The Role of Interleukin-1 in Corneal Inflammation

by

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Abstract

The fundamental premise of this work is that the inflammatory cytokine, interleukin-1 (IL-1), is an important component in the biology of corneal inflammation and wound healing. This dissertation covers studies on the expression of IL-1 in the cornea, how this expression is regulated, and the role of IL-1 during corneal inflammation. The initial hypothesis tested was that environmentally relevant levels of ultraviolet light induce the production of IL-1, as had been found with UV exposure of the skin. In support of this hypothesis, data presented in this dissertation show that irradiation with relatively low levels of ultraviolet B light (UVB) significantly increases the production of the two primary inflammatory cytokines, IL-1 and tumor necrosis factor α (TNFα) in corneal fibroblasts.

Because IL-1 in the eye is known to trigger inflammatory processes that are injurious to the cornea, the corollary, that the cornea possesses specific mechanism(s) of controlling the effects of IL-1, was tested. Whether or not human corneal cells can produce the endogenous IL-1 inhibitor, interleukin-1 receptor antagonist (IL-1ra), was examined. Immunohistochemical staining of human corneal tissue showed strong specific staining for IL-1ra. Northern Blot analysis of cultured corneal epithelial cells and stromal fibroblasts showed large increases in IL-1ra mRNA levels after stimulation with exogenous IL-1.

Furthermore, cultured corneal epithelial cells produced both secreted and intracellular isoforms of IL-1ra. This later finding was unexpected and has not been reported for any other epithelial cell types. This observation was based on ELISA data and later confirmed by immunoprecipitation of IL-1ra and RT-PCR analysis. This unique pattern of IL-1ra production has not been described in epithelial cells outside of those in the cornea.

Data in this dissertation show that corneal cells produce IL-1 in response to UVB irradiation and UVB irradiation causes a corneal inflammatory condition, photokeratitis. Because IL-1 has been implicated as a mediator of corneal inflammation, it was postulated that IL-1 is a key mediator of photokeratitis. If so, then increased levels of the endogenous inhibitor, IL-1ra, should reduce the inflammatory response to corneal UVB exposure. This was evaluated experimentally by inducing photokeratitis in transgenic mice that over-express IL-1ra. As postulated, these IL-1ra transgenic mice showed reduced photokeratitis when compared to nontransgenic littermate controls.

Many of the phlogistic properties of IL-1 arise through the induction of other components of an inflammatory cytokine cascade. To explore which element(s) of the inflammatory process were dysregulated, transgenic and nontransgenic mice were examined for changes in cellular adhesion molecules, secondary cytokines, and chemokines during the progression of photokeratitis.

RT-PCR analysis of IL-1 stimulated murine corneal stromal fibroblast (MCFB) cell cultures derived from transgenic and nontransgenic mice showed increases in the levels of chemokine mRNA following stimulation with IL-1. Irradiated MCFB showed increased production of the chemokine KC as measured by ELISA. The treatment of MCFB cultures with exogenous IL-1ra following UVB irradiation blocked any increase in KC protein found in MCFB tissue culture supernatants.

These data are consistent with the hypothesis that increased IL-1ra levels in the cornea decrease UVB-induced corneal inflammation by blocking IL-1 triggered chemokine production in corneal stromal fibroblasts.

Taken as a whole, these observations support the hypothesis that IL-1 plays a central role in the corneal inflammatory response.

Introduction

Interleukin-1: History and significance

Interleukin-1 (IL-1) is a pluripotent cytokine capable of eliciting responses from a wide variety of tissues and cell types.[1] IL-1 regulates the expression of a large number of genes that mediate the processes of inflammation. Many of the phlogistic properties of IL-1 occur via the induction of secondary mediators such as chemokines, interleukin-6 (IL-6), acute phase proteins, reactive oxygen intermediates and prostaglandins.[2] Production of IL-1 occurs early in the inflammatory response. There is a dramatic increase in the production of IL-1 in response to infection[3], microbial toxins[4], inflammatory agents, complement, and clotting components.[5] For example, in the response to infectious challenges, IL-1 recruits inflammatory cells by increasing expression of leukocyte adhesion molecules[6] and up-regulating the production of chemokines, such as interleukin-8 (IL-8)[7] and macrophage inflammatory protein-1 (MIP-1).[8] Systemically, IL-1 can modulate the hypothalamic pituitary axis inducing fever, anorexia, sleep and adrenocorticotrophin hormone release.[9] IL-1 release into the bloodstream produces changes in vascular permeability and hypotension.[10] Systemic IL-1 affects hematopoiesis by increasing the circulating levels of colony-stimulating factors that can result in neutrophilia.[11, 12]

Stimulation by exogenous IL-1 causes the up-regulation of IL-1 production

in many cell types, and this autocrine effect serves to amplify the inflammatory properties of IL-1.[13, 14] Increased IL-1 production is believed to be important in a number of human inflammatory diseases. Examples of IL-1-mediated injury include shock and organ failure associated with sepsis[15], bone destruction in rheumatoid arthritis[16-19] and islet destruction in pancreatitis.[20] In the eye, intravitreal injection of IL-1 produces an acute anterior uveitis in animals[21, 22] and release of IL-1 from intracorneal implants results in neovascularization.[23] Increased levels of IL-1 mRNA expression are found in animals with endotoxin-induced ocular inflammation.[24, 25] Transgenic mice engineered to produce high intraocular levels of IL-1α, exhibited symptoms of corneal inflammation and neovascularization.[26] Addition of exogenous IL-1 to cultured corneal cells causes increased production of matrix metalloproteinases[27], IL-6[28, 29], and IL-8.[30, 31]

At least seven major proteins are associated with the biologic effects of IL-1: two agonists, one receptor antagonist, two receptors, one receptor accessory protein and one receptor associated kinase.[32-34] IL-1α and IL-1β, the two agonist ligands, share only about 26% amino acid identity, yet they can bind to the same cell surface receptors and have similar biologic activities. Both IL-1α and IL-1β are translated as 31 kD propeptides and are enzymatically cleaved to 17 kD mature forms. Both the propeptide and mature forms of IL-1α possess biologic activity, whereas IL-1β requires proteolytic cleavage for activation. IL-1α is

myristylated after translation, remains in the cytosol, and is thought to be released only after cell death or injury.[35] Neither form of IL-1 contains a classic signal peptide, but IL-1β is secreted from cells by a mechanism that is not yet understood.[36] IL-1β release in monocytes has been reported to involve a novel secretory pathway separate from the endoplasmic reticulum-Golgi pathway.[37] Treatment of macrophages with calcium ionophores or ATP has been reported to trigger secretion of mature 17 kD IL-1β.[38] Secretion has also been associated with proteolytic processing of IL-1β by interleukin-1 converting enzyme.[39]

The two IL-1 receptors share about 28% amino acid identity in their extracellular domains and have slightly different binding affinities for IL-1α and IL-1β. These two receptors differ most in the cytoplasmic domain and signaling activity. IL-1 receptor type 1 (IL-1R1) is an 80 kD glycoprotein with a 215 amino acid cytoplasmic tail and a single transmembrane domain. This receptor is fully functional[40], and a number of different mechanisms for transmembrane signaling have been attributed to this receptor.[41] Most investigators agree that many of the genes downstream of IL-1 signaling are activated by the transcription factors, nuclear factor κB (NF-κB) and AP-1(Jun/Fos). The protein phosphorylation events needed to activate NF-κB occur by the formation of a multimeric receptor/ligand complex with IL-1 receptor accessory protein (IL-1RAcP) which then recruits the IL-1 receptor-associated protein kinase

(IRAK).[42] Both IL-1AcP and IRAK have been found to be necessary for IL-1R1 signaling. Over-expressing a truncated form of IL-1RAcP or treatment with antibodies against the extracellular portion of IL-1RAcP blocks IL-1 signaling in cells normally responsive to IL-1.[43] Dominant negative variants of IRAK also show total blockage of IL-1R1 signaling. The antiquity of the IL-1 pathway is suggested by the homology of IL-1R1/IRAK/NF-κB proteins to the Drosophila proteins Toll/Pelle/Dorsal.[44] The substrates for IRAK are likely to be other kinases that have been reported to phosphorylate IκB, the cytoplasmic inhibitor of NF-κB transcriptional activity.[45] The phosphorylation of IκB causes the addition of ubiquitin and proteasome-mediated degradation, freeing NF-κB to relocate to the nucleus and begin activating transcription.

IL-1 gene activation by transcription factor AP-1 (Jun/Fos) leads from the mitogen-activated protein kinase kinases, MKK3 and MKK4 [46] to p38 kinase [47] and the stress-activated protein kinases (SAPK) or c-Jun N-Terminal kinase (JNK), but other components that feed into this pathway are not yet clear.

Other activities that have been linked with IL-1R1 signaling include sphingomyelinase ceramide generation[48], Jak/Stat pathway[49], phosphatidylinositol 3-kinase (PI3-kinase)[50] and reactive oxygen intermediates.[51] It is unclear, however, whether these reports are due to the ability of IL-1R1 to recruit a variety of effector molecules into its multimeric signaling complex, or if these IL-1

signaling mechanisms are peculiar to the particular cells lines used in these studies.

Rates of IL-1 and IL-1R1 synthesis appear to be tightly regulated *in vivo.*[52, 53] However, most IL-1R1 positive cells show much higher levels of receptor on their surface when cultured *in vitro* and these artificially large numbers of receptor may contribute to IL-1 autocrine amplification.[54] IL-1 binding to IL-1R1 triggers receptor-mediated endocytosis[55] but the fate of endocytosed ligand-receptor complex is uncertain. Kuno et al.[56] reported that the receptor contains a nuclear localization sequence and that receptor-bound ligand is translocated to the nucleus. The nuclear localization of IL-1 may affect cell senescence in endothelial cells[57], because blocking IL-1β production with antisense RNA extends the life span of these cells.[58] At least one study has reported that receptor-ligand complex is disrupted in the endosome and the receptor is recycled to the cell surface prior to IL-1 nuclear translocation.[59] Nuclear IL-1 may regulate proliferation and differentiation in keratinocytes because they also localize IL-1 to the nucleus.[60] However, keratinocytes are believed to require production of new IL-1R1 to maintain a sustained IL-1 response.[61] IL-1 also has been shown to have IL-1R1 mediated effects on the growth and development of hemopoietic cells.[62]

The IL-1 receptor type 2 (IL-1R2) is a 68 kD glycoprotein with a

cytoplasmic domain of only 29 amino acids. The IL-1R2 is generally believed to be incapable of transmembrane signaling[63] and acts as a "dummy" receptor that decreases IL-1 responses by sequestering agonist. Levels of IL-1R2 are increased on the cell surface after IL-1 stimulation[64] and cells that are transfected with IL-1R2 display decreased IL-1 responsiveness.[65-67] This receptor can be proteolytically cleaved from the cell surface, releasing a soluble form that binds both agonists in solution.[64] Some studies have linked soluble IL-1R2 levels with a clinical reduction in disease severity.[68] These studies support a role for IL-1R2 as an inhibitor of IL-1 activity.

