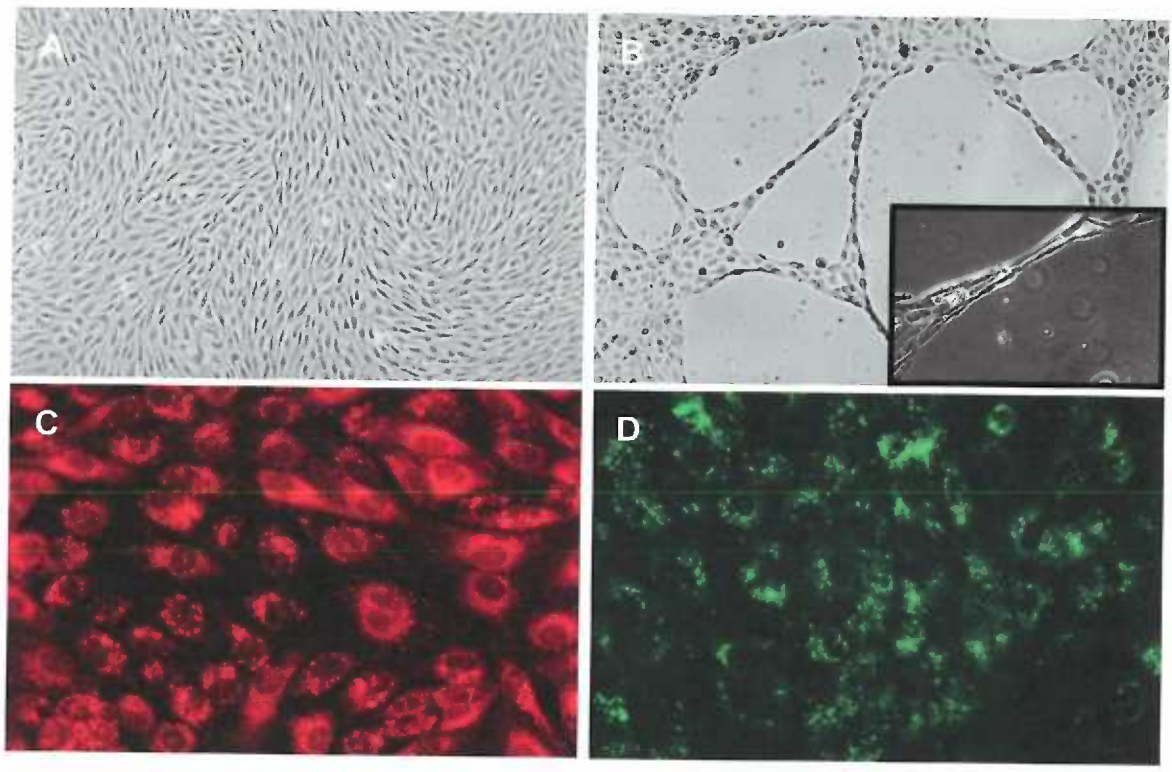

Figure 2. Biochemical and functional characterization of HIEC. Human iris EC grow in histotypical cobblestone monolayers **(A)**, form capillary-like networks when grown on the provisional extracellular matrix Matrigel **(B)**, take up Di-I-acetylated LDL **(C)**, and express von Willebrand's factor **(D).** Original magnifications: **(A, B)** x 25; **(B,** *inset***)** x 100; **(C, D)** x 100.

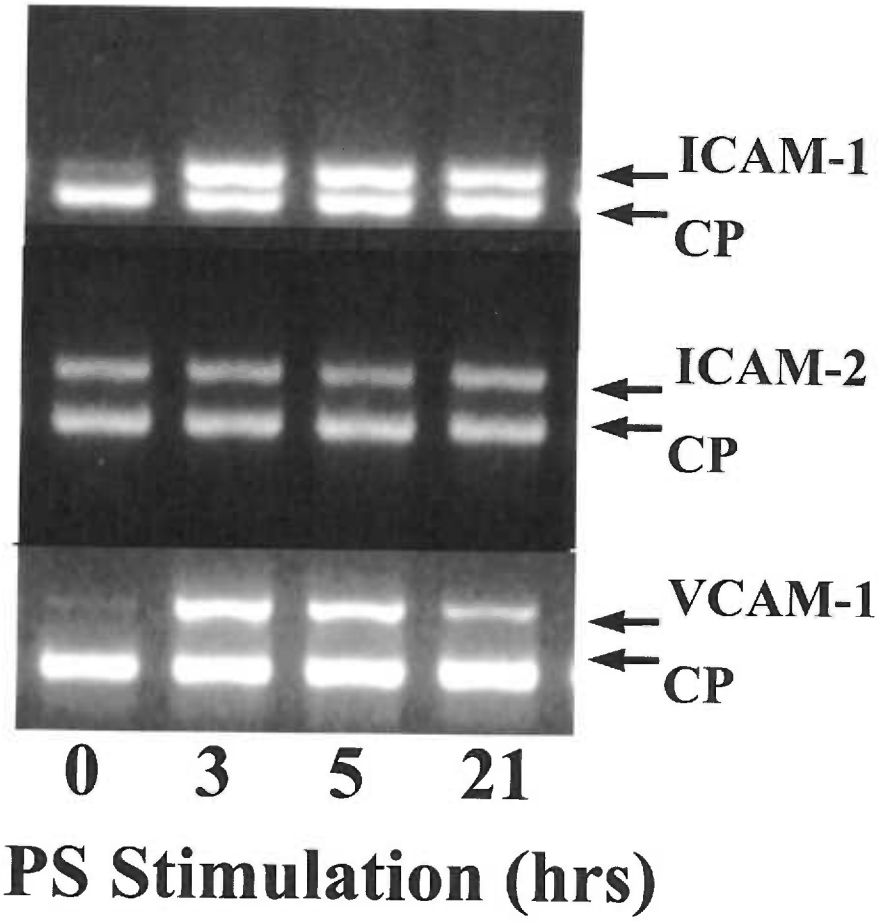

Figure 3. RT-PCR analysis of ICAM-1, ICAM-2, and VCAM-1 mRNA in LPS-stimulated HIEC cultures. HIEC cultures were stimulated for up to 21 hrs with 10 μg/ml LPS. While ICAM-1 and VCAM-1 mRNA levels significantly increased with LPS-stimulation, we did not detect any change in ICAM-2 mRNA expression under the same conditions by RT-PCR. ICAM-1, ICAM-2, VCAM-1, and cyclophilin (CP; internal control) amplicons displayed respective base-pair sizes of 319, 386, 618, and 292. Shown are data from one representative experiment of 3 each for ICAM-1 and ICAM-2, and 2 for VCAM-1.

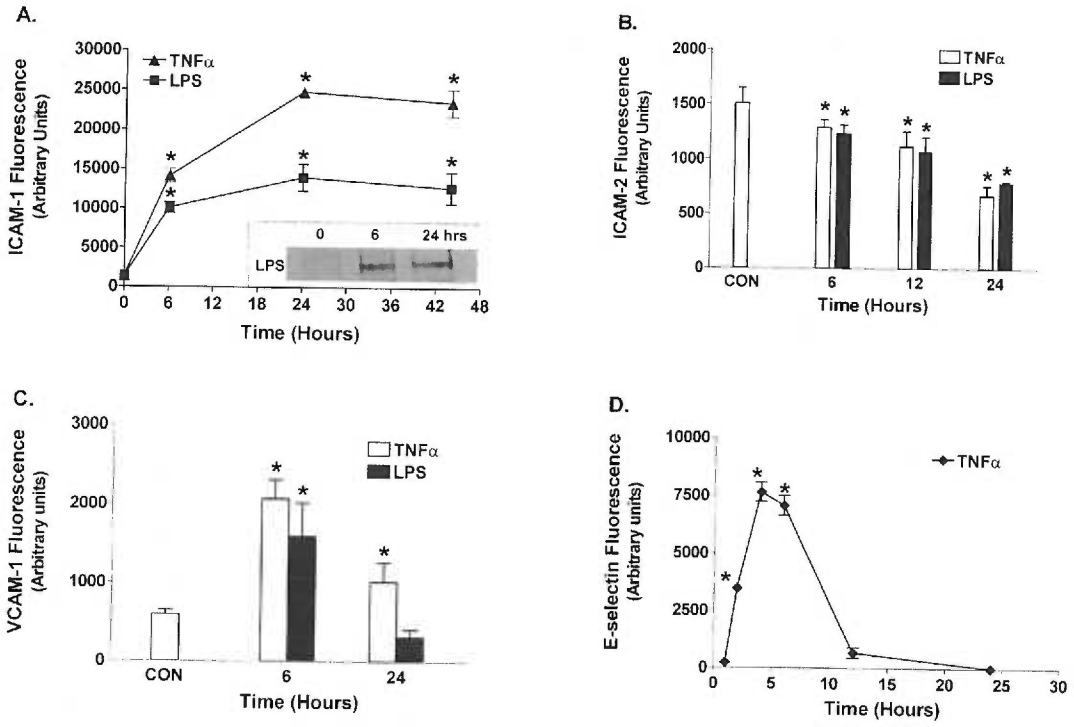

Figure 4. Adhesion molecule expression on HIEC cell surfaces.

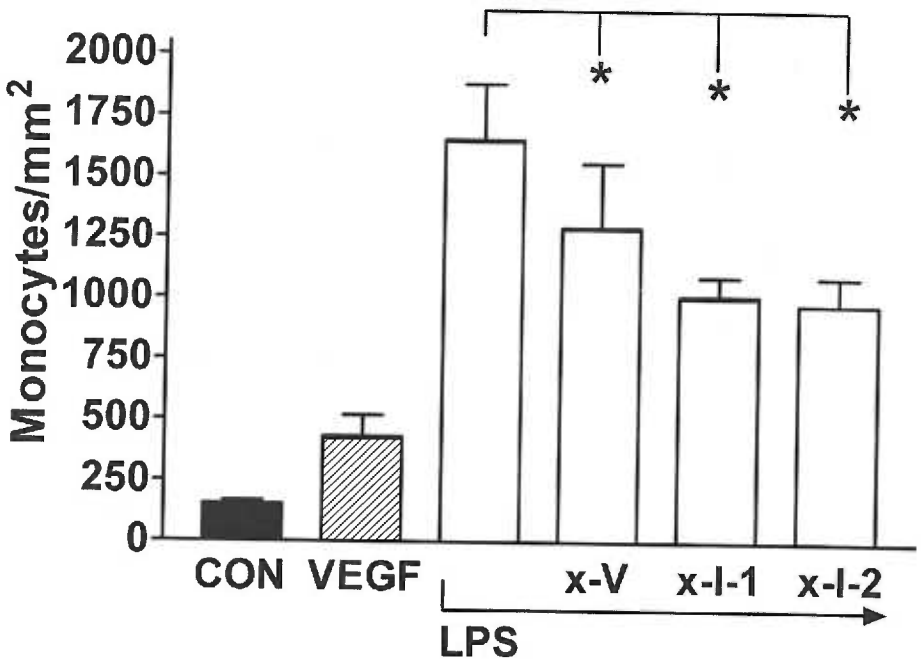

Figure 5. Monocyte adhesion to HIEC. Confluent monolayers of HIEC were stimulated with either VEGF (100 ng/ml) or LPS (10 μg/ml) for 12 hrs, followed by the addition of U937 monocytes for 15 minutes; Unstimulated HIEC served as controls (CON). Stimulation with VEGF and LPS resulted in significant 3- and 10-fold increases in monocyte adhesion, respectively ($p<0.01$ by ANOVA, in both cases) Anti-VCAM-1 (x-V), -ICAM-1 (x-I-1), or -ICAM-2 (x-I-2) neutralizing antibodies were added to some LPS-stimulated wells prior to monocyte incubations. All experimental variables were conducted in triplicate or quadruplicate wells per condition. Data represent mean monocytes bound/mm^2 HIEC monolayer surface area +/- S.D, and are presented for one of three representative experiments. Asterisk (*) indicates significant inhibition of monocyte attachment ($p< 0.05$) versus LPS-stimulated HIEC.

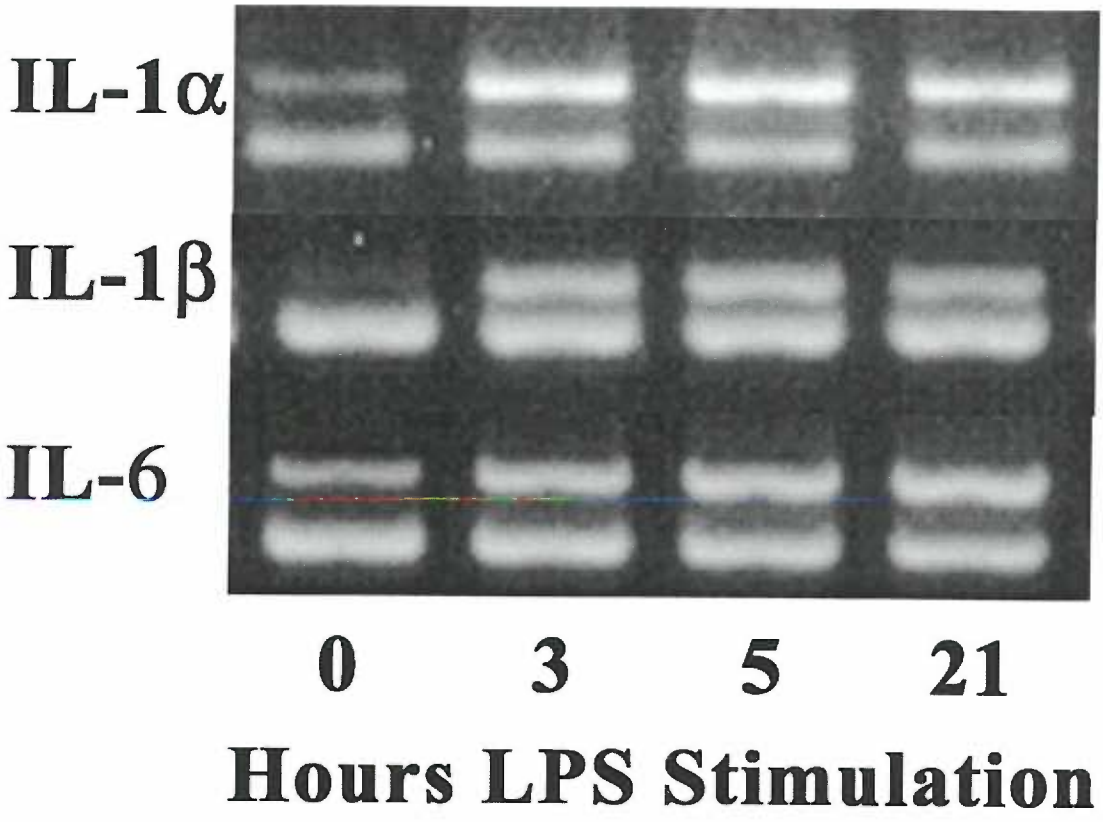

Figure 6. Cytokine expression in HIEC. Low but detectable levels of the inflammatory cytokines IL-1α, IL-1β, and IL-6 mRNA were potently upregulated in HIEC by exposure to LPS (10 μg/mL), with levels maximal by 3 hours and maintained through 21 hours. Shown are data from one representative of 2-3 independent experiments, all of which gave similar findings. Top lanes are indicated cytokine amplicons; bottom lanes represent cyclophilin amplicons as an internal control.

Chapter 4.

Constitutive and Inflammatory Mediator-Regulated Fractalkine Expression in Human Ocular Explants and Cultured Cells

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Summary

Fractalkine (FKN) is a dual adhesion molecule/chemokine that is important in inflammation but has not previously been explored in the eye. We investigated potential FKN expression in tissue sections of normal human iris and retina, and evaluated cultured EC and stromal cells from iris, retina, and choroid to determine whether they expressed constitutive and/or cytokine-modulated FKN.

By RT-PCR and immunohistochemistry, respectively, we identified constitutive FKN mRNA and protein expression in human iris and retina explants. TNFα treatment upregulated FKN message in iris explants. FKN protein was widely distributed throughout both tissues, but appeared more pronounced in microvascular EC and in several stromal cell types. By cDNA array and RT-PCR, EC cultured from iris, choroid, and retina typically expressed low constitutive FKN message that was upregulated by stimulation with either TNFα or LPS. Similar FKN mRNA upregulation was seen when iris and retinal stromal cell cultures were activated using the same agonists.

In HIEC, there was a polarized effect of stimulation with Th1 versus Th2 cytokines, with the classical Th1 cytokine, interferon-γ, upregulating FKN in synergy with TNFα. The Th2 cytokines IL-4 and IL-13 downregulated TNFα-induced FKN protein, while IL-10 was without effect. CD40 ligand upregulated basal FKN, but had no additional effect on TNFα-induced levels. Surprisingly, IL-17, a cytokine with only proinflammatory activities identified to date, downregulated TNFα-induced FKN protein in HIEC.

Thus, this is the first report of FKN expression in various ocular tissues and cells. Inflammatory mediator modulation of ocular FKN expression suggests that this adhesive

chemokine might play important roles in regulating leukocyte efflux in inflammatory eye diseases such as anterior uveitis and retinochoroiditis.

Introduction

During inflammation, the expression of multiple chemokines and adhesion molecules is upregulated in affected tissues.[9] These molecules are essential in mediating inflammatory disease progression by directing leukocyte recruitment to the microvascular wall and subsequent trafficking into the surrounding tissues. Fractalkine (FKN) is a recently characterized transmembrane glycoprotein adhesion molecule,[101] that can be proteolytically cleaved to yield a soluble chemoattractant form.[102] As a chemoattractant, FKN is unique in that it is so far the sole member of the CX_3C (or delta) subclass of chemokines, defined by two conserved cysteines in the primary amino acid sequence with three amino acids separating them.[59] Moreover, it is the first member of the classical chemokine superfamily to be ascribed adhesive as well as chemotactic and angiogenic functions.[103] Soluble FKN is a chemoattractive and adhesive for T cells, monocytes, and NK cells,[104-106] via their expression of the G protein-coupled receptor, CX_3CR1.[107] FKN is expressed by several cell types including endothelial cells (EC), vascular smooth muscle cells, dendritic cells, and neurons.[101, 108-110] In EC, it is also strongly induced by classical proinflammatory molecules such as tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1), [101] and is additionally modulated by cytokines such as IL-4 and interferon-γ (IFNγ).[111]

In the eye, inflammatory pathologies share many of the molecular mechanisms employed during inflammation elsewhere in the body.[73, 112] Only very recently has FKN-mediated leukocyte chemotaxis and adhesion been implicated as a major player in diverse inflammatory diseases. In these studies, FKN plays important roles in

mediating, for example, cardiac allograft rejection, arthritis, and psoriasis, and in modulating neuronal injury after a toxic insult.[113-116] To date, however, no investigations to our knowledge have sought to evaluate FKN expression in the eye, or whether such FKN expression might mediate ocular inflammation. In the current study, we demonstrate the presence of FKN in human ocular tissues, and also the constitutive and inflammatory agent-regulated expression of FKN in cultured ocular microvascular EC and stromal cells derived from human iris, choroid, and retinal tissues. These findings suggest that FKN may indeed play a regulatory role in the development and progression of ocular inflammatory disorders.

Results

FKN Expression in Human Ocular Tissues

By immunohistochemistry, FKN protein expression was observed in iris and retina/choroid tissues (Fig. 1A-E). In all ocular tissues examined, arteriolar, venular, and capillary EC tended to stain prominently, although occasional FKN-negative vessels were observed. A variety of stromal cell types also displayed strong constitutive FKN expression, including perivascular cells (presumed to be of pericyte/smooth muscle cell lineage; Fig. 1E), and several other stromal cell types, as well. We attribute such widespread staining to the fact that FKN exists in both a soluble and cell surface-bound molecule with a diverse expression profile. Similar staining patterns were observed in normal skin by others using the same methodology and monoclonal detection antibody.[14] We validated our findings in the same ocular tissues using a different polyclonal anti-FKN detection antibody (Santa Cruz #SC-7225), with identical results as shown in Fig. 1.

In the iris, the smooth muscle cells of the sphincter pupillae muscle were strongly positive (Fig. 1B), whereas the ciliary muscles stained much more faintly (not shown). We were unable to discern the extent of staining in the myoepithelial cells of the iris dilator muscle, due to obscuration by the large amounts of pigment in these cells and in the abutting pigment epithelial layer. Nearly all of the iris melanocytes scattered throughout the stroma, and more concentrated at the anterior iris border, however, were strongly positive for FKN.

Extensive cellular FKN staining was observed in a variety of cell types throughout the retina (Fig. 1C & D). Staining was most pronounced, however, in the vasculature, the inner nerve fiber layer and abutting ganglion cell layer (GCL), the outer

plexiform layer, the pigment epithelium, and within the outer limiting membrane, compared to the fainter staining of the nuclear and photoreceptor layers. Interestingly, Müller cells, discernable by their long transretinal processes, also strongly expressed FKN. The adjacent choroid showed strong FKN expression in EC and other stromal cells (Fig. 1D).

In concordance with our immunohistological findings, we observed clear constitutive FKN mRNA expression in both human iris and retina explants, using RT-PCR (Fig. 1F). Similar constitutive expression was also observed in murine iris and retina explants (not shown). With human iris explants, ex vivo TNFα (10 ng/ml) stimulation markedly increased FKN mRNA expression, with levels greatest between ≈3 to 5 hrs, and only beginning to decline after 24 hrs of stimulation (Fig. 2A).

TNFα and LPS Upregulate FKN mRNA in Ocular EC and Stromal Cells

In order to better elucidate potential FKN regulation in specific ocular cell types, we evaluated FKN message levels in EC cultured from human iris (HIEC), choroid (HCEC), and retina (HREC), as well as in EC-depleted stromal cell populations grown from human irises and retinas (Fig. 2B & C). By RT-PCR, we observed low but detectable FKN expression in all three ocular EC types, which was markedly and time-dependently upregulated by stimulation with either TNFα (10 ng/ml) or LPS (10 μg/ml), with TNFα causing a more persistent upregulation (Fig. 2B). IL-1α similarly upregulated FKN in these ocular EC (not shown). These findings were supported by parallel studies using cDNA arrays (Fig. 3). By gene array, we similarly detected weak constitutive FKN signals in HIEC and HREC that were markedly increased after 3 hr stimulations with

either LPS or TNFα. In human iris and retinal stromal cell cultures, we observed low but detectable basal expression of FKN mRNA that was, as with EC, upregulated by LPS and TNFα (Fig. 2C).

Constitutive and TNFα- and LPS-induced FKN Protein Expression in Ocular EC

Using an ELICA, we saw FKN protein expression profiles in ocular EC that correlated well with their observed mRNA profiles. In HIEC, HCEC, and HREC alike, we detected measurable albeit low constitutive FKN protein on cell surfaces (Fig. 4). Upon activation with TNFα or LPS, FKN protein was significantly upregulated in a dose- and time-dependent fashion in all three EC types, with TNFα being the more efficacious stimulus in this respect. For example, in HIEC, although both LPS (10 μg/ml) and TNFα (10 ng/ml) caused maximal induction after 6 hrs of stimulation, FKN levels were significantly higher with TNFα stimulation (Fig. 4A). Additionally, TNFα caused a more persistent elevation in cell surface FKN protein expression in these three EC types. In HIEC, while FKN levels began declining towards baseline after only 12 hrs of exposure to LPS, they remained maximally elevated through 24 hrs of TNFα exposure (Fig. 4A). Similar results were also obtained in several experiments using HCEC and HREC (not shown). We confirmed FKN protein upregulation by TNFα in HIEC by western blotting (Fig. 4B). In 2 experiments comparing HIEC to HREC with respect to concentration-dependent induction of FKN by TNFα, both EC types displayed a similar EC_{50} of ≈100 pg/ml (i.e., 5.7 pM; Fig. 4C).

Other Inflammatory Mediators Regulate FKN Expression in Ocular Cells

IFNγ and IL-4 are prototypical Th1 and Th2 cytokines, respectively, with important roles in regulating inflammation.[117] Thus, we investigated potential effects of these agents in mediating both constitutive and TNFα-induced FKN in ocular EC and stromal cells. Stimulating cultured HIEC with 10 ng/ml IFNγ modestly upregulated constitutive FKN expression, at both the protein level (Fig. 5A), and the mRNA level (Fig. 5B). Costimulation of HIEC with IFNγ and low dose (i.e., 1 ng/ml) TNFα resulted in a synergistic elevation in cell surface FKN expression (4 to 9-fold over sum effects of both cytokines acting alone), that was obvious at 6 hrs (Fig. 5A), and maintained through 24 hrs (not shown). Similar effects on FKN protein expression were obtained using HREC and HCEC (not shown). Iris stromal cultures responded similarly to IFNγ and TNFα stimulation, as was observed with EC (Fig. 5B).

In contrast to the inducing effect of IFNγ on endothelial FKN, the Th2 cytokine IL-4 (10 ng/ml), significantly decreased both constitutive and agonist-induced FKN protein expression over the same timeframe (Fig. 5A) Similar effects of IFNγ and IL-4 on FKN protein expression were also obtained using a large vessel endothelium representative, i.e., human aortic EC (not shown). Addition of IL-4 inhibited both TNFα-induced FKN as well as the synergistically elevated TNFα/IFNγ-induced FKN expression in HIEC by ≈50%, after 6 hrs of simultaneous stimulation with all 3 agents. This inhibitory effect was not, however, discernable at the mRNA level using standard RT-PCR (Fig. 5B).

We also tested the ability of other inflammatory reagents to modulate FKN expression in EC (Fig. 5C; all used at 10 ng/ml, 8 hrs). The Th2 cytokine IL-13, although having no significant effect on basal FKN protein levels in HIEC, caused a marked ≈65% decrease in TNFα-induced FKN after 8 hrs of concomitant stimulation. IL-10, another Th2 cytokine, however, was without effect on either constitutive or agonist induced FKN in HIEC and HREC (not shown). IL-17, generally considered to be proinflammatory,[118] had no effect on basal FKN expression in HIEC, but surprisingly caused a significant ≈40% decrease in TNFα-induced FKN protein levels. Similar results were obtained using IL-17 on TNFα-stimulated human dermal microvascular EC (not shown). IL-18, another proinflammatory cytokine related to the IL-1 family, had no effect on either basal or TNFα-induced FKN protein in HIEC (not shown), consistent with previous work showing a lack of involvement in experimental autoimmune uveitis.[119] CD40 ligand caused a significant 2-fold elevation in basal FKN protein levels in HIEC, but caused no observable additional increase when superimposed upon TNFα stimulation.

Discussion

In various studies of uveitis and retinitis, aberrant chemokine and adhesion molecule expression appear to play critical roles in disease initiation and progression.[73, 112, 120] FKN is a recently characterized multifunctional adhesion and chemoattractant molecule,[101] that is upregulated in the vasculature during a variety of inflammatory diseases, [113-116] wherein it plays a key role in regulating leukocyte influx into affected tissues. *In vitro*, FKN expression is modulated by inflammatory cytokines in EC and in smooth muscle cells.[101, 108] Given the mechanistic parallels between various ocular inflammatory diseases and inflammation elsewhere in the body, we sought to investigate whether FKN is expressed in tissues of the eye, and to better understand how such expression might be regulated by the local cytokine milieu. To this end, we tested ocular tissue explants, as well as cultured microvascular EC and stromal cells derived from iris, retina, and/or choroid.

By immunohistochemistry of ocular tissues, we observed clear constitutive FKN expression in the EC and in other stromal cells of the iris, retinal, and choroidal microvasculatures, consistent with a role in mediating leukocyte extravasation. Such widespread constitutive expression likely mediates normal leukocyte recirculation during routine immune surveillance. Its upregulation in cultured ocular EC by known inflammatory agents supports a further role for FKN in mediating ocular inflammation. That a variety of extravascular cell types throughout the eye strongly express FKN (e.g., Müller cells in the retina and the sphincter pupillae muscle in the iris), implies that additional functional roles may exist for FKN beyond its likely role in mediating the initial leukocyte efflux from ocular microcirculations. In the brain, for

example, neuron-derived soluble FKN binds its cognate receptor on nearby microglial cells, and subsequent paracrine interactions are neuroprotective.[116] It is possible that a similar relationship exists in the retina, wherein Müller cells, a glial cell type that strongly expresses FKN, might employ FKN-mediated cellular communication as a mechanism to protect/support juxtaposed retinal neurons.