Inhibitors of IL-1 activity were originally isolated from various sources. Dinarello et al. reported the presence of inhibitors of IL-1 activity in plasma and serum as assessed by thymocyte proliferation assay.[69] Rosenstreich reported an inhibitor of IL-1 activity obtained from the urine of febrile humans.[70] This febrile urine IL-1 inhibitor was subsequently shown to be the same as interleukin-1 receptor antagonist (IL-1ra). IL-1ra was also initially isolated from culture supernatants of human monocytes grown on adherent IgG or immune complexes.[5]

IL-1ra shares structural similarities and amino acid homology with IL-1α and IL-1β and is the third member of the IL-1 gene family. IL-1ra has been shown to be capable of blocking responses to IL-1α and IL-1β both *in vitro* and *in vivo*.[71, 72] IL-1ra competes effectively with both IL-1α and IL-1β for binding to

the IL-1 type 1 receptor. Crystal structures of IL-1R1 bound to agonist show folding of the third extracellular domain over the agonist after binding.[73] Whereas IL-1ra occupies the binding site with equal avidity, it fails to trigger the conformational changes in the third domain of the receptor.[74] Receptor occupancy by IL-1ra fails to elicit any detectable receptor signaling activities, including receptor internalization[75], NF-kB nuclear translocation[76] and binding of IL-1RAcP.[77]

There are three known isoforms of IL-1ra, two intracellular (icIL-1ra) and one secreted (sIL-1ra). All three isoforms of IL-1ra are encoded by a single gene and are produced through mRNA splicing of alternative exons. The primary sequence of the non-glycosylated 18 kD icIL-1ra differs from the 22-24 kD sIL-1ra only in lacking a leader sequence and 4 of its first 7 N-terminal amino acids.[78] Variation in post-translational processing leads to differences in molecular weight between the two larger isoforms.[79] The second isoform of icIL-1ra is a 16 kD protein resulting from a 13 amino acid deletion, due to the usage of an alternate exon II. All three isoforms of IL-1ra are functionally equal in their ability to inhibit IL-1 activities when added exogenously to cultured cells.[80] However, the biologic role of intracellular IL-1ra remains an area of speculation. Because IL-1α remains intracellular, icIL-1ra is thought to represent a counterbalance to the effects on cells in the immediate vicinity should the cell be

damaged and IL-1α released. Studies in keratinocytes suggest that the ratio of icIL-1ra to IL-1α helps regulate cell differentiation.[81] Some have reported icIL-1ra has a higher affinity for endocytosed IL-1R1 and may function in receptor turnover and maintenance of cell surface receptor numbers.[82]

IL-1ra has been shown to be protective and able to reduce the degree of inflammation in a number of experimental disease models. IL-1ra-transfected synoviocytes protected joints from inflammatory damage in a model of experimental arthritis.[83] Transgenic mice with lung-specific over-expression of IL-1ra, showed reduced neutrophil recruitment into lung following airway injury.[84] Treatment of murine corneal transplants with recombinant IL-1ra reduced corneal inflammation and the rate of corneal rejection.[85] Treatment with IL-1ra reduced neutrophil infiltration into the anterior chamber, but did not protect against increases in aqueous humor protein in a rabbit model of LPS-induced uveitis.[86]

Corneal Anatomy and Function

The cornea consists of three distinct layers and cell types (see Diagram 1, page 9). The outermost layer, the corneal epithelium is a stratified squamous epithelium made entirely of corneal epithelial cells. The basal layer of epithelial cells is less differentiated and adheres to a basement membrane by hemidesmosomes.[87] Replication of basal epithelial cells is necessary to maintain

epithelial thickness by replacing cells sloughed from the epithelial surface[88], but basal epithelial cells have a limited capacity to proliferate so this population of cells must be constantly renewed. New basal epithelial cells are believed to be produced by replicating stem cells located in the limbus, with older basal cells moving centripetally toward the central cornea. Epithelial cells flatten out as they undergo differentiation and move into the anterior layers of the corneal epithelium. The spinous cells of the intermediate epithelial layers are termed "wing cells" and adhere to the underlying basal cells and neighboring wing cells by demosomes and interdigitaion of plasma membranes. Apical corneal layers are composed of highly flattened "superficial cells" whose anterior surface is covered by microvilli to aid in oxygen uptake from the tear film. Tight junctions between superficial cells form a barrier to fluid movement into or out of the cornea.[89] Corneal epithelial cells express unique cytokeratin pairs, K5/K14 as basal cells and K3/K12 during differentiation[90], and the loss of these cytokeratins disrupts epithelial integrity.[91] The presence of a healthy intact epithelium suppresses pathologic processes in the cornea such as neovascularization. Corneas in which the epithelium has been fully denuded are resurfaced by epithelial cells from the adjacent conjunctiva. While histologically similar, these corneas have reduced clarity and a propensity for developing neovascularization.[92]

The underlying corneal stroma layer consists of collagen matrix (mostly

type 1) with dispersed corneal stromal fibroblasts (keratocytes). These collagen fibrils are tightly organized into orthogonal lamellae with uniform fibril spacing and diameter.[93] This organization of stromal ECM is necessary for corneal clarity and function. Organizational disruption by the deposition of collagen during wound healing is an important element in the development of corneal haze.[94] The stromal ECM also contains proteoglycans composed of keratan, chondroitin, and dermatan sulfate bound to core proteins. These proteoglycans are charged and are capable of imbibing large amounts of water causing swelling of the stroma and loss of corneal clarity. Only the fluid barrier in the upper epithelial layers and endothelial transport of water out of the stroma maintain these proteoglycans in a reduced state of hydration.

The keratocytes are the only cellular component of the corneal stroma, accounting for as little as 2% of the stromal volume. These fibroblast-like cells are found throughout the stroma and are responsible for the production and maintenance of the stromal extracellular matrix (ECM). The widely dispersed keratocytes are linked by gap junctions on long cellular processes that form an intricate network throughout the corneal stroma.[95] These connections allow intercellular communication amongst neighboring keratocytes and possibly across wide distances of the cornea.[96]

The innermost corneal layer is composed of a single layer of corneal

endothelial cells and a basement membrane (Descemet's membrane) secreted by these cells. The endothelium separates the stroma from the aqueous humor and is responsible for maintaining corneal clarity by actively pumping water out of the corneal stroma. Descemet's membrane contains two histologic and developmentally distinct layers. The first is made during fetal development, and is comprised of highly organized collagen lamellae that are striated in appearance. The less ordered second posterior layer is secreted after birth and continuously thickens throughout life.[97] Abnormal matrix deposition in this layer is seen in Fuchs' dystrophy and in the formation of retrocorneal fibrous membranes following penetrating injury to the cornea.

Cornea function requires that inflammatory events be restrained because edema and leukocyte infiltration can dramatically impair vision. As a result the cornea is an "immune privileged" tissue that lacks many components of immune surveillance. Leukocytes are normally absent in a healthy cornea and antigen presenting dendritic cells (Langerhans' Cells) are restricted to peripheral areas of the cornea. The migration of Langerhans' cells into the central cornea marks initial stages of inflammation.[98] Corneal tissues are avascular and lack the presence of class II major histocompatibility antigens (MHC II) in the healthy state.[99] Release of IL-1 into the cornea triggers MHC class II presentation and migration of Langerhans' and immune cells into the corneal tissue.[100, 101]

Corneal wound healing is a complex process that involves proliferation, migration and differentiation of both corneal epithelial cells and stromal fibroblasts.[102] After wounding, the basal cells at the edge of the defect rapidly begin spreading over the wound surface.[103] Basal cells further from the wound edge begin multiplying and migrating to cover the defect. This migration involves the disassembly of anchoring hemi-desmosomes[104], up-regulation of metalloproteinase production [105] and cell migration through focal adhesions to wound surface fibronectin and laminin.[106] IL-6 has been reported to stimulate the migration of corneal epithelial cells on fibronectin[107, 108] and IL-1 is believed to be a primary stimulator of IL-6 synthesis by corneal cells.[28, 109-111]

A number of growth factors, particularly Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF) and Transforming Growth Factor (TGF) family members, have been shown to increase corneal epithelial cell proliferation and migration.[112, 113] Effects of EGF on epithelial cell migration are enhanced by co-stimulation with IL-1.[114] Hepatocyte Growth Factor (HGF) and Keratinocyte Growth Factor (HGF) are produced by stromal keratocytes and are believed to stimulate the proliferation of overlying epithelial cells.[115, 116] Cultured stromal fibroblasts have been shown to increase HGF and KGF production to much higher levels after IL-1 stimulation.[117] The tears are another source of these growth factors for corneal epithelial cells. The levels of HGF and KGF in tears increase

following laser keratectomy, although which cell type(s) release these growth factors into the tears is unclear.[118]

Stromal fibroblasts outside the wound area convert into myofibroblast-type cells that migrate into the underlying matrix and begin the remodeling process that involves degrading old matrix and synthesizing new stromal matrix components.[119] Such phenotypic conversion and increased production of matrix degrading metalloproteinases can be triggered in cultured stromal fibroblasts by IL-1 stimulation.[120]

For the cornea to be functional it must retain its clarity and optical refractive qualities. The edema, cellular infiltrate and disruption of tissue structure common during inflammation in other tissues can irreparably damage corneal function. The presence of IL-1 in the cornea dictates that counteracting mechanisms be in place in order to control its inflammatory potential.

Several unique features of corneal biology offer an excellent opportunity to understand how IL-1 triggers inflammation. Corneal avascularity limits the rate that IL-1 can leave the immediate area after release. The time required for IL-1 to diffuse into the peripheral cornea delays immune cell infiltration into the corneal stroma. This slow diffusion places emphasis on the paracrine aspects in the activities of IL-1. Corneal avascularity also means infiltrating inflammatory cells must migrate through considerable distances of tightly packed collagen fibers of

the stroma to reach the injured area. The combination of these factors results in the recruitment of inflammatory cells into the central corneal stroma occurring over the course of days rather than hours. The limited number of cell types which comprise the cornea also helps to delineate the various components of the inflammatory cascade.