Cultured ocular EC expressed low amounts of FKN mRNA and protein, and these levels were potently upregulated by TNFα-stimulation, in partial concordance with previous observations using other EC types.[111] One notable difference is that in ocular microvascular EC we saw maximal FKN mRNA induction after 3 hours of stimulation, whereas the aforementioned study used HUVEC and maximal TNFα-induced FKN mRNA levels required 18-24 hrs, using a similar agonist concentration. This is possibly a reflection of site-derived endothelial heterogeneity.[71, 81] If so, this likely does not signify a gross difference between micro- and macrovascular EC, since in our hands, human aortic EC also showed a rapid upregulation of FKN in response to identical stimulation (not shown). Proinflammatory cytokines such as TNFα are significantly elevated in the local milieu in various ocular inflammatory scenarios.[73, 112] Thus, there may exist a functional link between upregulated FKN and the increased leukocyte efflux that is characteristic of ocular diseases such as anterior uveitis or retinochoroiditis. Our observation of both constitutive and inducible FKN expression in stromal cells suggests that FKN may be important not only in initial extravasation, but also in guiding leukocyte migration further into perivascular tissues once these cells have left the circulation, or in mediating their physical interactions with resident stromal cells following migration.

Our observations of a polarized FKN response in iris and retinal EC when stimulated with Th1 versus Th2 cytokines confirm and extend previous findings.[111] In both our report and in previous studies, the Th1 cytokine IFNγ acted both alone and in synergy with TNFα to upregulate FKN, while the Th2 cytokines IL-4 and IL-13 decreased both TNFα- and TNFα/IFNγ-induced FKN expression in these cells.[111] In our study, IL-4, but not IL-13, was able to significantly decrease the low basal expression of FKN protein in ocular EC, as well. The inductive effect of IFNγ versus the suppressive effect of IL-4 and IL-13 on FKN expression in ocular EC suggests that FKN may play a role in mediating ocular diseases such as iritis and uveoretinitis, which are considered to be primarily Th1-driven conditions.[121, 122] In support of this, both IL-4 and IL-13 have been shown to decrease inflammatory parameters in various experimental models of ocular inflammation,[122, 123] and to downregulate FKN in HUVEC.[111] Additionally, we observed no effect of IL-10, another Th2 cytokine known to be anti-inflammatory in some models of uveitis,[124] on either basal or TNFα-induced FKN expression in ocular EC. Local IL-10 levels increase in some ocular inflammatory states, and its levels are correlated with the downregulation of Th2 responses and with disease resolution.[124-126] In some models of experimental uveoretinitis, IL-10 appears to require the concerted action of IL-4.[124, 127] The requirement of the coordinated activity of multiple Th2 cytokines in mitigating inflammatory processes may be reflected in the observed lack of effect of IL-10, by itself, in modulating FKN expression in iris and retinal EC.

IL-17 is the prototype member of an emerging cytokine family.[118] IL-17's expression, to date, is limited to a subset of activated memory T lymphocytes that

appear to be distinct from the classical Th1/Th2 categorization.[128] All of the yet reported biological effects of IL-17 are of a proinflammatory nature, and IL-17 is elevated in diverse inflammatory pathologies including rheumatoid arthritis, asthma, psoriasis, transplant rejection, and systemic sclerosis.[129-133] At the cellular level, IL-17 stimulates chemokine and/or cytokine expression in EC, fibroblasts, and epithelial cells,[130, 134, 135] and induces adhesion molecules on EC and keratinocytes.[131, 133] To the best of our knowledge, ours is the first investigation of IL-17 action on tissues or cells derived from the eye, or on FKN expression. We hypothesized that IL-17 would act in a proinflammatory capacity and upregulate the expression of FKN in ocular EC. To our surprise, IL-17 alone did not alter basal FKN expression in HIEC, but in fact significantly antagonized TNFα-induced FKN expression in these cells. This novel observation using EC derived from the eye exemplifies the complex interplay of cytokine signals that govern microvascular involvement in inflammation, and raises the likelihood that IL-17 might play novel regulatory roles in ocular immune pathologies.

The CD40/CD40 ligand dyad is a well-studied proinflammatory signaling system.[136] In the eye, CD40 ligation has been linked to corneal transplant rejection, keratoconjunctivitis sicca, and Sjoren's syndrome, and causes IL-6 and IL-8 release by retinal pigment epithelium.[137-139] EC express both CD40 and its ligand, and ligation upregulates adhesion molecule expression,[140, 141] although controversy exists concerning the specific molecular responses. While one group previously reported the upregulation of FKN mRNA in CD40 ligand-stimulated HUVEC,[111] here we extend

these findings to the protein level, and also to the realm of the ocular microvasculature.

In summary, we describe for the first time the constitutive and cytokine-modulated expression of FKN in intact human iris and retinal explants, and in microvascular EC and stromal cell cultures derived from various ocular tissues. Considering the common expression of FKN in the eye and in other tissues, and its proven role in mediating leukocyte extravasation in inflammatory pathologies elsewhere in the body, these observations implicate FKN as a potential key regulator of ocular inflammation.

Acknowledgements

Gratitude goes out to Rory Dunnaway and the Lion's Eye Bank, Portland, OR, for providing donor tissues. This study is dedicated to the late Paul V. Texeira, a promising young colleague whose life was prematurely ended this past September, 2001. This project was supported by NEI grants EY06477, EY06484, and EY10572; by Research to Prevent Blindness awards to JTR, SRP, and the Casey Eye Institute; by Tartar Trust Research Fellowship grants (Oregon Science Foundation, Portland, OR) to MDS and to DOZ.

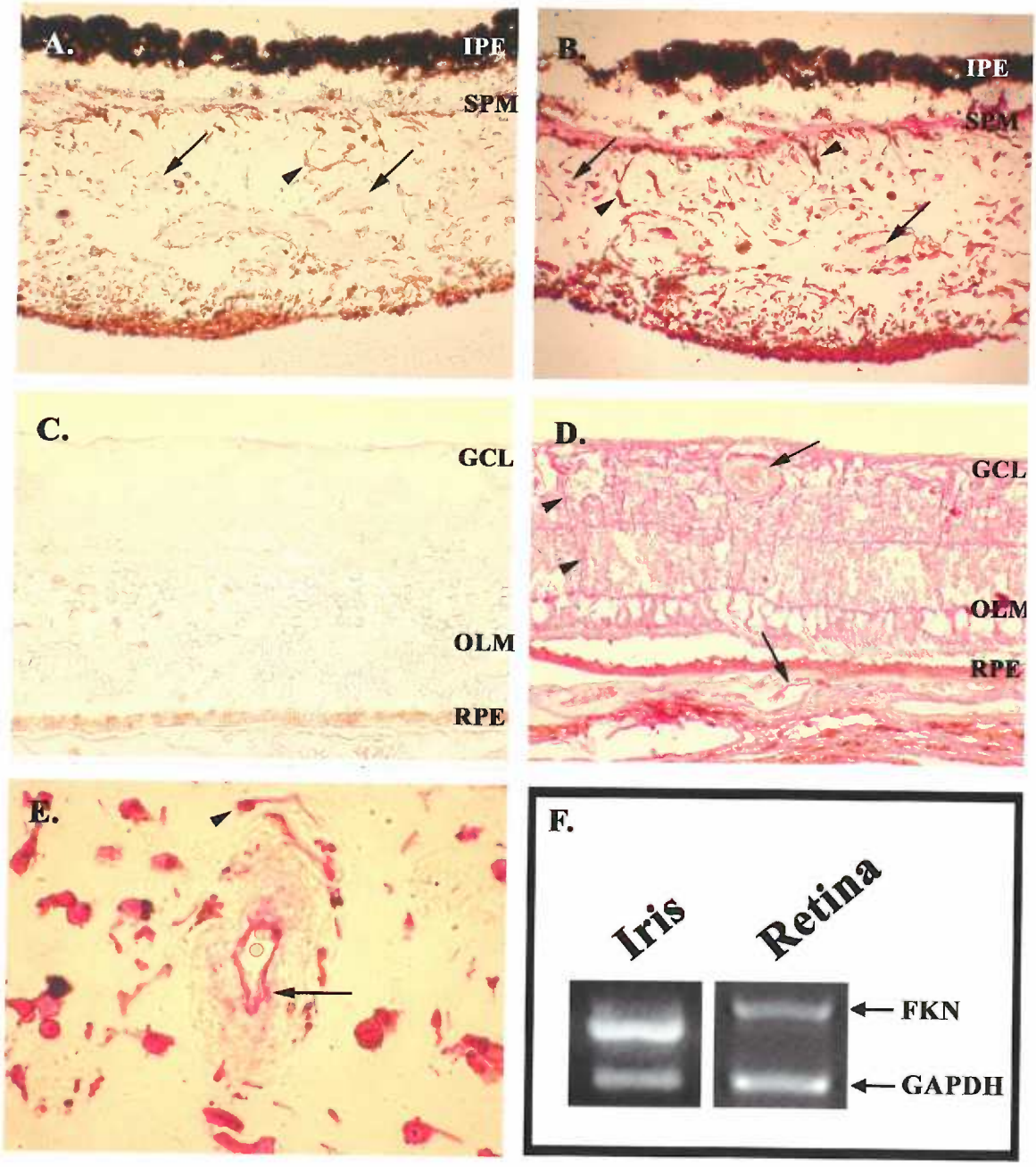


Figure 1. Fractalkine expression in ocular tissues.

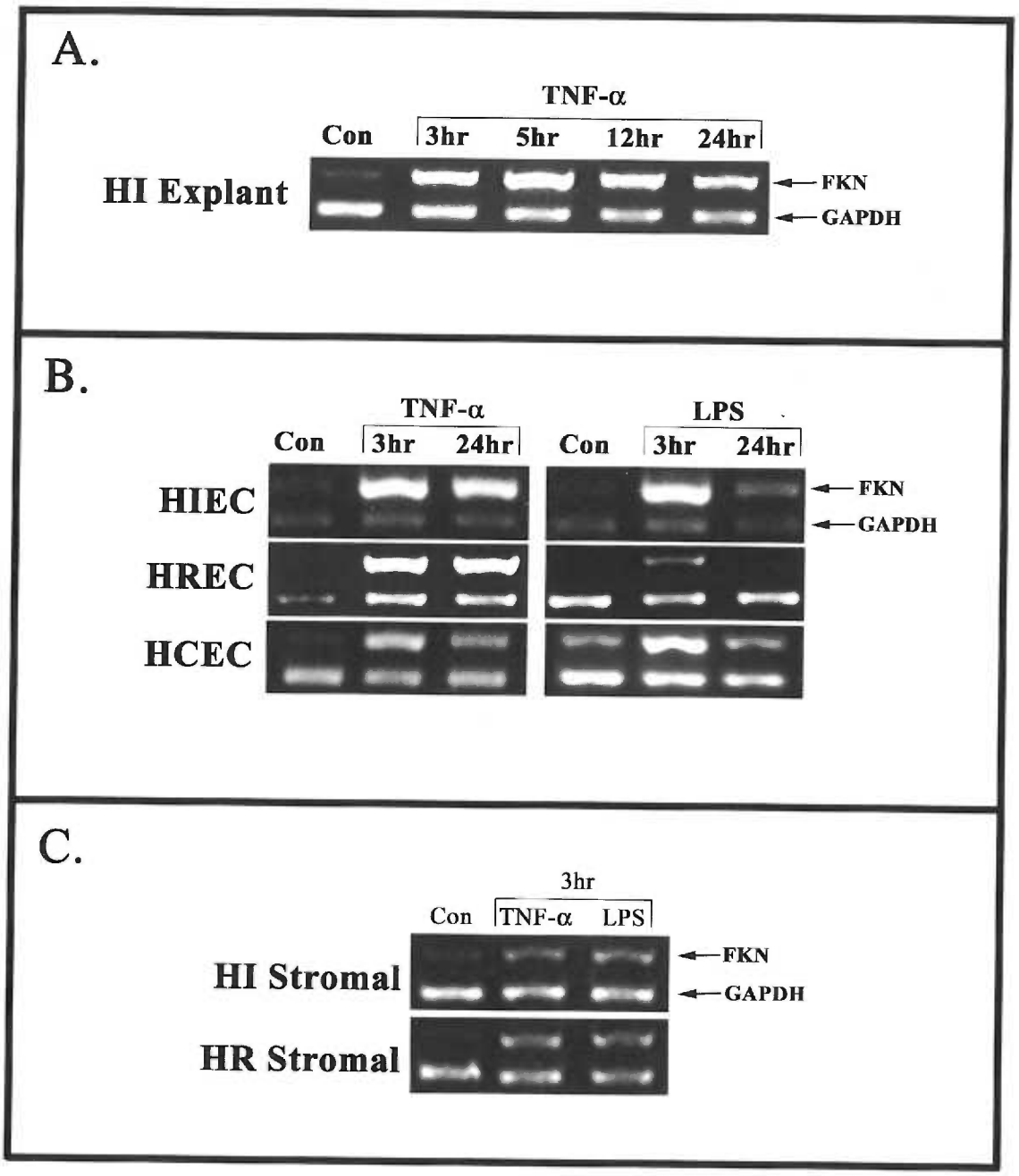


Figure 2. RT-PCR of fractalkine in ocular explants and cultured cells

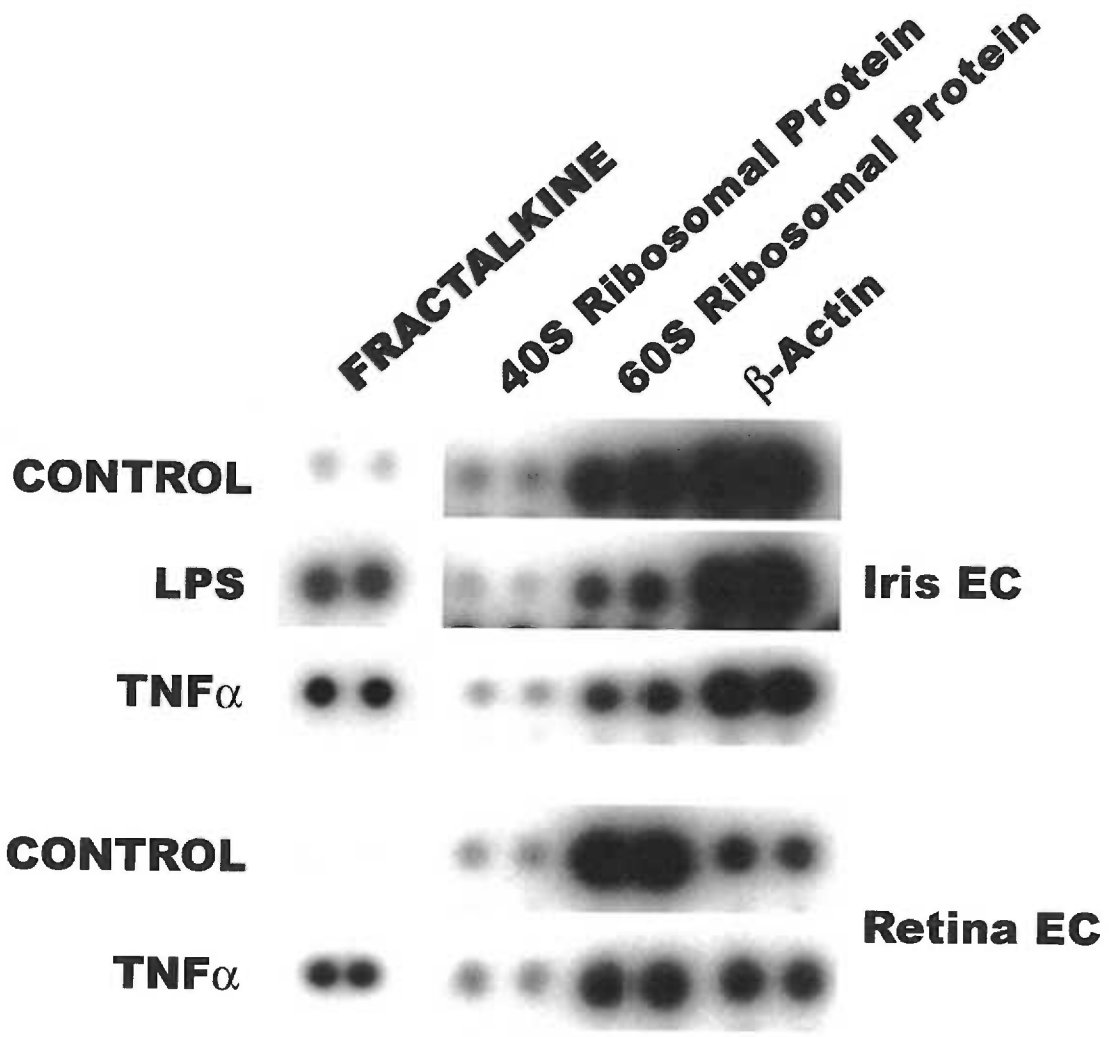
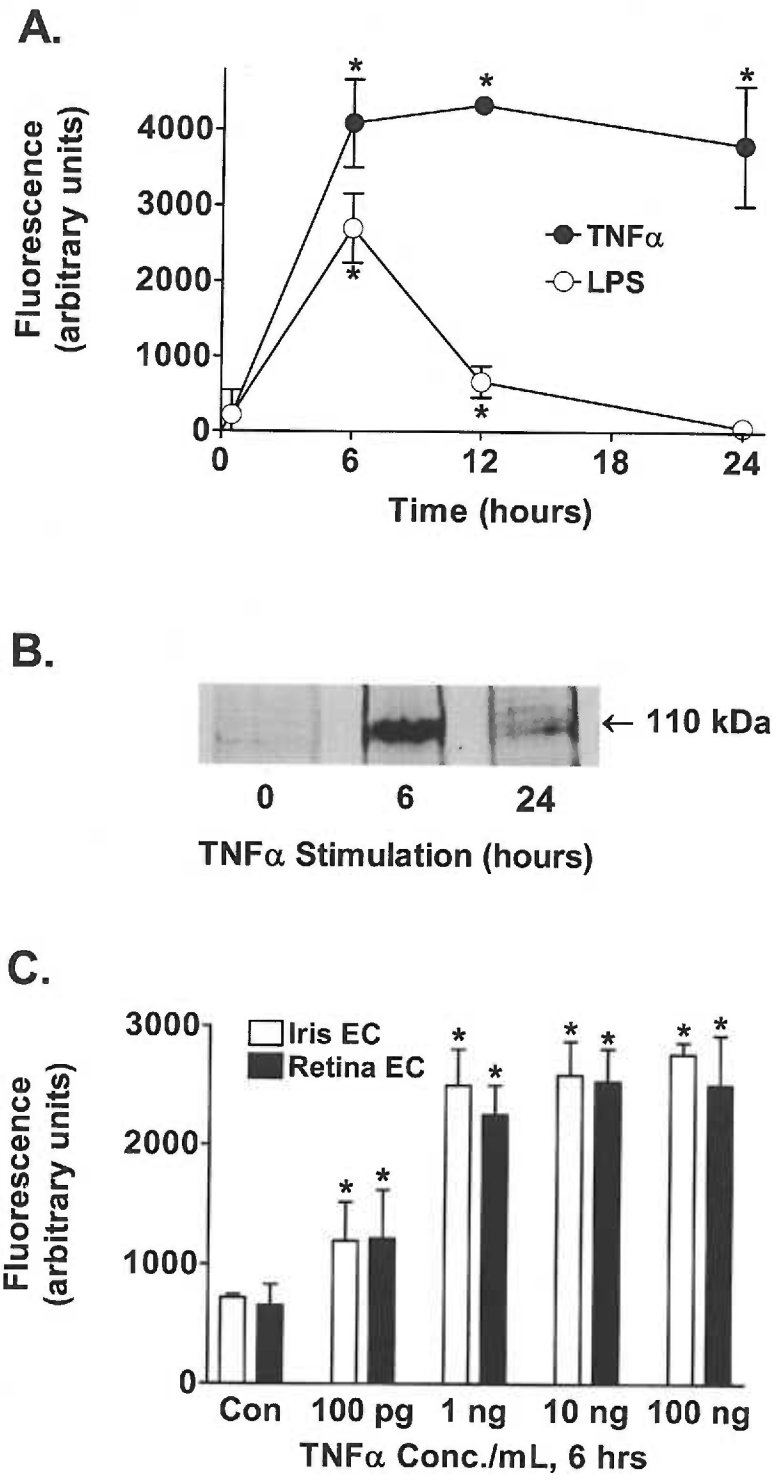
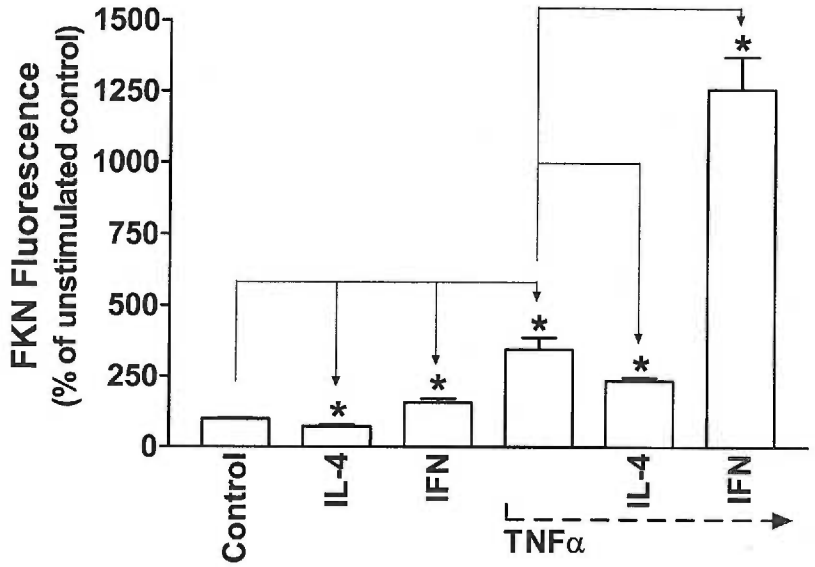


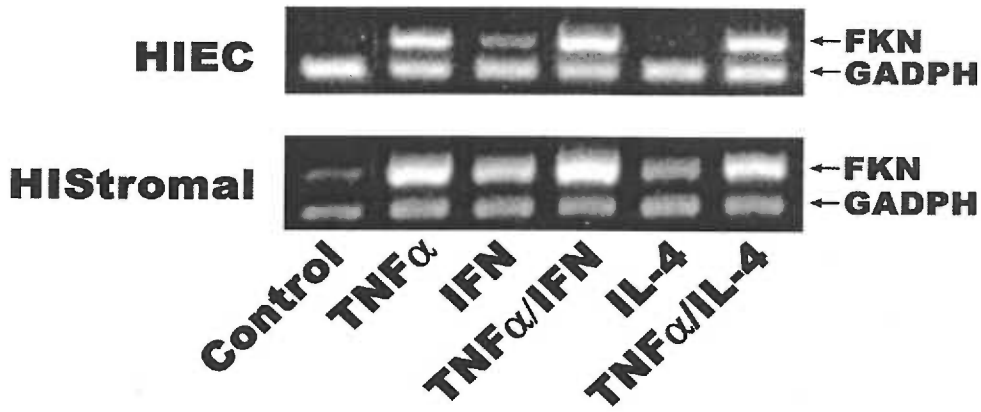
Figure 3. cDNA array analysis of LPS- and TNFα-induced FKN in ocular EC. Low constitutive FKN expression was markedly upregulated in iris and retinal EC after 3 hrs stimulation with LPS (10 μg/ml) or TNFα (10 ng/ml). Signals for ribosomal proteins 40S and 60S, and for β-actin (3 of the 8 housekeeping genes on the Clontech Atlas Arrays) are included as internal controls. Data are from one representative experiment of at least 3 independent experiments per condition. Similar results were also obtained in one experiment with human choroid EC (not shown).


A.
Fluorescence (arbitrary units)
4000
3000
2000
1000
0
0
6
12
18
24
Time (hours)
TNFα
LPS
B.
← 110 kDa
0
6
24
TNFα Stimulation (hours)
C.
Fluorescence (arbitrary units)
3000
2000
1000
0
Iris EC
Retina EC
Con
100 pg
1 ng
10 ng
100 ng
TNFα Conc./mL, 6 hrs

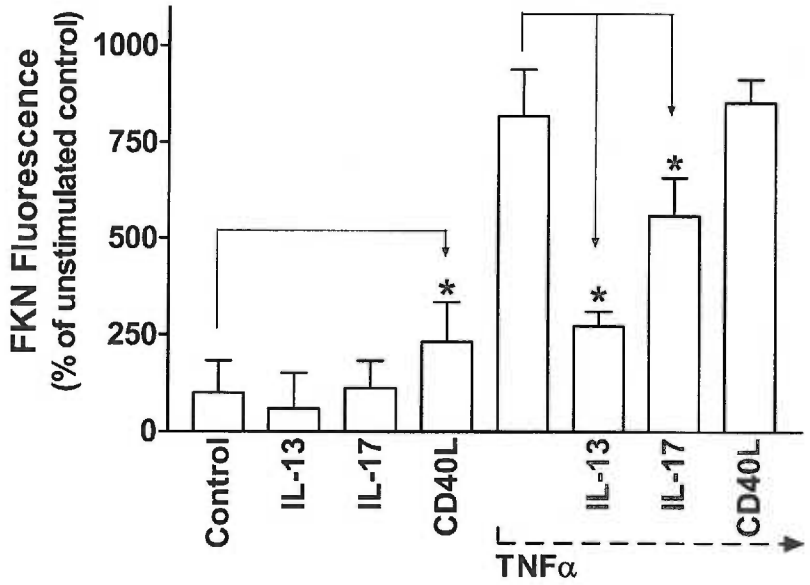
A


FKN Fluorescence
(% of unstimulated control)
1500
1250
1000
750
500
250
0
*
Control
IL-4
IFN
IL-4
IFN
TNFα

B


HIEC
HIStromal
←FKN
←GADPH
←FKN
←GADPH
Control
TNFα
IFN
TNFα/IFN
IL-4
TNFα/IL-4

C


FKN Fluorescence
(% of unstimulated control)
1000
750
500
250
0
*
Control
IL-13
IL-17
CD40L
IL-13
IL-17
CD40L
TNFα

Chapter 5

Comparing Inflammatory Mediator Expression Profiles in Human Iris versus Retinal Microvascular Endothelium using cDNA Arrays

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This work was presented, in part, as a poster at the Keystone Symposium on Molecular Mechanisms of Leukocyte Trafficking, April 12, 2002, Steamboat Springs, CO, and at the Association for Research in Vision Science annual conference on May 6, 2002, Ft. Lauderdale, FL. Cell culture work was performed by MDS and YP. Experiments for Figure 1 and Tables 1 and 2 were conducted by MDS and PVT. Experiments for Figures 2 were conducted by MDS and YP, and experiments for Figures 3-6 were conducted by MDS.

Summary

Inflammatory disorders often occur selectively in tissues of the eye, while other ocular tissues in close proximity remain completely uninvolved. The endothelium plays an essential role in mediating inflammation, in large measure by regulating leukocyte trafficking into inflamed tissues. Given that different ocular tissues have different predispositions for becoming inflamed, we hypothesized that this variable propensity towards inflammation is due, at least in part, to inherent biological differences within the endothelium that comprises these tissues' respective microcirculations.

Iris and retinal microvascular EC (HIEC and HREC) cultures were developed as a model system to directly compare gene expression between the iris and retinal endothelia. In appreciation of the biological diversity that exists even amongst different individuals within a species, we established donor-matched pairs of human iris and retinal EC. Using cDNA expression arrays for mRNA analysis, and protein quantitation methods for validation of array findings, we compared the expression profiles of these two EC types, with special interest given to adhesion molecules and chemokines that have potential importance in ocular inflammation.

Overall, constitutive gene expression profiles of HIEC and HREC were remarkably similar. By gene array, we observed message for several potential inflammatory mediators (*e.g.*, the adhesion molecules SQM and MUC-18), that had not previously been reported in the eye. Following stimulation with TNFα or LPS, both cell types responded by upregulating mRNA for a variety of adhesion molecules, chemokines, and cytokines. Representative inflammatory mediators (*e.g.*, IL-6, IL-8) were validated at the protein level.

From gene array screens, two adhesion molecules (E-selectin and VCAM-1), and one chemokine (MCP-1) were consistently and significantly several-fold elevated in TNFα-activated HREC versus HIEC. At the protein level, we were unable to confirm differences in VCAM-1 expression between these EC types. Secreted MCP-1 levels tended to be higher in HREC vs HIEC conditioned media, although this difference was not statistically significant. E-selectin protein, however, was significantly and reproducibly elevated both on HREC cell surfaces and in their cell culture supernatants, compared to HIEC derived from the same individuals. Neutralizing antibody against E-selectin, but not recombinant human soluble E-selectin, inhibited leukocyte binding to TNFα-activated microvascular EC.