Much of the work on understanding the role of IL-1 in epithelial biology has involved skin keratinocytes, which share many aspects of physiology and morphology in common with corneal epithelial cells. Both cell types respond to exogenous IL-1 as well as being able to synthesize IL-1. Changes in the intracellular levels of IL-1 and icIL-1ra occur in keratinocytes during differentiation, and appear to regulate several differentiation associated proteins.[121-123] These aspects of IL-1 in relation to corneal epithelial cells have not been investigated. Unlike the skin, the cornea is not protected by a keratinized upper layer nor can it utilize the photo-protective properties of pigmentation and melanocytes. The precise organization of corneal extracellular matrix and the tightly controlled state of stromal hydration cannot experience the disruptions of inflammation and edema without the danger of irreparably damaging its clarity and refractive properties necessary for function.

Ultraviolet irradiation and its effects on the eye and immune system

Ultraviolet light has long been recognized to induce a number of biological effects. Erythema, cell death, inflammatory infiltrate and nuclear condensation are characteristics of the "UV effect".[124] Investigators believed these effects were due only to UV-induced damage of nucleic acids and/or cellular damage by the generation of free radicals. Recent studies recognize that these effects arise from specific intracellular signaling pathways leading to the induction of apoptotic cell death and inflammation.[125]

Ultraviolet light is divided by wavelengths into three basic types: UVA (320-400 nm), UVB (280-320 nm), and UVC (180-280 nm). UVC radiation has far more penetrating power than UVB or UVA, but most is absorbed by the ozone layer and little reaches the Earth's surface. Because of this, UVC radiation does not represent a significant environmental hazard. However, UVC is probably the most frequently studied type of ultraviolet radiation in biologic systems. Whereas a sufficient dose of UV irradiation induces strand nicking and pyrimidine dimerization in DNA, later studies have shown ultraviolet responses in the absence of DNA damage[126] and in enucleated cells.[127, 128] Recent studies have reported UV irradiation-induced, ligand-independent oligomerization and activation of cell surface receptors for IL-1[129], TNFα[130], epidermal growth factor[131] and Fas.[132] Reports of UV irradiation immediately activating

serine/threonine kinases, such as Src[133] and JNK 1[134], leading to the induction of NF-κB and AP-1 mediated transcription have also been published. In addition, irradiation with UV has also been reported to trigger biologic responses by interactions with cytoplasmic organelles such as mitochondria[135] and ribosomes.[136] Many of the pathways activated by UV irradiation also occur in other cellular stress responses such as osmotic[129], hypoxic and low glucose stress.[137] Many UV radiation-activated pathways display delayed and/or biphasic kinetics, which suggest at least partial mediation through secondary products such as cytokines and growth factors.[138, 139] There are reports of UV responses being mediated by alternate pathways such as reactive oxygen intermediates[140, 141], protein kinase C [142] and ceramide signaling.[143]

Photokeratitis

Exposure to ultraviolet radiation is an environmental hazard for everyone who spends time out of doors. The condition of "snow blindness" occurs in skiers and mountain climbers as a result of excessive exposure to UV radiation from natural sunlight. Acute UV exposure resulting in photokeratitis is usually associated with artificial UV sources such as tanning lamps, welding equipment or UV germicidal lamps. The clinical findings associated with acute UV photokeratitis are tearing, pain, foreign body sensation, corneal edema and punctate epithelial erosions. Fortunately, the prognosis for visual recovery is

excellent, and the eye generally heals within 48-72 hours.

Chronic exposure to UV radiation is associated with the development of a number of degenerative changes on the surface of the eye. Climatic droplet keratopathy is one such condition that is characterized by the bilateral deposition in the superficial corneal stroma and Bowman's layer of translucent material. This material may accumulate until visual disturbance results. Strong epidemiological evidence supports the idea that UV radiation is a major etiologic factor in this corneal condition. The link between UV light and the disease severity has been established in a number of population studies conducted in northern latitudes.[144-146] Other degenerative changes that have been associated with chronic ultraviolet light exposure include pterygia and pingueculae. Pterygia are typically raised triangularly shaped lesions with the apex extending into the corneal surface. Histopathologically, pterygia can destructively invade Bowman's layer and large pterygia may encroach upon the visual axis, requiring surgical removal.

Experimental studies in mice show that large doses of UV radiation produce epithelial hyperplasia, degeneration of Bowman's layer, and corneal neovascularization.[147] In rabbit corneas, full-thickness pathologic damage has been observed following acute exposure to UV light.[148] Other experimental studies have also demonstrated changes in corneal stromal hydration and

endothelial morphology.[149]

The murine studies presented in this dissertation involve a lower dosage of UV radiation exposure with a limited spectrum of UV wavelengths and restriction of the injury to the central cornea. This results in a burn that causes sufficient epithelial loss to expose underlying stroma, triggers a transient stromal neutrophilic infiltrate, and resolves with no apparent long term damage. Photokeratitis offers a means to study factors which may contribute to corneal diseases of a chronic and more severe nature.

Many inflammatory corneal diseases, such as herpetic stromal keratitis (HSK), initially display a mild transient neutrophilic infiltrate that resolves and is followed by a more severe and destructive inflammatory event.[150] Differences in the level of corneal damage between photokeratitis and chronic ulcerative corneal conditions may represent differences in neutrophil activation in the wound area. Activated neutrophils produce chemotactic signals that recruit other immune cells into the area, multiplying the potential for tissue damage. Evidence suggests that the initial transient neutrophil influx of HSK affects the recruitment of T-cells and other leukocytes which mediate the later, more serious, inflammatory events.[151] The recruitment of small numbers of unactivated neutrophils into a wound site to "stand guard" over a situation of potential infection offers the advantage of speeding immune responses should a pathogen attempt invasion. However,

activation of neutrophils can release high levels of degradative proteases[152], cytotoxic granules[153], and reactive oxygen compounds[154] capable of damaging the surrounding tissue and killing nearby corneal cells. Experimentally recruiting neutrophils into the CNS, by local expression of chemokine transgenes, showed no immediate damage to the surrounding brain tissue even though the levels of infiltrating neutrophils were high.[155, 156] Studies in the skin have shown that an apoptotic "die off" of neutrophils recruited into a wound, which occurs during epithelial closure and epithelial healing, is hindered if this apoptosis is blocked or delayed.[157] This suggests that leukocyte recruitment is independent of leukocyte activation and that systems are in place for the removal of quiescent leukocytes after the potential for infection has passed.

The studies presented in this dissertation offer some insights into the regulation of corneal leukocyte recruitment. I believe that this model of photokeratitis could be used to study other aspects of corneal disease such as the mechanisms of activation or removal of immune cells recruited into the cornea.

MATERIALS AND METHODS

Isolation and culture of corneal cells.

Human corneal cells: Primary human corneal epithelial cell cultures were established from transplant quality human corneas obtained from the Oregon Lions Eye Bank (Portland, OR). Corneal donors were 40-68 years old and had no underlying ocular disease. The epithelium was aseptically scrapped with a No. 15 scalpel after 1 hour Dispase (grade II, 2.4 Units/ml, Boehringer Mannheim, Indianapolis, IN) treatment at 37 °C. Cells were then cultured in medium 154 supplemented with bovine pituitary extract, bovine insulin, hydrocortisone, human transferrin, and human epidermal growth factor (Cascade Biologics, Portland, OR). The culture medium was changed every 48 hours until the cells were nearly confluent. All cultures were maintained at 37 °C in a humidified, 5% CO_2 atmosphere.

Primary human corneal stromal cell cultures were established from tissue obtained as described above from the Oregon Lions Eye Bank. The central cornea was trephined with an 8-mm biopsy punch after the epithelial layer had been scrapped away. The endothelial cell layer and Descemet's membrane were removed with forceps. The corneal stroma was minced with scissors and cultured in medium MCDB 402 (JRH Biosciences, Lexena, KS) with 10% heat-inactivated

fetal bovine serum (GibcoBRL Life Technologies, Gaithersburg, MD). Culture medium was changed every 72 hours until cells were nearly confluent. All cultures were maintained at 37 °C in a humidified, 5% CO_2 atmosphere.

Primary human corneal endothelial cells were isolated by scraping this layer of cells from Descemet's membrane. Endothelial cells were pooled from three donor corneas, and total RNA was extracted for use in RT-PCR studies.

For human IL-1ra studies, cells were obtained from three sets of freshly removed corneas (six corneas). Corneal fibroblasts used in UVB-stimulated cytokine studies were obtained from four different donors (eight corneas). All studies were carried out at least three times and results from representative experiments are presented.

Murine corneal cells: Murine corneal fibroblast cultures (MCFB) were established from freshly excised corneas after the epithelial and endothelial cells had been scraped off. The stroma was minced and cultured in medium MCDB 402 supplemented with 10% heat-inactivated fetal bovine serum. All cultures were maintained at 37 °C in a humidified, 5% CO_2 atmosphere. The genotype of IL-1ra transgenic and congenic nontransgenic MCFB cultures was determined by PCR analysis of genomic DNA extracted from monolayers.

Animals

IL-1ra mice: Heterozygous IL-1ra transgenic breeding males were supplied by D. Hirsch (Columbia University). These IL-1ra transgenic mice contain six additional copies of the IL-1ra gene under the control of endogenous promotor.[158] These animals appear phenotypically normal and can be only distinguished from nontransgenic littermates by genotyping. Mice were mated to normal C57Bl/CBA F1 congenic females obtained from Jackson Laboratories (Bar Harbor, ME). Only adult mice 6-10 months of age, of either sex were used and experimental controls were nontransgenic littermates.

Preparation of tissue culture supernatants and cell lysates

Cell monolayer supernatants were collected, centrifuged at 3,000 xg for 20 minutes at 4 °C, and stored as aliquots at -80 °C. Cultured cells were lysed on ice for 20 minutes in a buffer containing 20 mM Tris pH 7.8, 1% Nonidet P-40 (NP-40), 100 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (PMSF), and 10 µg/ml each of Leupeptin, Pepstatin A, Antipain and Chymostatin (Sigma Chemical, St. Louis, MO). Cell lysates were clarified by centrifugation at 10,000 xg for 20 minutes at 4 °C, and stored as aliquots at -80 °C.