These data demonstrate that inherent diversity exists in inflammatory gene regulation between two microvascular EC types derived from different but closely situated tissues. The most pronounced difference that we detected was in E-selectin expression, an EC/leukocyte adhesion molecule with a well-known role in propagating inflammation. Endothelial surface expressed E-selectin played an important role in mediating leukocyte attachment to activated EC, while soluble E-selectin was without effect. The EC type-specific difference that we observed in cell surface E-selectin expression supports our hypothesis that tissue-specific microvascular EC heterogeneity might be an important determinant of a tissue's susceptibility to developing inflammation.

Introduction

The biological activities of the endothelium determine whether leukocytes are permitted exit from the circulation to sites of inflammation.[2, 8, 9] Extravasation of leukocytes across the endothelial monolayer does not occur uniformly along the vascular tree, but instead occurs preferentially in some vascular regions compared to others.[70, 72] Tissue-specific leukocyte extravasation is exemplified in inflammatory diseases that selectively target certain tissues while sparing other nearby ones.[67, 73] Tissue-specific susceptibility to inflammation could be due to intrinsic biological differences amongst the EC that reside in these divergent tissues. Alternatively, EC from different regions of the vascular tree might comprise a functionally homogenous cell population, but whose activity is differentially regulated by tissue-specific variations in local environmental cues (i.e., the cellular and molecular milieu, or hemodynamics). Many tissues are incredibly diverse in terms of the number and complexity of the microenvironments that are contained within them,[65] and the EC investing these tissues are exquisitely responsive to the molecular and cellular components that surround them.[5, 142-144] The primary aim of this study was to explore whether innate biological differences exist amongst human microvascular EC derived from different tissues of the same individuals

In a standardized cell culture environment, we compared for the first time the gene expression profiles of two different microvascular EC types that were derived from the same human donors, with special emphasis placed on characterizing genes with a high potential for mediating inflammation. Using cDNA arrays, we observed that although gene expression profiles are largely similar between donor-matched HREC and HIEC, mRNA levels for several known inflammatory mediators were differentially

regulated after exposure to TNFα. At the protein level, one of those genes identified, the adhesion molecule E-selectin, was confirmed to be significantly higher in TNFα-activated HREC vs HIEC. In adhesion assays, EC surface E-selectin was more important than soluble E-selectin in mediating leukocyte adhesion to TNFα-activated EC. The observed difference in E-selectin expression supports the idea that innate biological variability exists amongst EC from distinct microvascular beds, and suggests that EC heterogeneity might be reflected at the functional level by tissue- or site-specific variations in EC/leukocyte interactions. If this paradigm holds true, vascular regions with enhanced EC/leukocyte interactions would likely facilitate greater local leukocyte extravasation, and thus render the tissues surrounding those vessels more prone to developing inflammation.

Results

Donor-Matched Ocular EC Cultures

Using the protocols detailed in the Methods section,[145] we established donor-matched cultures of HIEC and HREC for comparison studies. In early efforts, we tested a variety of enzyme-based digestion protocols to maximize initial EC yields from retina and iris tissues. Using these protocols, we typically obtain approximately 50 x 10^3 viable EC per iris or retina pair. Starting with similar cell yields from both tissues is useful, since we wanted these cultures to be population doubling-matched to minimize the biological variability that might arise amongst differently aged cultures. Proliferation rates and confluent cell densities were very similar for these donor-matched cultures (not shown), and all cells were used between passage 3 and 5 in experiments.

Inflammatory Mediator Expression in Ocular EC

By gene array (Figure 1), we evaluated a host of known adhesion molecules and chemokines for their constitutive or TNFα- or LPS induced expression in HIEC and HREC (Tables 1 and 2). These studies provided a comprehensive profile in these cells, of the expression of many genes with known involvement in leukocyte trafficking. Figure 1 shows typical raw data generated by this approach, with some of the molecules of interest highlighted. The expression of some of the cell adhesion molecules that were identified (*e.g.*, ICAM-1 and VCAM-1), had previously been evaluated at the protein and functional levels in these cultured EC (Chapter 3, Figures 3-5),[145]. Several adhesion molecules whose expression was identified in ocular EC do not appear to have been previously investigated in the ocular microcirculation by others (*e.g.*, ICAM-2, MUC18, SQM1,

fractalkine). We also evaluated the protein expression of representative members of the cytokine and chemokines families that have known roles in ocular inflammation (*e.g.*, IL-6,[146] and IL-8,[147, 148] respectively), in ocular EC (Figure 2).

Constitutive gene expression profiles were remarkably similar in donor-matched HIEC and HREC. Nearly 1000 known genes were surveyed by gene array, and, of the 60 or so adhesion molecules and chemokines included, none showed at least a two-fold difference in basal expression level between the cell types. Constitutive platelet-derived growth factor-A[149] (PDGF-A subunit, Genbank accession #X06374) expression was 3.3 ± 1.6-fold higher in HIEC than in HREC ($p=0.012$, n=4 experiments using different donor-matched EC pairs), and mutL DNA mismatch repair protein homolog-1[150] (MLH-1, Genbank accession #U07418) expression was 2.6 ± 0.74-fold higher in HIEC vs HREC ($p=0.019$, n=3 matched pairs). Since neither of these two molecules plays a known role in leukocyte trafficking, they were not studied further.

Upon activation with TNFα or LPS, a number of genes with proven roles in inflammation were significantly upregulated after 3 hrs, in both EC types (Figures 1 and 2; Tables 1 & 2). TNFα (10 ng/ml) and LPS (10 μg/ml) resulted in qualitatively similar gene changes when either EC type were stimulated with these agents, although at these concentrations TNFα appeared to be a more potent and persistent stimuli than LPS in inducing chemokine and adhesion molecule expression (*e.g.*, fractalkine regulation is discussed in detail in Chapter 4). For donor-matched iris vs retina comparisons, we used only TNFα, and not LPS, as the activating agent. Of the adhesion molecules studied by gene array, message for two molecules, E-selectin and VCAM-1, was significantly upregulated in TNFα-stimulated HREC vs HIEC (≈ 2- and 3-fold elevated at 3 hrs,

respectively; Figure 3). Neither of these molecules was detectable in unstimulated EC. Only one chemokine, monocyte chemoattractant protein-1 (MCP-1), which was constitutively and similarly detectable in unstimulated donor-matched HREC and HIEC, was elevated by TNFα (3 hrs) to significantly different levels in activated HREC vs HIEC (Figure 4). In these experiments, TNFα-stimulated HREC expressed approximately 2.5-fold more message for MCP-1 than did similarly activated HIEC.

Validation of Gene Array Results at the Protein Level in Ocular EC

We measured E-selectin protein on both cell surfaces and in conditioned media of donor-matched HREC and HIEC, using a cell monolayer-based immunodetection method (ELICA) and a standard capture ELISA, respectively. After 6 hrs TNFα stimulation, E-selectin on cell surfaces was significantly elevated in HREC to levels approximately 40% greater than in donor-matched HIEC (Figure 5A). In one experiment comparing cell surface E-selectin on endotoxin-stimulated (10 μg/ml, 4 hrs) retinal and iris EC from unmatched donors, and in one experiment comparing E-selectin on donor-matched interferon γ-stimulated (100 ng/ml, 4 hrs) HREC and HIEC, retinal EC expressed 2- and [150]observations suggest that retinal EC might produce greater amounts of E-selectin than do iris EC, in response to a variety of pro-inflammatory stimuli. In culture media that was conditioned by donor-matched EC that had been activated by TNFα (10 ng/ml, 24 hrs), the amount of soluble E-selectin was significantly elevated to levels ≈3-fold higher in HREC vs HIEC (Figure 5B), consistent with the array results.

We also measured MCP-1 in conditioned media from these activated EC. TNFα significantly elevated immunodetectable soluble MCP-1 in both cell types. In two donors

tested, TNFα-induced MCP-1 levels exhibited higher levels in HREC vs HIEC media, but this difference did not attain statistical significance (n=2 experiments using 2 different donor matched pairs, not shown). We were also unable to discern any differences in cell surface VCAM-1 in HREC and HIEC that had been stimulated with TNFα for 6 and 24 hrs (n=2 experiments with different matched EC pairs, not shown).

Investigating the Functional Significance of Soluble E-Selectin

We tested the ability of soluble E-selectin to mediate the binding of U937 monocytes, Jurkat T cells, and normal human polymorphonuclear cells to cultured HREC monolayers. Background binding of U937 monocytes to unstimulated HREC was low, but attachment was markedly upregulated when the EC had been pre-stimulated with TNFα (Figure 6A). Similar results were obtained using Jurkat T cells or freshly isolated human peripheral blood neutrophils (not shown).

In earlier experiments, E-selectin expression on TNFα-activated EC was approximately half-maximal at 2 hrs, maximal at 4 hrs, and had decayed to control levels by 12 hrs (see Chapter 3, Figure 4D), and the EC_{50} for inducing E-selectin was approximately 100 pg/ml TNFα (not shown). Other inducible EC adhesion molecules (*i.e.*, ICAM-1, VCAM-1, and fractalkine) are also upregulated by TNFα, but maximal cells surface expression of these molecules appears later than does E-selectin (see Chapter 3, Figure 4A and Chapter 4, Figure 4A)[81, 145]. Thus, in order to maximize E-selectin-mediated leukocyte binding and minimize the contribution of these other adhesion molecules in adhesion assays, subsequent experiments were performed with HREC that had been stimulated for 2 hrs with TNFα.

U937 monocyte binding to TNFα-stimulated (0-1 ng/ml, 2 hrs) HREC increases in a cytokine concentration-dependent manner (Figure 6A). The majority of this binding is mediated by E-selectin, since monocyte adhesion is almost completely inhibited by the inclusion of neutralizing anti-E-selectin antibody (Figure 6A). This antibody similarly inhibited the adhesion of Jurkat T cells and neutrophils to TNFα-activated HREC (not shown). The inclusion of recombinant soluble E-selectin (10 μg/ml) did not have any effect on U937 monocyte binding to unstimulated HREC (Figure 6B). Soluble E-selectin (up to 10 μg/ml) did not affect U937 monocyte binding to TNFα-activated HREC, as well. Similar to the results obtained using U937 monocytes, soluble E-selectin did not affect the binding of Jurkat T cells or neutrophils to either unstimulated or TNFα-activated HREC (not shown).

Discussion

The primary finding of this experimental series is that human microvascular EC derived from iris and retina, although largely alike in overall gene expression profiles, do indeed exhibit heterogenous expression patterns of some genes. All of the genes that displayed tissue-specific variability in regulation by TNFα are known modulators of the inflammatory response, with important roles in mediating EC/leukocyte interactions. If maintained in the *in vivo* scenario, this observation would be a strong indication that inherent biological differences amongst EC assist in determining whether a particular tissue will respond to activating stimuli with an inflammatory response.

This is not the first study to investigate or support the idea of EC heterogeneity,[69, 71, 85, 144] but is the first to compare microvascular EC derived from different sites within the same individuals. Indeed, after close scrutiny of the 277 references that were retrieved from a Pubmed search cross-referencing "endothelial cell" and "heterogeneity" (08/14/02), only five other studies appear to have compared any human EC types cultured from the same individuals,[81, 151-154] three by members of our team, and the numbers of donors used in all of these studies were very low. None of these studies compared different microvascular EC from the same individuals. Previous reports have described marked biological heterogeneity, including of adhesion molecule and chemokine expression, when different EC types were compared (summarized in references [71, 144]). Almost always, however, and understandably given the technical hurdles that need be overcome in generating donor- and passage-matched EC cultures, these studies compared cells from different donors.

In our hands, remarkable variation occurs in both constitutive and TNFα-activated EC, when cells from the same tissues (*e.g.*, irises) of different individuals were compared (not shown). Only when we switched to a donor-matched system did potentially important differences emerge from the high background caused by person-to-person biological variability. Thus, in future studies that address EC heterogeneity, we issue caution regarding the interpretation of observations made when comparing EC that originate from different individuals.

The significant difference we observed is that both cell surface-expressed and secreted/shed E-selectin protein expression levels are greater in activated retinal versus iris EC. E-selectin is a well known mediator of ocular inflammation,[93, 155, 156] and this molecule's unequal expression by iris and retinal EC might be reflected in differing propensities for these tissues to become inflamed in response to locally elevated inflammatory cytokine levels. E-selectin mRNA and protein were undetectable in unstimulated EC. After stimulation with TNFα, cell surface E-selectin protein expression was markedly but disproportionately elevated in these two microvascular EC types, with levels approximately 40% greater in retinal EC versus iris EC. The physiological implications of this observed difference are currently unclear. In another study using a cultured EC adhesion model similar to ours, small increases in expression of the adhesion molecule VCAM-1 were reflected in proportionately increased monocyte/EC binding.[53] In our assay system, TNFα-activated EC bind monocytes, neutrophils, and T lymphocytes at levels 10 to 20-fold above adhesion to unstimulated EC. If similar E-selectin-dependent adhesion differences occur within the microcirculations of different

tissues *in vivo*, a 40% elevation in leukocyte extravasation might greatly exacerbate inflammation in those tissues that express more E-selectin.

While cell surface E-selectin was elevated by approximately 40%, secreted/shed E-selectin levels were 3-fold higher in retinal EC. Locally elevated levels of soluble E-selectin in the retinal microvasculature might limit an inflammatory response by binding and occupying the cognate receptor(s) on circulating leukocytes, thus precluding these leukocytes from engaging E-selectin on the vascular wall. Alternatively, previous reports have described a chemotactic/activating role of both EC-surface bound and soluble E-selectin on neutrophils,[157-159] and soluble E-selectin reportedly has a chemotactic effect on monocytes.[160] Results from these studies suggest that elevated endothelial E-selectin expression, whether cell-bound or soluble, might perpetuate an inflammatory response.

In our hands, recombinant human soluble E-selectin neither potentiated nor inhibited monocyte, neutrophil, and T cell adhesion to unstimulated or TNFα-activated HREC. Similar results were obtained by other groups, wherein although soluble E-selectin almost completely inhibited HL-60 human myeloid leukemia cell adhesion to soluble E-selectin-coated microtiter plates,[161] soluble E-selectin did not affect the binding of these same HL-60 cells to cultured EC.[162] Taken together with our data, these latter observations suggest that additional E-selectin adhesive ligands[163-168] are present on the leukocytes that we tested, and that these ligands are not blocked by exogenous recombinant soluble E-selectin. In our experimental series, we tested the effects of soluble E-selectin on two cell lines (U937 monocytes and Jurkat T cells) and freshly isolated neutrophils. Some of the earlier work describing an activating effect of soluble E-selectin used freshly isolated monocytes.[160] Thus, leukocytes might respond to soluble

E-selectin in a cell type-specific fashion that was not discernable with the leukocyte types and/or assays that we employed.

The disparity in the reported effects of soluble E-selectin on leukocyte activation and adhesion might also be attributable to varied sources for the recombinant protein used in different studies. The protein that we used was the extracellular domain of E-selectin produced in Chinese hamster ovary (CHO) cells, while other studies have used recombinant E-selectin extracellular domain/Protein A domain chimeras from a baculovirus insect cell expression system.[157, 169] It is conceivable that these different expression systems yield a protein product with distinct structural conformations or glycosylation patterns that might underlie the reported functional differences. In support of a lack of effect of soluble E-selectin on leukocyte activation/adhesion, preliminary gene array experiments testing U937 monocytes, Jurkat T cells, and human neutrophils that were exposed to soluble E-selectin (1 μg/ml, 3 hrs), have not revealed any changes in mRNA expression for the 588 molecules evaluated (unpublished observations).

Soluble E-selectin has also been reported to have pro-angiogenic activity.[170] Although we did not test this possibility, it is interesting to speculate that the markedly elevated E-selectin that we observed shed from retinal EC versus iris EC might cause or exacerbate the neovascularization that accompanies some retinopathies. Along these lines, increased levels of soluble E-selectin have been measured in vitreous humor of persons with proliferative diabetic retinopathy.[171]

Besides regulating leukocyte migration, adhesion molecules play important roles in infection. For example, ICAM-1 expression by the upper respiratory epithelium is a primary mechanism by which rhinoviruses associated with the common cold gain entry to

the body.[172] In the eye, certain pathogenic microbes preferentially infect one tissue over another. Cytomegalovirus (CMV), for example, tends to infect the retina but not the iris.[74] Although CMV has been clearly shown to activate infected EC and upregulate E-selectin expression in these cells,[173] whether E-selectin potentiates CMV infections in ocular tissues is unknown.

Dissemination of a variety of neoplasms has also been shown to utilize adhesion molecules,[174-176] including E-selectin.[177, 178] B cell lymphomas that invade the eye do so almost exclusively in the retina, and are almost never observed in the iris.[88] It will be important in the future to evaluate whether innate heterogeneity of adhesion molecule expression in the eye, including of E-selectin, plays any role in determining the affected tissues in any of these ocular metastatic disorders.

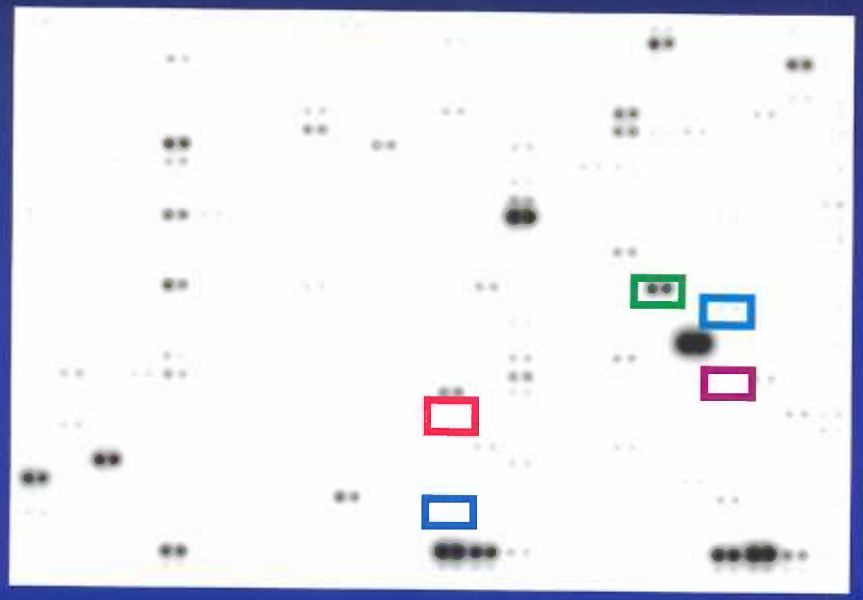
Although we have solidly confirmed only one difference in gene/protein expression amongst HREC and HIEC in this study, we have only investigated a limited spectrum of the total genome, in studies that compared tissues from 4 individuals. If current estimates of the complexity of the human genome are accurate,[179] we would expect that many more ocular EC genes might show tissue-specific regulation. A thorough understanding of innate functional differences in the microvasculatures of discrete tissues might allow us the opportunity to selectively regulate them.

Acknowledgements

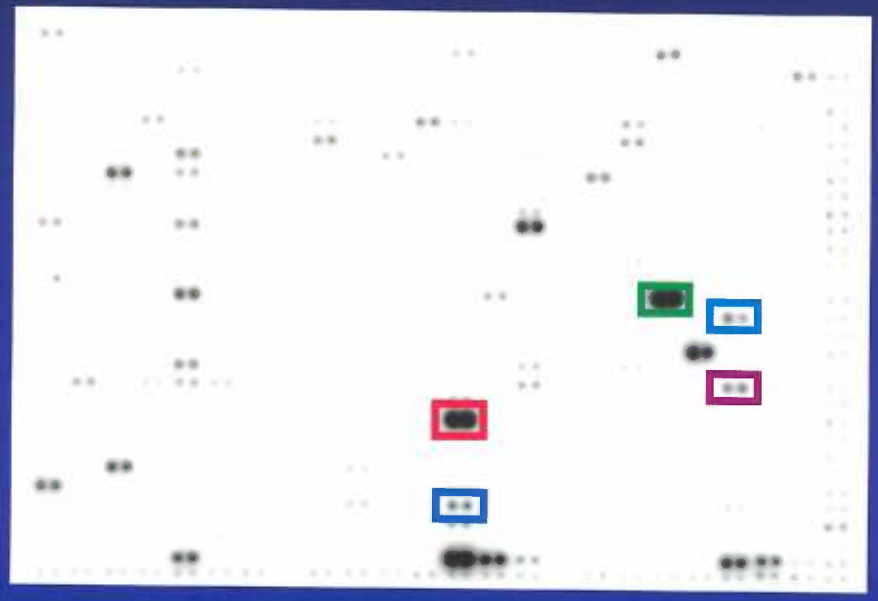
We thank Rory Dunaway and Tracy Gerlach of the Lions Eye Bank, Portland, OR, for providing donor tissues. This project was supported by NEI grants EY06477, EY06484, and EY10572; by Research to Prevent Blindness awards to JTR, SRP, and the Casey Eye Institute; by Tartar Trust Research Fellowship grants (Oregon Science Foundation, Portland, OR) to MDS and to DOZ; and by a Retina Research Foundation Travel Scholarship Award and a Keystone Symposia Travel Scholarship to MDS.

Iris EC Gene Expression Profiles

Control



LPS, 3 hrs.



ICAM-1 VCAM-1 MCP-1 MCP-2α IL-8

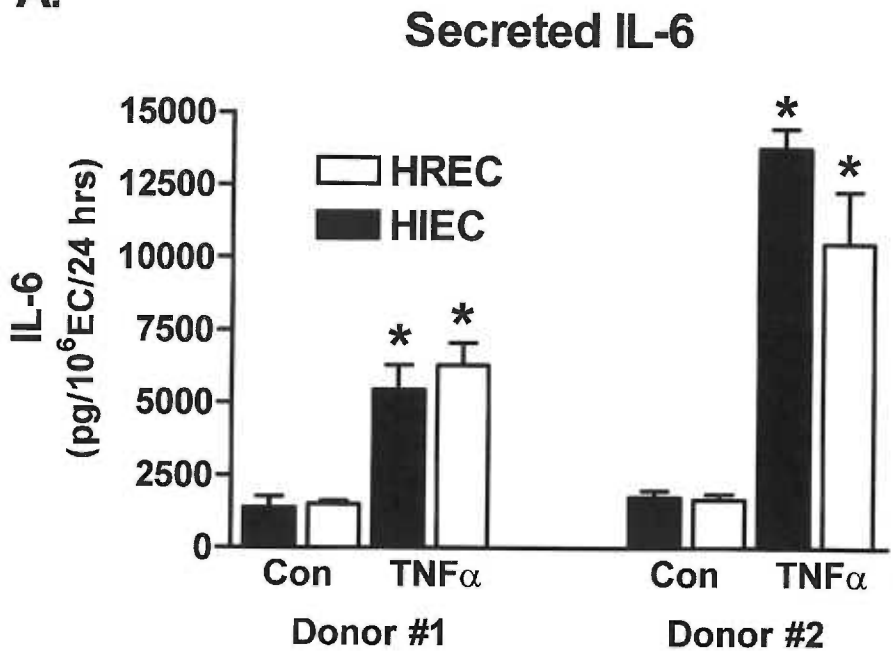
GenBank Accession #	Adhesion Molecule	Constitutive Expression	TNFα, 3hrs	TNFα, 24 hrs
J03132	Intercellular Adhesion Molecule-1 (CD54)	+	↑↑↑	↑↑↑
X15606	Intercellular Adhesion Molecule-2 (CD102)	+	nc	↓
X69711	Intercellular Adhesion Molecule-3 CDW50)	—	nc	nc
M30257	Vascular Cell Adhesion Molecule-1 (CD106)	+/-	↑↑↑	↑↑↑
M28526	PECAM-1 (CD31)	+	nc	nc
L20471	Basigin (CD147)	+	nc	nc
M28882	MUC18 (CD146)	+	nc	nc
S71824	NCAM-1 (CD56)	—	nc	nc
M30640	E-Selectin (CD62E)	—	↑↑↑	nc
M25280	L-Selectin (CD62L)	+	nc	nc
M25322	P-Selectin (CD62P)	+	nc	nc
M33374	Cell Adhesion Molecule SQM1	+	nc	nc
M81104	CD34	+	nc	nc
M59040	CD44H	+	nc	nc
U91835	Fractalkine	+/-	↑↑↑	↑↑↑
X68742	Integrin alpha 1 (VLA1a, CD49A)	—	nc	nc
M34480	Integrin alpha 2B (CD41)	—	nc	nc
M59911	Integrin alpha 3 (VLA3a, CD49C)	+	nc	nc
L12002	Integrin alpha 4 (VLA4a, CD49D)	—	nc	nc
X06265	Integrin alpha 5 (VLA5a, CD49E)	+	nc	nc
X53586	Integrin alpha 6 (VLA6a, CD49F)	+	nc	nc
X74295	Integrin alpha 7b	—	nc	nc
L25851	Integrin alpha E (CD103)	+	nc	nc
Y00796	Integrin alpha L (CD11a)	+	nc	nc
J04145	Integrin alpha M (CD11b)	—	nc	nc
M14648	Integrin alpha V (CD51)	—	nc	nc
M81695	Integrin alpha X, (CD11c)	—	nc	nc
X07979	Integrin beta 1 (CD29)	+	nc	nc
M15395	Integrin beta 2 (CD18)	—	nc	nc
J02703	Integrin beta 3 (CD61)	+	nc	nc
X53587	Integrin beta 4 (CD104)	—	nc	nc
J05633	Integrin beta 5	+	nc	nc
M35198	Integrin beta 6	—	nc	nc

Table 1. Adhesion molecule expression in control and TNFα-stimulated ocular EC.

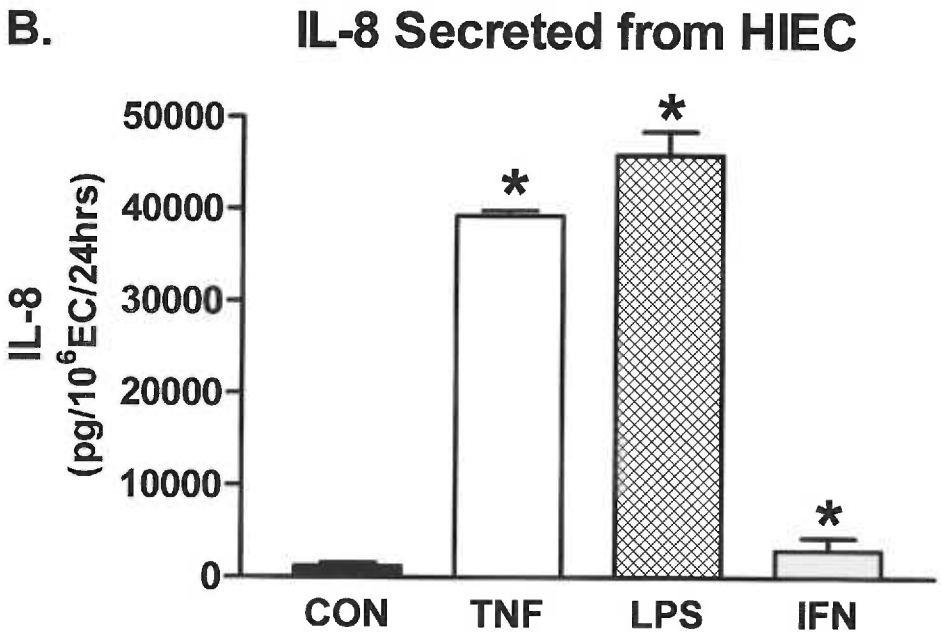
GenBank Accession #	Chemokine Class	Chemokine	Constitutive Expression	TNFα, 3 hrs	TNFα, 24 hrs
D49372	CC Class	Eotaxin, SCYA11	—	nc	nc
U88320		SLC, SCYA21	+	nc	nc
M57502		I-309, SCYA1	+/-	nc	nc
M24545		MCP-1, SCYA2	+	↑↑	↑↑
Y10802		MCP-2, SCYA8	—	↑	↑
X72308		MCP-3, SCYA7	—	↑	↑
U83171		MDC	+	nc	nc
M23452		MIP-1α, SCYA3	—	nc	nc
J04130		MIP-1β, SCYA4	—	nc	Nc
X53799		MIP-2α, GROβ	+/-	↑↑↑	↑↑
U77180		MIP-3β, ELC	+/-	nc	↑
M21121		RANTES, SCYA5	+	nc	nc
D43767		TARC, SCYA17	—	nc	nc
X78686	CXC Class	ENA-78, SCYB5	—	↑	↑
Y00787		IL-8	+	↑↑↑	↑↑↑
X02530		IP-10, SCYB10	—	nc	nc
X72755		MIG, SCYB9	—	nc	nc
U16752		SDF-1, SCYB12	+	nc	nc
U91835	CX_3C Class	Fractalkine	+/-	↑↑↑	↑↑↑
U23772	C Class	Lymphotactin	—	nc	nc
U10117	Other	EMAP-II	+	nc	nc
M25639		MIF	+	nc	nc
D10925	Receptors	CCR1, MIP1α-R	+	nc	nc
U03905		CCR-2, MCP1-R	+/-	nc	nc
U28694		CCR-3, eotaxin-R	—	nc	nc
U60000		CCR-6	—	nc	nc
Y12815		CCR-9	—	nc	nc
D10924		CXCR-4, SDF1-R	—	nc	nc

Table 2. Chemokine Expression in Control and TNFα-Stimulated Ocular EC. Shown are cumulative data from 4-8 experiments using HIEC (control, and 10 ng/ml TNFα, 3 & 24 hours). Chemokine expression profiles of HREC were essentially identical to HIEC, with one important exception (see Fig. 4). "↑" indicates a 2- to 5-fold induction; "↑↑" indicates a 5- to 10-fold induction; and "↑↑↑" indicates >10-fold induction, versus unstimulated controls. "↓" indicates inhibition by >50% versus unstimulated controls. "**nc**" indicates not changed from constitutive expression levels.