Extraction of Genomic DNA

Genomic DNA was isolated from tails by Protease K digestion (0.5 mg/ml) in 1 ml of buffer containing 1% SDS, 400 mM NaCl, 20 mM Tris pH 8.0, 5 mM EDTA at 55 °C for 18 hours. After tissue digestion, the DNA was purified by extraction with 0.5 ml buffer saturated phenol, 0.5 ml chloroform:isoamyl alcohol (49:1). The organic and aqueous layers were separated by centrifugation (3000 xg for 20 minutes at 4 °C). The upper aqueous layer was removed and the DNA precipitated by mixing with 1 volume of cold 100% isopropyl alcohol at -20 °C overnight. Precipitated DNA was recovered by centrifugation (16,000 xg for 15 minutes at 4 °C), washed in cold 70% ETOH, and dissolved in sterile water. DNA was quantitated spectrophotometrically by the absorbance at 260 nm, and sample purity determined by the ratio of absorbance at 260 nm divided by absorbance at 280 nm. Template DNA was stored at -20 °C and diluted to 25 ng/µl prior to use.

Enzyme-Linked Immunosorbent Assay (ELISA)

General Protocol: Primary capture antibody at 4-10 µg/ml (in 100 mM $NaHCO_3$ buffer, pH 9.6) is added to 96-well ELISA plates (100 µl/well), and incubated for 2 hours at 25 °C. Wells were washed 2 times with Tris buffered saline (TBS) and incubated with 200 µl/well of TBS containing 0.01% Tween 20 and 3 % bovine serum albumin (BSA), for 2 hours at 25 °C. Wells were washed 3

times (400 µl/well) with wash buffer (TBS with 0.01% Tween 20, 0.1% BSA). Then sample or standard was added to triplicate wells (100 µl/well) and incubated for 2 hours at 25 °C. Wells were washed 3 times with wash buffer (400 µl/well). Then 100 µl of enzyme-conjugated secondary antibody was added to the wells and incubated for 2 hours at 25 °C. The secondary antibody was diluted 1:4000 in TBS containing 0.01% Tween 20 and 2% serum of same animal species as secondary antibody. Wells were washed 4 times (400 µl/well) with wash buffer, then 200 µl/well of substrate was added and the color allowed to develop (for 10-20 minutes). Substrate used was dependent on the enzyme conjugated to the secondary antibody (disodium p-nitrophenyl phosphate for alkaline phosphatase conjugates and o-phenylenediamine dihydrochloride for horseradish peroxidase conjugates). After sufficient color developed, 50 µl of stop solution (3M H_2SO_4) was added to the wells. The absorbance was read with a 96 well plate reader set at the proper wavelength (405 nm for disodium p-nitrophenyl phosphate and 492 nm for o-phenylenediamine dihydrochloride).

Commercially available primary and secondary antibodies for human cytokines were used for all ELISAs (R&D Systems, Minneapolis, MN), except that human IL-lra which was tested with a commercially available ELISA kit (R&D Systems). Antibodies for murine IL-6 ELISA were from Pharmingen (San Diego, CA) and murine KC ELISA was a commercially available kit (R&D

Systems).

Bioassays

IL-1: The D10.S cell line, an IL-1-dependent murine cell line (gift of C. Dinarello, Tufts University, Boston, MA), was used to measure cellular IL-1 bioactivity.[159, 160] D10.S cells were washed three times and suspended in RPMI 1640 (Gibco BRL) containing 10% fetal bovine serum, 1% L-glutamine, and 5 x 10^{-5}M 2-mercaptoethanol (2-Me). Cells were added to wells (5 X 10^3 cells/well; final volume, 100 µl) in 96-well plates (Corning, Corning, NY). Dilutions (ranging from 1:2 to 1:624) of cell culture supernatants from UV-irradiated and nonirradiated cells were added to triplicate wells (100 µl/well), bringing the final volume to 200 µl/well. Cells were then incubated at 37°C in 5% CO_2 for 48 hours and then labeled with 1 µCi ^{3}H-thymidine per well (Amersham, Arlington Heights, IL) for 6 hours. Incorporation of ^{3}H-thymidine was measured as an index of cellular proliferation. Concentration of IL-1 in samples was determined by comparison of ^{3}H-thymidine incorporation in triplicate wells containing standard concentrations of recombinant human IL-1α (1-100 pg/ml)(Genzyme Corp., Cambridge, MA). IL-1-specific proliferation was confirmed by the use of anti-IL-1 neutralizing antisera.

IL-6: The IL-6 bioactivity was measured by proliferation of the

IL-6-dependent murine hybridoma B9 cell line (gift of L. Aarden, University of Amsterdam, The Netherlands[161]) and this method has been described previously.[159] Cells were washed three times and suspended in RPMI 1640 with 10% fetal bovine serum, 1% L-glutamine, and 5 x 10^{-5}M 2-Me and plated at 3 X 10^5 cells/ml cells (final volume, 100 µl) in 96-well plates, 100 µl dilutions of cell culture supernatants were added, bringing the final volume to 200 µl/well. Cells were then incubated at 37°C in 5% CO_2 for 66 hours and then labeled with 1 µCi ^{3}H-thymidine per well for 6 hours. Cellular proliferation was measured as a function of incorporated ^{3}H-thymidine in triplicate wells at dilutions of supernatants ranging from 1:4 to 1:256. Concentration of IL-6 in unknown samples was determined by comparison of ^{3}H-thymidine incorporation with that of wells containing standard concentrations of recombinant human IL-6 (Genzyme Corp.) of 1-1,000 pg/ml for human culture supernatants and recombinant murine IL-6 (Genzyme Corp.) for supernatants from murine cells. The specificity of this response was determined by the use of anti-IL-6 neutralizing anti-sera (Genzyme Corp.) in human supernatant assays. Presence of murine IL-6 was corroborated by specific ELISA.

Cultured cell, *in vivo*, and *ex vivo* ultraviolet irradiation

UVB radiation source: A bank of 4 Westinghouse (Somerset, NJ) FS-40

bulbs was the UV source in all experiments. These bulbs emit UVB and UVA, with an emission spectrum ranging from 280 nm to 350 nm and a spectral peak at 315 nm.[162]

To investigate cytokine production in cultured cells after UV irradiation, nearly confluent cultures of corneal fibroblasts (HCFB or MCFB) grown in 150 mm Petrie dishes were washed in Hank's balanced salt solution (HBSS). Cultures were then fed serum-free MCDB 402 supplemented with transferrin, insulin and selenium (ITS) for 18 hours. Immediately prior to irradiation, plates were washed 2 times in HBSS (with out phenol red, Ca^{+2}/Mg^{+2} free), covered in HBSS (4 ml/150mm plate), then irradiated by Westinghouse (Somerset, NJ) FS-40 bulbs. After irradiation, HBSS was replaced with 8 ml serum free MCDB 402 with ITS, and cultures were incubated at 37°C in 5% CO_2. Supernatants from irradiated human corneal fibroblast monolayers and whole human corneas were tested for secreted IL-1α, IL-1β, IL-6, IL-8, and TNFα. Lysates from irradiated human corneal fibroblast monolayers were tested for intracellular IL-1α, IL-lβ, and TNFα.

For cytokine Northern blot studies, cells were harvested for RNA isolation at 3 hours after UV irradiation. For RT-PCR studies, cells were harvested at 4 hours after UV irradiation. For cytokine secretion studies, cell supernatants and lysates were prepared as previously described at 24 hours after UVB irradiation

and stored at -80°C.

For IL-1ra-treated samples, in the KC ELISA experiments, plates received 1 μg/ml of recombinant human IL-1ra (gift of D. Hirsch, Columbia University) immediately after irradiation. In RT-PCR studies of IL-1ra-treated cultures, monolayers were treated as above except that all plates were washed 2 hours prior to irradiation and fed with 8 ml serum free MCDB 402 with ITS. IL-1ra samples received a pretreatment with 1μg/ml of recombinant human IL-1ra at this time. Immediately before irradiation monolayers were washed as before, covered with 4 ml/ 150mm dish of HBSS, and irradiated (15 mJ/cm^2). Monolayers were fed 8 ml of serum free MCDB 402 with ITS. In IL-1ra-treated cultures 1μg/ml of fresh IL-1ra was added.

***Ex vivo* irradiation:** In separate experiments, pairs of whole, intact human corneas were removed from Optisol, rinsed in HBSS, and placed individually in moist culture dishes with the epithelial side oriented upward. Corneas were left untreated or were UV irradiated *ex vivo* with 100 mJ/cm^2 UVB, and then incubated in 2.5 ml serum-free media (RPMI 1640+ITS) for 24 hours at 37°C. Organ-cultured human corneas irradiated with 100 mJ/cm^2 of UVB have been shown to undergo few ultrastructural changes with the exception of some epithelial sloughing.[163] After irradiation, supernatants were centrifuged (at 3,000 xg for 20 minutes at 4 °C), and stored as aliquots at -80°C. The whole corneas

were embedded in O.C.T. (Tissue-Tek, Miles Inc., Elkhart, IN), snap frozen in liquid N_2, and stored at -80 °C prior to sectioning for immunohistochemical study.

***In vivo* irradiation:** Mice were anesthetized with a cocktail of ketamine, xylazine, and acepromazine. The mice were laid on their side, the upward eye was proptosed, and the head covered by a triangular mask of heavy paper. A 2.5 cm disk with a 1.5 mm aperture was used to restrict the UV rays to the center of the cornea and protect adjacent limbal and conjunctival tissue during the UVB exposure. UVB doses were 50 mJ/cm^2, given with an exposure time of 2 minutes 24 seconds. Mice were treated with topical antibiotics (Ciprofloxacin 0.3%, Alcon Laboratories, Fort Worth, TX) and sterile ophthalmologic ointment after UVB exposure. During the 72 hour course of the experiment, mice were observed for signs of discomfort or eye irritation. Mice were anesthetized by isoflurane gas in oxygen, examined with operating microscope (Ziess, Germany) and treated with topical Ciprofloxacin three times daily. At 72 hours post-UV irradiation, mice were sacrificed by CO_2 narcosis and their eyes enucleated.

Immunohistochemistry- human cells and tissues

Cultured human cells: For immunohistochemistry studies, 2-3 week old primary cultures of human corneal epithelial cells and stromal fibroblasts were harvested by trypsinization. Cells were washed, re-plated onto 22-mm coverslips

in six well plates, and cultured until nearly confluent. Cell monolayers were stimulated with 100 pg/ml of recombinant human IL-1α for 24 hours. Cell monolayers were washed three times in cold HBSS, then fixed for 10 minutes at 4 °C in phosphate-buffered saline (PBS) containing 1% paraformaldehyde, and then washed three times in cold PBS. Fixed cell monolayers were incubated for 30 minutes in PBS containing 5% normal horse serum (Sigma) to inhibit non-specific antibody binding. Cell monolayers were incubated (for 1 hour at 25 °C) in PBS containing 0.01% Tween 20, 3% BSA, and either 5 μg/ml murine monoclonal antibody (Mab) specific for IL-lra (gift of W. Arend, University of Colorado Health Sciences Center, Denver, CO) or 5 μg/ml of purified preimmune mouse IgG. Monolayers were washed three times and incubated for 1 hour with a 1:200 dilution of alkaline phosphatase conjugated horse anti-mouse IgG (Vector Laboratories, Inc. Burlingame, CA). Monolayers were washed 4 times and developed in 100 mM Tris (pH 8.2) containing 1 mg/ml of Fast Red TR substrate (Pierce, Rockford, IL). This substrate stains cells by forming a red precipitate where alkaline phosphatase activity is localized.

Human corneal tissue: Transplant quality corneas were harvested within 1 hour postmortem, (Lions Eye Bank) and held in Optisol at 4 °C for 6-8 hour before embedding. Corneas were washed three times with HBSS (Ca^{+2}/Mg^{+2} free) then embedded in O.C.T. (Miles Inc., Elkhart, IN), frozen in liquid N_2, and stored

at -80 °C until sectioned. Tissue blocks were warmed to -12 °C and 8 µm sections were cut on a Minotome (International Equipment Company, Needham, MA). Sections were mounted on Superfrost slides (VWR Scientific, Plainfield, NJ), fixed for 5 min in acetone at 4 °C, and then washed three times in PBS. Sections were incubated for 1 hour in PBS containing 5% normal horse serum to inhibit non-specific antibody binding. Sections were incubated with anti-IL-1ra Mab or preimmune IgG, secondary antibody, and substrate as described above. After developing with Fast Red TR, sections were fixed for 5 min in 2% glutaraldehyde in PBS at 4 °C, counterstained with hematoxylin, and photographed under brightfield illumination with a Nikon inverted microscope.

Immunohistochemistry - murine tissues

Paraffin tissue sections: Prior to immunohistochemical staining paraffin embedded sections were de-paraffinized by soaking in xylene (3 times, 5 minutes each) and rehydrated in graded alcohols (100% ethanol 2 times, 5 minutes each, 95% ethanol 5 minutes, 80% ethanol 5 minutes, deionized H_2O (dH_2O) 5 minutes, TBS). Sections were enzymatically digested before immunostaining if required for the antigen of interest. Sections were incubated in TBS containing 3% BSA plus 0.01% Tween 20 for 1 hour at 25°C, to inhibit non-specific antibody binding. Sections were washed 2 times (5 minutes each) in wash buffer (TBS with

0.1%BSA and 0.01% Tween-20) and incubated for 1 hour at 25 °C with the appropriate concentration of primary antibody diluted in TBS containing 3% BSA plus 0.01% Tween-20 for 1 hour at 25 °C. Sections were washed 3 times (5 minutes each) in wash buffer, and incubated for 1 hour at 25 °C with a biotinylated rabbit anti-rat IgG (mouse adsorbed, Vector labs.) diluted 1:200 in wash buffer containing 2% normal rabbit serum. Sections were washed 3 times (5 minutes each) in wash buffer and incubated with alkaline phosphatase anti-alkaline phosphatase antibody-avidin conjugate (Vectastain ABC-AP , Vector lab.) for 45 minutes at 25 °C. Sections were washed 4 times in wash buffer and stained with Fast Red TR as previously described.

Neutrophils: De-paraffinized murine sections were digested with 0.1% trypsin for 5 minutes at 25 °C. Washes and antibody incubations were conducted as described previously. The primary antibody used was a neutrophil-specific rat monoclonal antibody, Ly-6G (Gr-1), at 1:500 dilution (Pharmingen, San Diego).

Macrophages: De-paraffinized murine sections were digested with 0.1% trypsin for 5 minutes. Washes and antibody incubations were conducted as described previously. The primary antibody used was a rat monoclonal antibody against the murine macrophage-specific antigen F4/80 (at 1:750 dilution)(Serotec, Oxford, U.K.).

ICAM: Frozen sections of mouse corneas (5 μm) were fixed and incubated to inhibit nonspecific antibody binding as previously described. Sections were incubated with 1:200 dilution of a rat monoclonal antibody against murine ICAM (Caltag Laboratories, San Francisco, CA). Sections were washed and incubated with secondary antibody, Vectastain ABC-AP, and substrate as described above.

IL-6: Frozen sections of mouse corneas (5 μm) were fixed and incubated to inhibit nonspecific antibody as previously described. Sections were incubated with a 1:200 dilution of rat monoclonal antibody against mouse IL-6 (Pharmingen, San Diego). Sections were washed, incubated with secondary antibody, Vectastain ABC-AP, and substrate as described above.

P-Selectin: Frozen sections of mouse corneas (5 μm) were fixed and incubated to inhibit nonspecific antibody binding as previously described. Sections were incubated with 1:500 dilution of rat monoclonal antibody against mouse P-selectin (Pharmingen, San Diego). Sections were washed, incubated with secondary antibody, Vectastain ABC-AP, and substrate as described above.

Histochemical staining for Neutrophils

Neutrophils in de-paraffinized serial corneal sections were histochemically stained by incubating in a buffered solution containing LB Violet tetrazolium dye, sodium nitrite, and Naphthol AS-D chloroacetate (Sigma Diagnostics, Saint

Louis, MO). Neutrophil esterase activity generates a naphthol compound, which reacts with the tetrazolium dye forming a bright red precipitate that stains the cells. All reagents are obtained in kit form from Sigma Diagnostics. This histochemical technique will also show strong positive staining for promyelocytes and mast cells.[164] The number of neutrophils found by this histochemical staining was confirmed by immunohistochemical methods using a monoclonal antibody against a neutrophil specific marker (Gr-1) as detailed earlier.

Histology

Sets of 10 serial 5 μm sections, spaced at 50 μm intervals, were cut throughout the paraffin embedded eyes with a Spencer microtome (American Optical Co., Buffalo, NY). Sections were mounted on Superfrost slides (3 slides per 10 sections), 1 slide per set was de-paraffinized and stained with hematoxylin and eosin, and 1 slide per set was de-paraffinized and histochemically stained for esterase.

Paraffin embedded eyes: Enucleated eyes were fixed overnight in neutral buffered formalin at 4 °C, washed briefly in deionized water, and stored in 60% ethanol until tissue processing. Eyes were paraffin embedded in an automated Citadel tissue processor (Shandon Products Ltd., Cheshire, UK) set as follows: 1 hour 80% alcohol (Flex, Richard-Allan, Kalamazoo, MI), 1 hour 95% alcohol (3

times), 1 hour 100% alcohol (3 times), 1.5 hours xylene (3 times), 2 hours paraffin at 56 °C (2 times).

Immunoprecipitation of IL-1ra

Human corneal epithelial and stromal cells were ^{35}S cysteine/methionine labeled as previously described.[166] Nearly confluent cultures of epithelial cells and stromal fibroblasts were stimulated for 3 hours with 100 pg/ml of IL-1α (Genzyme Corp.), washed two times with cysteine and methionine free media 154XP (Cascade Biologics), and labeled for 18 hours at 37 °C with EXPRE^{35}S^{35}S labeling mix (NEN Dupont, Wilmington, DE) at 100 μCi/ml in 154XP media. After labeling, cell supernatants were collected and cells were washed three times in HBSS. Cells were lysed in buffer containing 1% NP-40, 100 mM NaCl, 10 mM Tris, pH 7.4, 5 mM EDTA, and 2 mM PMSF for 20 minutes at 4 °C, and then clarified by centrifugation at 10,000 xg for 20 minutes at 4 °C. Culture supernatants and cell lysates were combined, and the volume reduced to 1.5 ml by Centri-prep 10 concentrators (Amicon Corp., Beverly, MA). Before immunoprecipitation, samples were precleared once with 25 μl of protein A-Sepharose (Pierce) and preimmune rabbit IgG (25 μg/sample) followed by protein A-Sepharose two times. For immunoprecipitation, 25 μg of rabbit polyclonal anti-IL-lra antibody per sample (Genzyme Corp.) was bound to protein

A-Sepharose with an antibody orientation kit (Pierce). We have previously used this antibody in immunoprecipitation and Western blot assays and have observed that it precipitates a 17 kD band from solutions of recombinant human IL-lra (data not shown). After binding antibody, protein-A Sepharose (Pierce) was recovered by centrifugation (1000 xg for 10 minutes at 4 °C) and washed three times in TBS with 0.01% Tween 20. Samples were then eluted into 25 µl of reducing sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 10% glycerol, 50 mM Tris pH 6.8, 5% 2-Me) by heating for 5 min at 95 °C, and then centrifuged for 1 minute at 16,000 xg. Eluted proteins were separated by electrophoresis through 15% polyacrylamide SDS gels and autoradiographed at -80 °C with Kodak X-OMAT AR film. Phorbol 12-myristate 13-acetate diester (PMA)-differentiated U937 monocytic cells (IL-lra producer)[167] served as a positive control and the G361 human melanoma cell line (non-IL-lra producer) was used as a negative control for these studies.

Northern Blot Analysis Human studies

Poly (A)+ RNA isolation: Poly (A)+ RNA from cell monolayers was isolated by oligo (dT) cellulose affinity chromatography as follows: Near confluent cultures were lysed in 10 ml of buffer containing 200 mM NaCl, 200 mM Tris pH 7.5, 1.5 mM $MgCl_2$, 2% SDS, and 200 µg/ml Proteinase K at 42 °C

for 90 minutes. After lysis, the NaCl concentration of buffer was raised to 500 mM, and then samples were mixed with 50 mg of oligo (dT) cellulose (GibcoBRL Life Technologies) per sample and incubated 2 hours 25 °C. Oligo (dT) cellulose bound poly (A)+ RNAs were recovered by centrifugation, washed 3 times in binding buffer (500 mM NaCl, 10 mM Tris pH 7.5), and then eluted with 10 mM Tris pH 7.5. Poly (A)+ RNA was precipitated overnight at -20 °C by the addition of 0.1 volume 3M sodium acetate and 2.5 volume of cold 100% ethanol. RNA was recovered by centrifugation (30,000 xg for 40 minutes at 4 °C) and dissolved in 10 mM Tris, pH 7.2 with 1 mM EDTA.