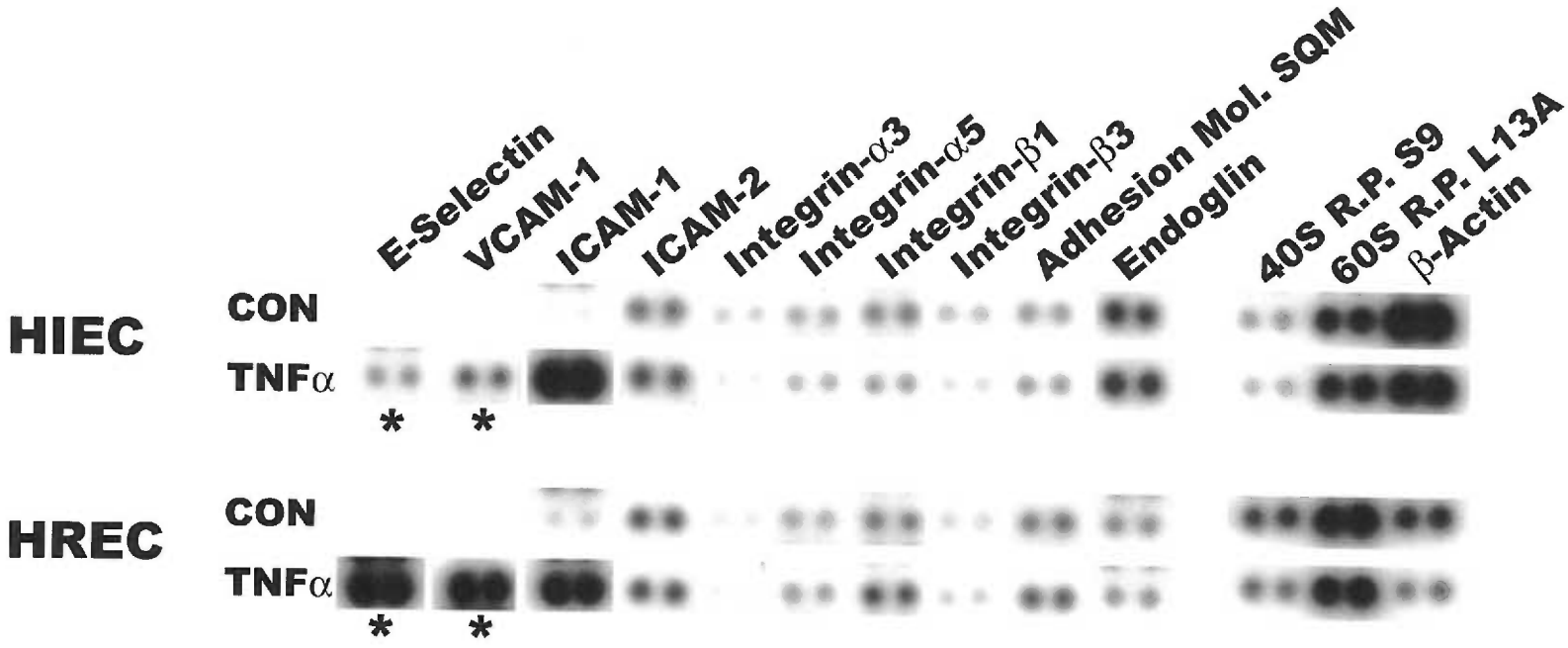
A.


Secreted IL-6
IL-6
(pg/10^6EC/24 hrs)
15000
12500
10000
7500
5000
2500
0
HREC
HIEC
*
Con
TNFα
Donor #1
Donor #2

B.


IL-8 Secreted from HIEC
IL-8
(pg/10^6EC/24hrs)
50000
40000
30000
20000
10000
0
*
CON
TNF
LPS
IFN

Cell Adhesion Molecules in Ocular EC



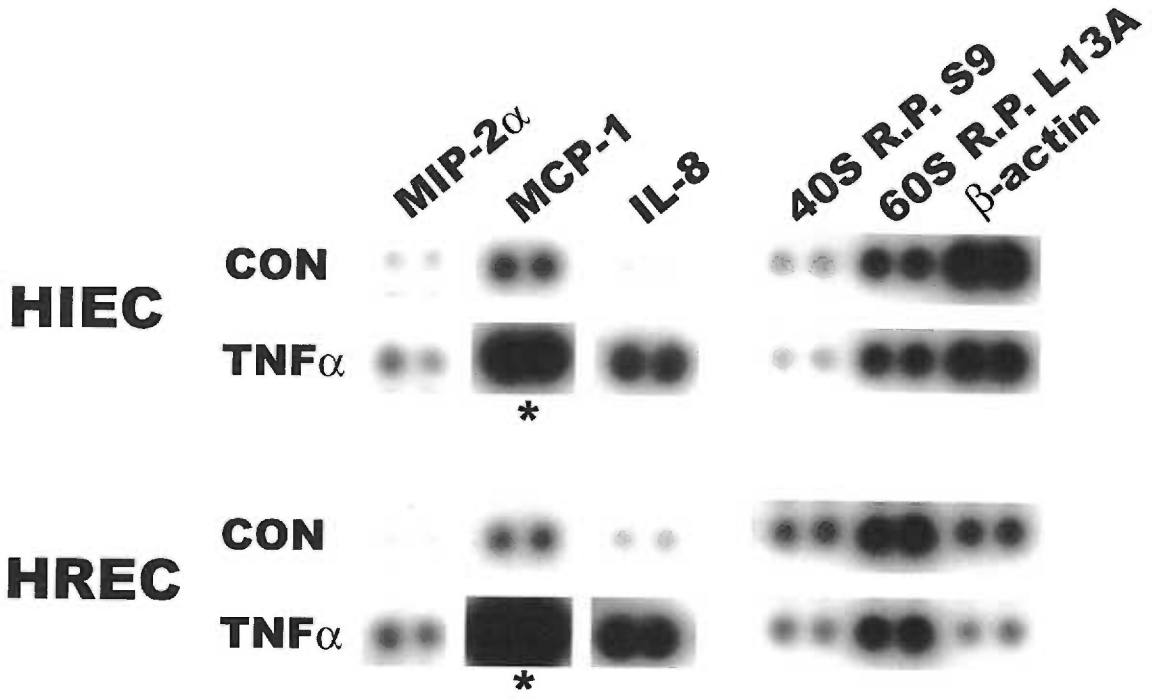


Figure 4. Comparison of chemokine expression in donor-matched iris and retina EC. Shown are gene array data on select genes from one donor-matched experiment (out of 4 different donor pairs). MCP-1 was constitutively expressed by both human iris and retinal EC (HIEC and HREC), at similar levels. After TNFα-stimulation (10 ng/ml, 3 hours), however, MCP-1* was significantly elevated to levels ≈2.5-fold over that in activated HIEC (n=4 experiments using different donor pairs, p=0.03 by ANOVA).

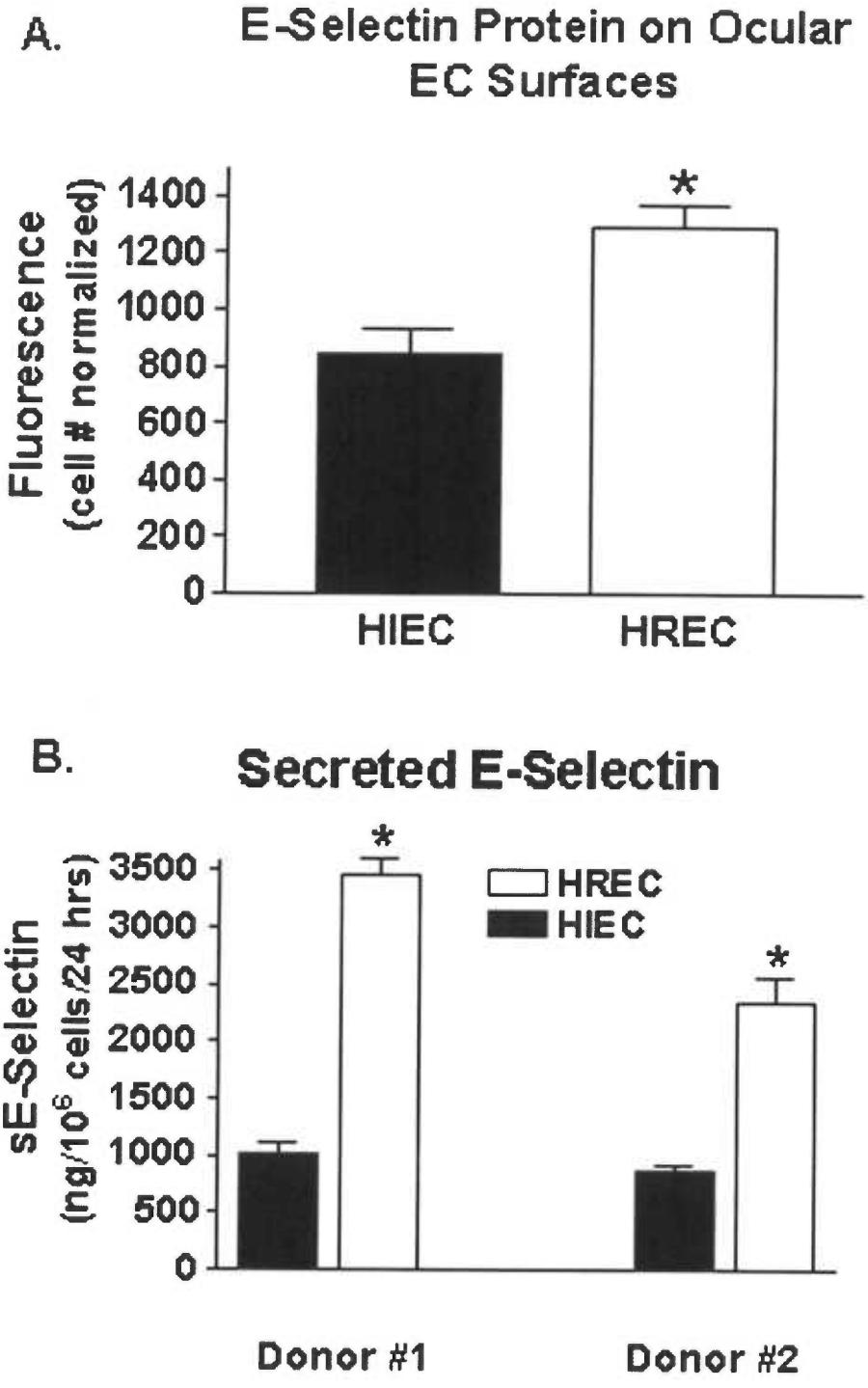
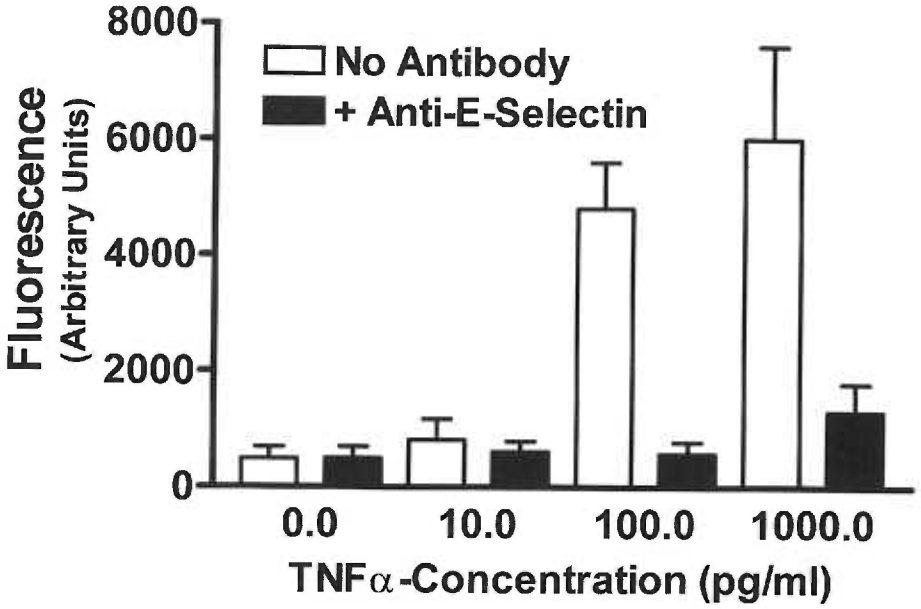
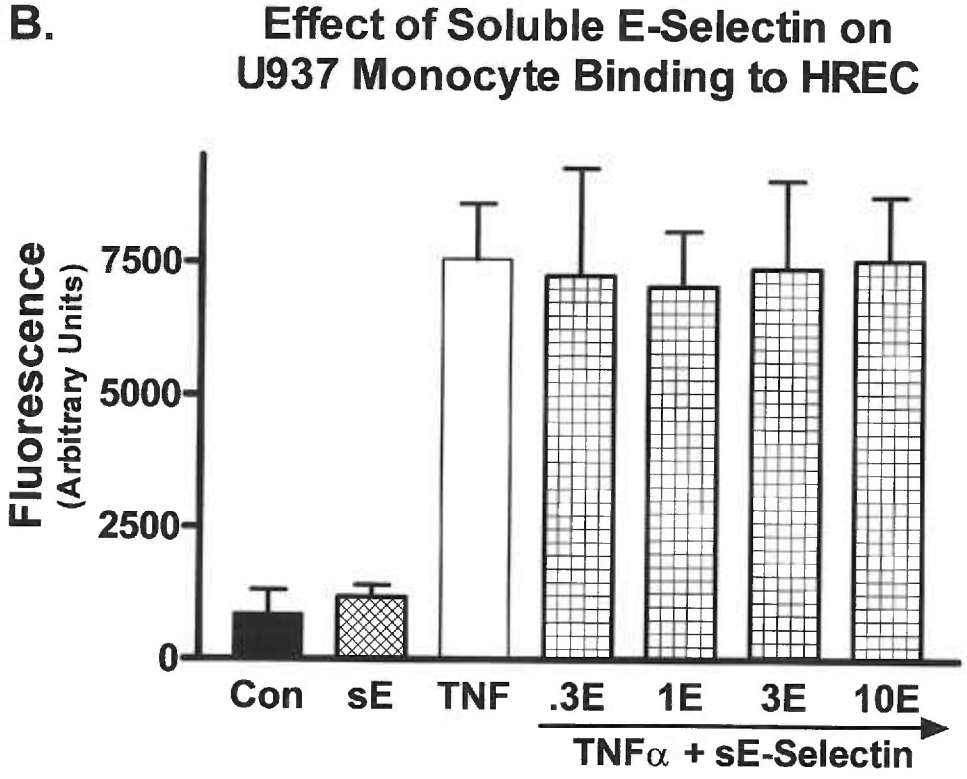


Figure 5. Cell surface and secreted E-selectin

A.