Electrophoresis and Blotting: Poly (A)+ RNA (4 µg per lane) was separated by electrophoresis through 1.0% agarose denaturing formaldehyde gels containing 2 mM 3-N-morholinopropanesulfonic acid (MOPS), and then blotted to nitrocellulose membrane (0.45 µm pore size) by capillary transfer in 10X SSC (1.5M NaCl, 150 mM sodium citrate).

^{32}P-labeled probes and hybridization: The following cDNA probes were used for the Northern blot studies: human IL-1α (gift of P. Lomedico, Hoffmann-LaRoche, Nutley, NJ [168]); human IL-6 (gift of T. Hirano, Osaka University, Japan [169]); human IL-8 (gift of K Matsushima, NCI-Frederick Cancer Research Facility, Frederick, MD [170]); human TNFα (gift of R. Derynck, Genentech, South San Francisco, CA [171]); human IL-1ra (detects both icIL-1ra and

sIL-lra)(gift of D. Carter, Upjohn Inc., Kalamazoo, MI); cyclophilin, (gift of J. Douglass, Vollum Institute for Advanced Biomedical Research, Portland, Oregon [172]).

Denatured complementary DNA (cDNA) probes were ^{32}P-labeled by replication with DNA polymerase I Klenow fragment and random hexamer priming. Northern blots were hybridized for 18 hours at 65 ° C in a buffer containing 50% formamide, 5X SSC, 5X Denhardt's, 25 mM sodium phosphate pH 7, 0.5 mg/ml herring sperm DNA, and 5% SDS. After hybridization blots were washed 2 times (in 2X SSC, 0.1% SDS) for 30 minutes at 25°C, 1 time (in 0.1X SSC, 0.1% SDS) for 15 minutes at 25 °C, and 2 times (in 0.1X SSC, 0.1% SDS) for 15 minutes at 56-60 °C. Blots were autoradiographed at -80 °C with Kodak X-OMAT AR film. Equivalent loading of RNA in each lane of the Northern blots was confirmed by hybridizing the blots with the cDNA probe for the gene, cyclophilin. Scanning densitometric analysis with normalization to cyclophilin messenger RNA (mRNA) band densities was used to quantitate differences in the mRNA expression of corneal cell cytokines. Preparation of ^{32}P-labeled probes and hybridization of Northern blots was performed by J Brown.

Reverse transcriptase-polymerase chain reaction analysis (RT-PCR)

RT-PCR for human iclL-lra and slL-lra (Performed by X. Huang)

PCR analysis of steady-state mRNA from human corneal epithelial, stromal, and endothelial cells was conducted as follows: Poly (A) + mRNA (15 ng) or total RNA (200 ng) was reverse transcribed into first strand cDNA by oligo(dT)-primed reverse transcriptase. The reaction mixture (20 µl final volume) containing 20 U of M-MLV reverse transcriptase (GibcoBRL) with 125 µM each dNTP, 1 U RNasin (Promega, Madison,WI), and 200 ng of oligo $(dT)_{12-18}$ primer (GibcoBRL) was incubated for 2 hours at 37°C. Reverse transcriptase products (1.5 µl) were PCR amplified in a reaction mixture containing 3.5 mM Mg^{2+}, 1 mM each dNTP, 0.6 U of TFL polymerase (Epicentre Technologies Corp., Madison, WA) and 20 pmole of IL-1ra specific primers and/ or 2.0 pmole of cyclophilin primers. Each 25 µl reaction mixture was incubated for 25 cycles in a Perkin Elmer (Norwalk, CT) model 9600 thermocycler set as follows: denaturation, 15 seconds, 94°C; annealing, 1 minute, 55°C; extension, 2 minutes, 72°C. PCR products were separated by electrophoresis through 3% agarose gels and detected by ethidium bromide staining. The PCR primers were designed by S. R. Planck, PhD., and made by Operon Technologies Inc. (Alameda, CA) as follows,

icIL-lra (sense) 5'-GAA GTT GAG TTA GAG TCT GAA A

	(antisense)	5'-TGA CAT TTG GTC CTT GCA AGT A
sIL-1ra	(sense)	5'-GAA TGG AAA TCT GCA GAG GCC TCC GC
	(antisense)	5'-TGA CATTTG GTC CTT GCA AGT A
IL-1ra	(sense)	5'-GCA AGA TGC AAG CCT TCA GAA TCT GGG
	(antisense)	5'-GCT GGT CAG CTT CCA TCG CTG TGC A.
Cyclophilin	(sense)	5'-TGT TCT CGA CAT TGC CGT CGA C
	(antisense)	5'-GCA TTT GCC ATG GAC AAG ATG CCA GGA

RT-PCR Analysis of MCFB

RT-PCR analysis of steady-state mRNA from murine corneal fibroblast cells was conducted as follows. Total RNA (150 ng) was reverse transcribed into cDNA as previously described. Amplification of cDNAs was done by touchdown PCR[173, 174] with a Perkin Elmer model 9600 thermocycler set as follows: initial denaturation, 5 minutes, 95°C; + denaturation, 15 seconds, 94°C; annealing, 1 minute, 69°C; extension, 2 minutes, 72°C; for 2 cycles. This 3 temperature PCR protocol was repeated 9 times with a 1 degree C decrease in the annealing temperature each time (annealing temperatures from 69°C to 60°C). This was followed by 20 cycles: denaturation, 15 seconds, 94°C; annealing, 1 minute, 55°C; extension, 2 minutes, 72°C. The total volume of the reaction mixture was 25 µl containing 4 mM Mg^{2+}, 1 mM each dNTP, 0.6 U of Taq polymerase (Perkin

Elmer Corp.) and 20 pmole of specific primer and/ or 2.5 pmole of GAPDH primer. Separation of PCR products, primer design, and primer manufacture were as previously described. The sequences of primers used are as follows:

IL-1ra	(sense)	5'-GAT GCA AGC CTT CAG AAT CTG G
	(anti-sense)	5'-ATC CTG GAC AGG CAG CTG ACT C
IL-6	(sense)	5'-CGG AGA GGA GAC TTC ACA GAG GAT A
	(anti-sense)	5'-CCA CTC CTT CTG TGA CTC CAG CTT A
KC	(sense)	5'-CTC AAG AAT GGT CGC GAG GCT
	(anti-sense)	5'-GCA CAG TGG TTG ACA CTT AGT GGT CTC
MIP-2	(sense)	5'-CAG TGA ACT GCG CTG TCA ATG C
	(anti-sense)	5'-CAG ACA GCG AGG CAC ATC AGG TA
GCP-2	(sense)	5'-TTC TTT CCA CTG CGA GTG CAT TCC
	(anti-sense)	5'-GGT TCC AGC TCG CCA TTC AT
GAPDH	(sense)	5'-CAT CAA GAA GGT GGT GAA GCA GG
	(antisense)	5'-CCA CCA CCC TGT TGC TGT AGC C

PCR Typing of IL-1RA transgenic mice.

Template genomic DNAs (100ng) were subjected to 35 rounds of PCR amplification with a Perkin Elmer model 9600 thermocycler set as follows: denaturation 15 seconds, 94°C; annealing, 1 minute, 55°C; extension, 2 minutes,

72°C. The total volume of the reaction mixture was 30µl with 4 mM Mg^{2+}, 1 mM each dNTP, 0.625 U of Taq polymerase and 20 pmole of specific primers. Separation of PCR products, primer design, and primer manufacture were as previously described. The sequences of primers used are as follows:

IL-1ra (sense) 5'-TGG AGG TCT GGT TCA CTG TA

IL-1ra (anti-sense) 5'-ATC CTG GAC AGG CAG CTG ACT C.

After PCR amplification, 17 µl of sample were removed, combined with 2 µl of 10X L buffer (Boehringer Mannheim) and 1 µl of Kpn 1 exonuclease (5 U/µl , Boehringer Mannheim) and digested for 2 hours at 37°C. Digested PCR products were separated by electrophoresis through 3% agarose gels. These primers anneal at position 3191 in intron 2 and 4462 in exon 4 of the murine IL-1ra gene. The amplification product is a 1,271 bp fragment from both the endogenous IL-1ra gene and the IL-1ra transgene. However, the transgene amplification product contains an engineered Kpn I site and digests with Kpn 1 into 261 bp and 1010 bp fragments.

Electron Microscopic Analysis (performed by C. Meshul PhD., VAMC, Portland, OR)

To confirm that the corneal stromal cells were keratocytes, nonirradiated corneal cells were fixed overnight in 2.5% glutaraldehyde-3% paraformaldehyde

in 0.1M sodium cacodylate buffer, pH 7.35. The cells were washed in buffer, placed in 1% osmium tetroxide-1.5% potassium ferricyanide at room temperature for 1 hour, dehydrated in a series of increasing alcohols, cleared in propylene oxide, and polymerized in Embed 812/Araldite (Electron Microscopy Sciences, Fort Washington, PA) overnight at 60°C. Blocks of cells were sectioned on an RMC (Tucson, AZ) ultramicrotome with a Diatome diamond knife, and the sections were placed in nickel mesh grids and counter-stained with lead and uranyl salts. The sections were viewed and photographed on a JEOL 1200 TEMSCAN electron microscope (Peabody, MA) and photographed at an initial magnification X4000.

Data analysis

Quantitative data was analyzed for statistical significance by application of Student t-test or Mann-Whitney rank sum test.

CHAPTER 1

Ultraviolet irradiation induces multiple cytokines.

Introduction:

Exposure to ultraviolet (UV) light is a significant environmental and occupational hazard capable of causing acute and chronic inflammatory changes in the cornea.[145, 148, 175-177] It is well established that UV irradiation of the skin induces the production of inflammatory cytokines and alters cutaneous immune function.[178-182] However, the ability of UV irradiation to induce similar effects in the cornea has not been examined. A number of studies by our group and others have established that the release of such cytokines as interleukin (IL-1), IL-8, and tumor necrosis factorα (TNFα) can contribute to ocular inflammation and uveitis.[21, 22, 183-186] In addition, human corneal stromal cells can produce a number of cytokines that mediate inflammatory activities in immune and nonimmune cells.[30, 31, 109, 187-192] In this study, we examined the effect of environmentally relevant amounts of UV irradiation on human corneal stromal cell cytokine production. Our results indicate that cultured corneal stromal cells and whole human corneas respond to even moderate levels of UV irradiation with a significant increase in the production of the inflammatory cytokines, IL-1, IL-6, IL-8, and TNFα. The local release of these cytokines by cells in the irradiated cornea may be responsible for UV-mediated acute and chronic inflammation of the cornea

Results:

Electron Microscopic Identification of Corneal Stromal Cells

Fresh human corneal stromal cells were isolated and cultured for the UV studies. Cultured cells were analyzed by electron microscope to verify that the cells isolated for these studies were corneal keratocytes and were not contaminated by epithelial or endothelial cells. As indicated in Figure 1.1, the cultured cells used in these studies demonstrate the typical ultrastructural features of stromal keratocytes, including the absence of desmosomal contacts and only a moderate accumulation of intermediate filaments (tonofilaments).

Effect of Ultraviolet Irradiation on Corneal Stromal Cell Cytokine Expression

The effect of low-dose (10 mJ/cm^2) and high-dose (100 mJ/cm^2) UV on corneal stromal cell IL-1, IL-6, IL-8, and TNFα mRNA expression was determined by Northern blot analysis 3 hours after irradiation (Fig. 1.2). Equivalent loading of RNA on Northern blots was assessed by measuring cyclophilin mRNA levels (Fig. 1.2). Differences in mRNA expression for each cytokine after UV irradiation were quantitated by scanning densitometric analysis and were normalized to cyclophilin mRNA levels, thus allowing comparison to constitutive cytokine levels in untreated cells. Cells exposed to low-dose UV (10

mJ/cm^2) have an 11-fold increase in IL-1α mRNA levels compared with that in nonirradiated cells, whereas exposure to higher amounts of UV (100 mJ/cm^2) results in a 7-fold increase in IL-1α mRNA expression compared with that in nonirradiated cells. Because the higher dose of UV used in these studies is accompanied by significant cell death, there may be a reduction in cytokine mRNA induction compared with that in cells treated with the lower dose of UV.

The effect of UV irradiation on corneal stromal cell IL-6 mRNA expression was examined. Northern blot analysis of cells irradiated with 10 mJ/cm^2 UV demonstrates that this dose of UV induces an eight fold increase in IL-6 mRNA expression compared with that at constitutive levels (Fig. 1.2). Likewise, exposure to 100 mJ/cm^2 UV irradiation results in an eightfold increase in IL-6 mRNA levels (Fig. 1.2). When corneal stromal cells are irradiated with 10 mJ/cm^2 UV, there is a 30-fold increase in IL-8 mRNA expression compared with that in non-UV-exposed cells (Fig. 1.2). Exposure of cells to 100 mJ/cm^2 UV results in a 35-fold increase in IL-8 mRNA expression compared with that in nonirradiated cells. The effect of UV on corneal stromal cell TNFα mRNA expression was then examined (Fig. 1.2). The level of TNFα mRNA increases slightly after low dose UV irradiation of corneal stromal cells, with up to a sixfold increase in TNFα mRNA after high-dose UV. Thus, UV irradiation results in a significant increase in corneal stromal cell mRNA for IL-1α, IL-6, IL-8, and

TNFα inflammatory cytokines.

Effect of Ultraviolet irradiation on Cytokine Secretion by Corneal Stromal Cells.

Corneal stromal cell cytokine secretion after UV irradiation was examined. The effect of UV irradiation on IL-1 production was measured by ELISA and bioassay. Because IL-1 activity is predominately cell associated, we measured the production of this cytokine in cellular lysates 24 hours after UV exposure. As indicated in Figures 1.3 A and 1.3 B, increased quantities of IL-lα (x15) and IL-1β (x2) are detected in stromal cell lysates as measured by ELISA after exposure to 10 mJ/cm^2 UV and are further increased (x40 and x5, respectively) after exposure to higher amounts of UV irradiation. In a parallel study, increased corneal stromal cell IL-1 biologic activity (x8.5) was detected in 24 hour supernatants of UV-irradiated stromal cells, measured in the D10.S proliferation assay (Fig. 1.3 C). Thus, IL-1 production is significantly increased in corneal stromal cells after UV exposure.

The effect of UV irradiation on corneal stromal cell IL-6, IL-8, and TNFα secretion was examined in 24-hour culture supernatants. Ultraviolet irradiation of corneal stromal cells causes a significant dose-dependent increase in secreted IL-6 (a greater than 300-fold increase after 20 mJ/cm^2 UV) as measured by ELISA

(Fig. 1.4 A). In addition, UV irradiation induces a dose-dependent increase in the release of IL-6 biologic activity by corneal stromal cells, measured in the B9 proliferation assay (Fig. 1.4 B). The effect of UV on corneal stromal cell IL-8 production was measured. Similar to that of IL-1 and IL-6, increased IL-8 secretion was detected in 24 hour supernatants of corneal stromal cells exposed to UV in a dose-dependent fashion, measured by ELISA (Fig. 1.5).

The effect of UV on stromal cell TNFα production was examined. We measured the production of this cytokine in cellular lysates 24 hours after UV exposure. Similar to increases seen in the other inflammatory cytokines, UV irradiation induces a dose-dependent increase in cell-associated TNFα production, measured by ELISA (Fig. 1.6). Thus, UV exposure induces increased production of IL-1, IL-6, IL-8, and TNFα mRNA and protein.

Effect of Ultraviolet Irradiation on Whole-Cornea Cytokine Production.

We evaluated the effect of UV irradiation on cytokine production by whole human corneas in an *ex vivo* tissue culture system. As indicated in Figure 1.7, there are significant increases in the secretion of IL-6, IL-8, and TNFα in 24-hour supernatants after whole human cornea irradiation. As expected, because IL-1α is primarily cell-associated, there is less secretion of IL-1α into the organ culture media after UV irradiation, compared with the secretion of other cytokines,

although secreted IL-1α increases after UV irradiation. No secreted IL-1β is detected in cornea explant supernatants, with or without UV irradiation. Our results indicate that higher doses of UV (100 mJ/cm^2) are most effective for the induction of these cytokines in the *ex vivo* tissue culture system, compared with their level of induction in cultured corneal stromal cells. Thus, as in cultured corneal stromal cells, UV induces increased production of inflammatory cytokines by irradiated whole human corneas.

Discussion:

It has recently been established that human cornea cells are capable of producing a number of inflammatory cytokines.[30, 31, 109, 187-192] The role of these cytokines in normal corneal biology and in corneal disease is an area of increasing investigation. The effect of UV irradiation on corneal cytokine production has not been carefully evaluated. We examined the effect of UV light on cornea cytokine production because this environmental agent can lead to significant ocular inflammation and pathologic changes. The ability of UV irradiation to induce cytokine production in the skin is well established. We and others have demonstrated that UV exposure results in augmented IL-1, IL-6, IL-8, and TNFα production in human skin keratinocytes.[178-182] In the current study, we demonstrate that UV exposure from Westinghouse FS-40 sunlamp bulbs induces

human corneal stromal cells and whole human corneas to produce increased quantities of IL-1, IL-6, IL-8, and TNFα, which induce significant inflammatory activities in lymphoid and nonlymphoid cells. The fact that UV irradiation can induce the production of these cytokines in cultured corneal cells and in whole-cornea tissue is of significant biologic importance and may provide a mechanism by which UV is capable of inciting acute and chronic pathologic inflammatory changes in the cornea.

Results of previous studies indicate that exposure of the cornea to excessive UV irradiation leads to damage on cellular and molecular levels. Acute UVB exposure causes edema and photokeratitis and is associated with an inflammatory reaction in the anterior eye segment, particularly in the cornea.[148, 177] Irradiation with UVB also leads to elevated shedding of corneal epithelial cells that is postulated to contribute to the characteristic pain that accompanies photokeratitis, caused by the exposure of subsurface nerve endings in the cornea.[193] In vivo, UV irradiation is also associated with rapid activation of c-fos and c-jun expression in the epithelial cell layers of the cornea in rats.[194] Expression of these protooncogenes in the irradiated cornea suggests they may play a role in the initiation of cell death after UV. There is also evidence that UV irradiation can alter corneal immune function by reducing the number of Langerhans' cells in the corneal epithelium, which can have a beneficial effect on

corneal graft survival.[195]

In the current study, we have chosen the FS-40 Westinghouse sunlamp bulb as the UV light source. These bulbs emit UVA and UVB radiation, with an emission spectrum ranging from 280 to 350 nm and a peak at 315 nm.[162] At a distance of 50 cm, the UVB irradiance of the FS-40 bulbs is equivalent to natural noontime sunlight, whereas the UVA irradiance is approximately 10% of natural sunlight.[162] This UV source is relevant to human ocular disease, because similar artificial radiation sources are used for tanning purposes and are a significant source of acute UV keratitis.[177] Natural sources of UV light can also cause acute UV keratitis if people are exposed to large amounts of reflected light from snow, sand, or water. Chronic exposure to solar UV irradiation is also associated with external eye disease including pinguecula, pterygium, climatic droplet keratopathy, and squamous metaplasia or carcinoma.[145, 175-177] Data in a recent study quantify human UV radiation ocular exposure by using induction of photokeratitis in the human cornea as a biologic dosimeter for chronic ocular exposure to UV radiation.[196]

The observation that corneal cell cytokine production is increased after UV exposure may help explain the mechanism of photoinflammation in the cornea. The role of cytokines in ocular inflammation has been investigated in a number of recent studies.[21, 22, 30, 31, 109, 183, 186-191, 197] The inflammatory cytokines IL-1α, IL-1β,

IL-8, and TNFα have been shown to induce ocular inflammation.[21, 22, 183, 186, 197] The ability of corneal cells themselves to produce IL-1, IL-6, IL-8, and TNFα after stimulation by various agents has also been clearly established.[30, 31, 109, 187-189] There is evidence that particular inflammatory cytokines are important in different corneal diseases, including pseudophakic bullous keratopathy.[190, 191] Thus, in the current study, we have demonstrated that UV irradiation of human corneal stromal cells and intact corneal tissue results in the production of multiple inflammatory cytokines, all of which have a role in inflammatory processes in the cornea.