E-Selectin-Dependent U937
Monocyte Binding to HREC
Fluorescence
(Arbitrary Units)
8000
6000
4000
2000
0
No Antibody
+ Anti-E-Selectin
0.0
10.0
100.0
1000.0
TNFα-Concentration (pg/ml)

B.


Effect of Soluble E-Selectin on
U937 Monocyte Binding to HREC
Fluorescence
(Arbitrary Units)
7500
5000
2500
0
Con
sE
TNF
.3E
1E
3E
10E
TNFα + sE-Selectin

Chapter 6. Summary and Conclusions

6A. Summary of Findings and Relation to Existing Knowledge

Leukocyte extravasation is a key event in the inflammatory response, and this cell migration is critically dependent upon the nature of EC/leukocyte adhesive interactions.[2, 8, 9] Inflammation, and the adhesive interactions that promote it, do not occur homogenously throughout the vascular tree, but instead in many diseases occurs preferentially in some sites compared to others.[70, 72] We hypothesized the existence of EC heterogeneity, manifested in differences in the innate propensity of iris versus retinal EC to express adhesion molecules and chemokines.

In order to test this hypothesis, we first established a well-controlled *in vitro* system, *i.e.*, pure EC cultures derived from the iris, choroidal, and retinal microvasculatures, and used gene arrays to generate gene expression profiles in these cells under basal and cytokine-stimulated conditions. Since human iris EC had not previously been cultured by others, we established their endothelial nature by evaluating them for a variety of characteristic phenotypic traits,[71] such as their uptake of LDL[77] and their ability to undergo *in vitro* angiogenesis given the proper environment.[78] We also tested their ability to express a variety of cell adhesion molecules and to support leukocyte adhesion in static culture assays.

Ocular tissues employ a diversity of adhesion molecules and chemokines in regulating leukocyte traffic during inflammation.[75] By cDNA array screening, we identified in ocular EC the constitutive expression of a wide variety of inflammatory mediators, several of which had not previously been explored in the eye by others (*e.g.*, ICAM-2, MUC18, SQM1, fractalkine; Pubmed searches 05/23/02). Both

MUC18 and SQM1 were constitutively expressed in ocular EC, and were not upregulated by TNFα. MUC18 (CD146, Mel-CAM, S-Endo-1) is an adhesion molecule of the immunoglobulin superfamily that is present on both endothelial and melanoma cells.[180] Similarly, SQM1 is an adhesion molecule with homology to the β integrin family, that is expressed by squamous cell carcinomas of the head and neck, and by EC.[181] Both of these molecules were reported to mediate homotypic as well as heterotypic cell interactions, suggesting a role in regulating tumor metastasis. It will be interesting in the future to evaluate whether these adhesion molecules also mediate homotypic microvascular EC-EC interactions during an inflammatory response.

We have also identified for the first time in ocular EC several adhesion molecules that are constitutively expressed but, unlike MUC18 and SQM1, are additionally regulated in these cells by the inflammatory agents LPS and TNFα. These include the molecules ICAM-2 and fractalkine (FKN), both of which are known to play important roles in inflammation.[49, 96, 97, 113-116] ICAM-2 is constitutively expressed in ocular EC, and both its mRNA and protein expression on cell surfaces is downregulated to 50% of constitutive expression levels after 24 hrs of TNFα stimulation in these cells, consistent with previous observations.[95] Even after appreciable cytokine-mediated downregulation, however, endothelial ICAM-2 is still functional on cell surfaces, as indicated in monocyte adhesion assays employing neutralizing anti-ICAM-2 antibodies.[145] These data suggest a likely role for ICAM-2 in mediating leukocyte trafficking within the eye.

The expression of FKN, a combination chemokine/adhesion molecule, was examined in significant depth in ocular tissues. FKN was constitutively expressed by

both ocular EC and stromal tissues, and was upregulated by LPS and TNFα. In agreement with earlier work on FKN expression by EC, TNFα-induced FKN was synergistically potentiated by Th1 cytokines (*i.e.*, interferon-γ), and was inhibited by classical Th2 cytokines (*i.e.,* IL-4 and IL-13). Additionally, we observed the novel and unexpected downregulation of FKN by the typically proinflammatory cytokine IL-17. The observed expression of FKN in ocular microvascular EC and stromal tissues, and the complexity of this molecule's regulated expression by diverse inflammatory mediators, suggests that FKN plays previously unrecognized roles in leukocyte trafficking in the eye both in noninflamed states as well as during inflammation.

After characterizing our *in vitro* model and exploring the regulated expression of several inflammatory mediators, we then used cDNA arrays to compare gene expression profiles in donor- and passage-matched iris and retinal EC cultures. By this approach, we identified three known inflammatory mediators (E-selectin, VCAM-1, and MCP-1) that were differentially expressed in TNFα-stimulated iris and retinal EC, with mRNA levels for all three molecules higher in HREC vs HIEC. Validation studies were then conducted to evaluate protein expression of these three molecules in HIEC and HREC. At the protein level, cell surface and secreted/shed E-selectin levels were approximately 40% and 300% greater, respectively, in HREC versus HIEC, and these differences were statistically significant. In two donor-matched EC pairs, MCP-1 levels measured in the conditioned media of TNFα-activated HREC cultures were higher than those secreted by HIEC, although this did not attain statistical significance. We were unable to discern a significant difference in VCAM-1 protein on activated HREC and HIEC paired cultures.

In order to gain insight into the functional significance of 3-fold differences in soluble E-selectin released from these cells, we evaluated the effects of soluble E-selectin on leukocyte adhesion to cultured ocular EC. Adhesion of U937 monocytes, Jurkat T cells, and human neutrophils to TNFα-stimulated HREC was highly dependent in these assays on EC cell surface expression of E-selectin. This dependence was evidenced by nearly complete inhibition of leukocyte/EC binding when neutralizing anti-E-selectin antibody was included in the adhesion assay. Leukocyte binding to unstimulated and TNFα-activated HREC monolayers was unaffected by the inclusion of recombinant human soluble E-selectin in leukocyte suspensions during the adhesion assays. The lack of effect of soluble E-selectin on leukocyte adhesion in our hands, compared to the leukocyte activating effects of soluble E-selectin previously reported by others, could be due to either differences in leukocyte types employed in these studies,[160] or to distinct receptor binding/activating characteristics of recombinant proteins that were produced in different expression systems.[157]

6B. Experimental Limitations

In vivo, EC are closely juxtaposed to other cell types such as smooth muscle cells, pericytes, and other stromal cell types that reside in the vascular wall and surrounding tissues. A multitude of EC biological functions are regulated by complex interactions with the extracellular matrices and soluble bioactive molecules that are secreted by these neighboring cells.[182-185] EC activities are also mediated by direct cell/cell interactions with surrounding cells.[186-189] Additionally, EC actively respond to mechanical forces

such as shear stress, cyclic strain, and hydrostatic pressure that are inherent to the circulating blood that bathes the endothelium.[153, 154, 190-193] Given that all of these stimuli vary considerably from tissue to tissue, and even within different vascular segments of the same tissues' circulation, it is likely that these highly diverse influences are together a major causative factor in EC biological heterogeneity that has been observed *in vivo*. For EC that are grown statically in polystyrene tissue culture plates, none of these physiological influences remain. We removed these normal stimuli and grew diverse EC in a standardized artificial environment, in order to allow any inherent biological differences that might exist amongst these cells to be more easily observed. In doing so, we are operating under the assumption that the culture conditions that we have selected will not cause dedifferentiation of these EC to a basal state in which intrinsic biological differences are masked. We initially tested several biological criteria to establish that these cultured EC are functionally endothelial in nature. That we only discerned one clear difference in inflammatory mediator expression in TNFα-activated EC from iris and retina *(i.e.*, E-selectin) suggests that either: A) The number of intrinsic differences in inflammatory gene expression that exist amongst diverse microvascular EC are limited, although these differences may be sufficient by themselves to cause important tissue-selectivity in inflammation; or that B) more tissue-specific differences actually exist than are discerned by this model, although they are masked by artificially induced EC dedifferentiation. Future studies should address these possibilities.

In this experimental series, we only compared the effects of one inflammatory mediator, TNFα, on gene expression in microvascular EC derived from different tissues. Considering the vast numbers of known molecules that govern vascular function during

inflammation, it will be important in the future to evaluate potential tissue-specific EC responses to an expanded selection of bioactive agents.

The microcirculatory EC of a given tissue are not necessarily a functionally homogenous population. In many tissues examined, including the iris[147, 194] and retina,[195] leukocyte extravasation during inflammation occurs primarily in the postcapillary venules, with considerably less migration occurring in arterioles and capillaries. Thus, biological heterogeneity might exist amongst the EC that comprise different blood vessel types within a given microvascular bed. A limitation of our system is that we do not know the relative percentages of arteriolar, capillary, and venular EC that comprise our microvascular EC cultures. If intrinsic differences exist amongst EC from different anatomical regions of the microcirculation, and our paired iris and retinal EC cultures contain unequal proportions of these EC types, this could skew experimental interpretations of innate EC heterogeneity.

6C. Conclusion

In light of the inherent limitations of our experimental system, we believe that our approach constitutes a step forward in better understanding the biology of the microcirculation as it relates to inflammation. For three decades, cultured EC have provided a wealth of insight into the complex molecular mechanisms at work in vascular biology, with many findings from these models having been confirmed in *in vivo* models.[196] The idea of EC heterogeneity is not new, and previous studies have compared the *in vitro* functioning of EC from diverse tissues,[69, 71, 85, 144] Our study, however, is the first study to directly compare microvascular EC that were cultured

from different, closely situated tissues obtained from the same donors. Given the great person-to-person biological variability that exists, we believe that this constitutes a significant advance in the investigation of potential EC diversity.

In screening nearly 1000 known genes with cDNA arrays, it became obvious that, when removed from their normal *in vivo* environment and cultured in an artificial system, the two ocular microvascular EC types that we tested had largely similar constitutive gene expression profiles. Nonetheless, clear differences in inflammatory mediator expression were revealed when these cells were stimulated with the proinflammatory cytokine TNFα. The most clearcut difference that we observed was in the expression of the adhesion molecule E-selectin, a known modulator of inflammation in the eye and elsewhere in the body. Here, activated retinal EC displayed increased protein on their surfaces compared to iris EC, and total secreted E-selectin protein was even more greatly elevated in HREC relative to HIEC. We further speculate that, if these differences in responsiveness to inflammatory stimuli are found to exist in the *in vivo* condition, that this biological variability might constitute the basis of one tissue being inherently more prone to developing inflammation. This hypothesis will need to be evaluated in future work.

6D. Future Directions

Although the experiments described within this thesis have provided new knowledge about EC function, and has confirmed and extended the findings of others regarding EC biology, the current observations have also created new questions to be addressed. Foremost is whether the observed differential regulation of E-selectin (or of

other inflammatory mediators) in diverse EC occurs in intact microvessels, and if so, what might be the biological implications? Leukocyte adhesion assays performed *in situ* on frozen sections[197] of cytokine-activated human or animal retinae and irises and including neutralizing anti-E-selectin antibodies would provide a comparison of the relative involvement of E-selectin in binding leukocytes in these tissues. Intravital microscopy[198] and/or scanning laser ophthalmoscopy[199] could be used to noninvasively evaluate the extent of leukocyte binding to the different microvascular structures in these tissues, in animal models of ocular inflammation.[73] Antibodies against newer markers used to differentiate arteriolar and venular EC (*e.g.*, ephrins [200-203] could be employed in both the *in situ* and *in vivo* studies described above to discern the specific microvessel types to which leukocytes bind. These markers might also be used as the basis to selectively isolate and culture pure arteriolar and venular EC cultures from the microvasculature, for *in vitro* comparisons with one another, or for comparisons with corresponding cultures derived from a different tissue.

If the biological differences that are observed in diverse cultured EC are confirmed to exist in *ex vivo* and *in vivo* models microvascular beds, then the question will arise as to what additional differences might exist. Improved and expanded genomic[204] and proteomic[205, 206] methodologies can be applied to rapidly evaluate the possibility that additional biological differences amongst various EC cultures, or among microvessels that are isolated intact from different tissues using laser capture microdissection techniques.[200]

It will also be important to investigate the responses of diverse EC to an expanded repertoire of biological stimuli. *In vitro*, a variety of environmental cues can be

individually or concomitantly reproduced. Coculture,[207] provisional extracellular matrix,[183] and simulated hemodynamic models[153, 191] already exist, and can be used to compare the effects of diverse environmental stimuli on the physiology of different EC types. Acting together, these experimental approaches will likely provide important new knowledge about the intricate molecular activities of EC, and how these activities contribute to both normal vascular functioning and to pathologies involving the circulatory system.

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