Ultraviolet induction of cytokines in whole cornea explants is of particular clinical relevance, because this experimental approach more closely models human UV exposure *in vivo*. It is interesting, but not unexpected, that whole corneas tolerate higher doses of UV irradiation (100 mJ/cm^2) than do cultured keratocytes, which begin sloughing off the culture dish at UV doses of 50 mJ/cm^2. This is probably related to the whole corneas having an intact epithelial cell layer that absorbs a significant amount of this irradiation. By determining cytokine levels in organ culture supernatants, we may be measuring secreted cytokines, not only from corneal keratocytes, but perhaps from endothelial cells and epithelial cells as well. The low levels of IL-1α and undetectable levels of IL-1β that are found in whole cornea supernatants are probably because these cytokines are primarily intracellular rather than secreted cytokines. In addition, results in

previous studies by us and others indicate that higher doses of UVA than of UVB are needed to induce cytokine production in skin cells.[198] Ongoing studies in our laboratories will also differentiate the effect of UVA versus UVB irradiation on corneal cytokine production and the UV induction of corneal epithelial cell and endothelial cytokines. Future studies of the patho-physiology of corneal UV-induced cytokines may lead to better approaches to the treatment of the acute and chronic effects of corneal UV damage.

Acknowledgments

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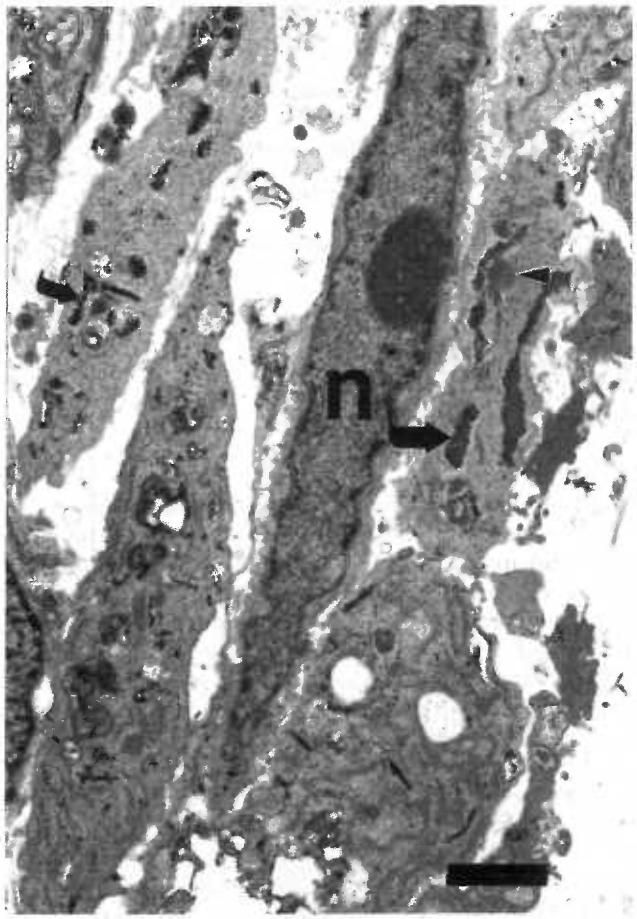

FIGURE 1.1 Ultrastructural features of human corneal stromal cells. Photomicrograph of cultured corneal stromal cells demonstrating long, slender keratocytes, which contain a nucleus (n), mitochondria, lipid droplets (arrowhead:), rough endoplasmic reticulum, and an accumulation of glycogen (arrow). Desmosomal contacts between adjacent cells are not evident. Calibration bar = 2 μm. Electron microscopy done by C. Meschul.

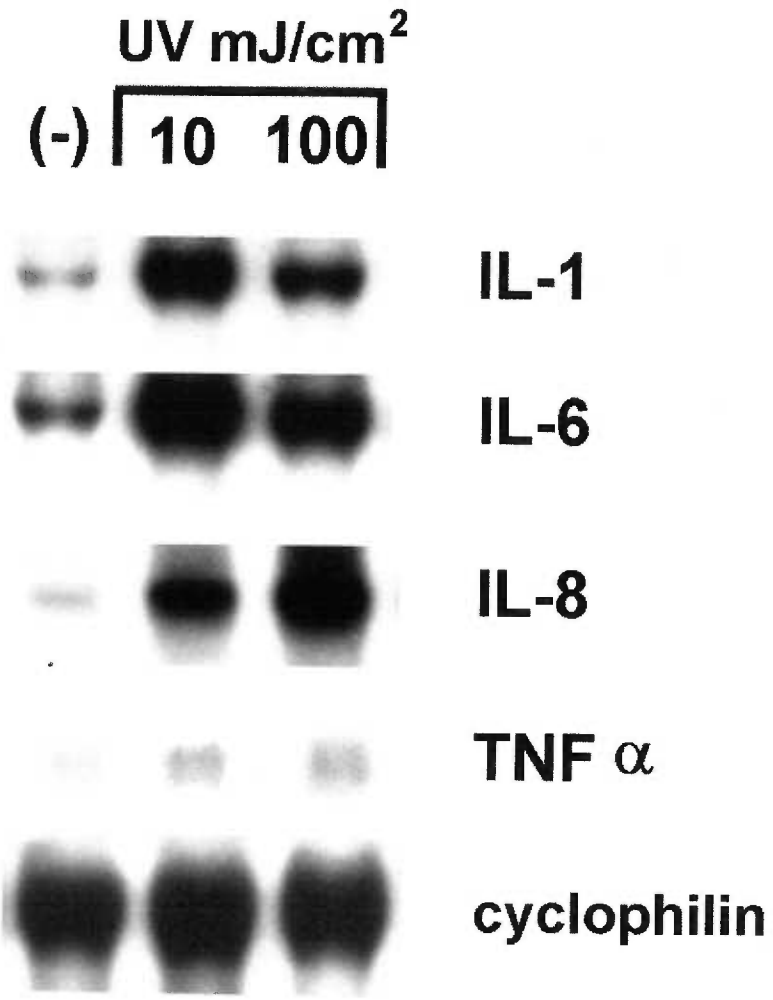

FIGURE 1.2 Ultraviolet induction of human corneal stromal cell cytokine mRNA expression. RNA was extracted from corneal stromal cells and interleukin-1α, interleukin-6, interleukin-8, and tumor necrosis factorα, expression was determined by using ^{32}P-labeled complementary DNA probes specific for each human cytokine. Each lane contains 5 μg poly A+ RNA from unstimulated cells (-) or cells stimulated with 10 mJ/cm^2 or 100 mJ/cm^2 UVB irradiation (Westinghouse F20 bulbs). Cells were harvested 3 hours after UV irradiation. Equivalent amounts of mRNA in each lane were verified by hybridizing with a ^{32}P-labeled complementary DNA probe for cyclophilin. These results are representative of triplicate experiments. Production of ^{32}P-labeled probes and hybridization done by J. Brown.

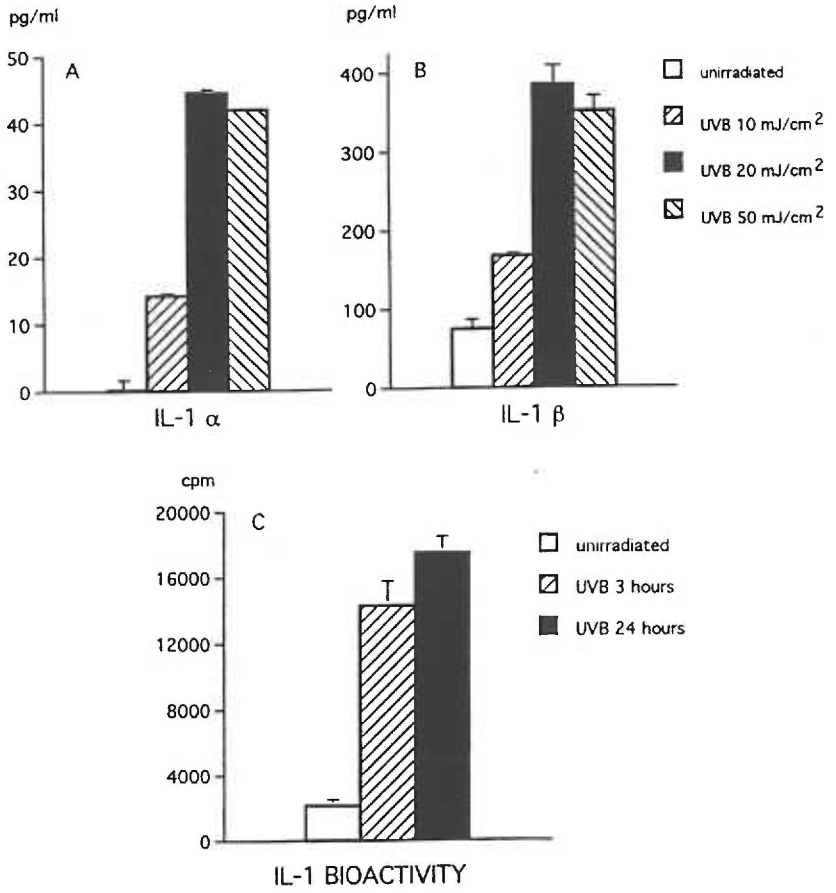

FIGURE 1.3 (A and B) Ultraviolet induction of interleukin-lα and interleukin-β in human corneal stromal cells. Subconfluent cultures, of early passage human corneal stromal cells were irradiated with 0, 10, 20, and 50 mJ/cm^2 UVB (Westinghouse FS-40 bulbs), and interleukin-lα and interleukin-1β expression were measured by enzyme-linked immunosorbent assay in cell lysates collected 24 hours after irradiation. Standard deviations of values for triplicate samples are indicated by error bars. Nonirradiated cells produced 0.3 pg/ml interleukin-lα. These results are representative of experiments conducted three times. (C) Ultraviolet induction of interleukin-1 bioactivity in human corneal stromal cells. Subconfluent cultures of early passage human corneal stromal cells were irradiated with 50 mJ/cm^2 UVB (Westinghouse FS-40 bulbs), and secreted interleukin-1 activity was measured in supernatants collected 3 and 24 hours after irradiation, with the DlO.S interleukin-l-specific proliferation bioassay. Data are shown at a supernatant dilution of 1:4, with standard deviations of values for triplicate samples indicated by error bars. These results are representative of experiments conducted three times.

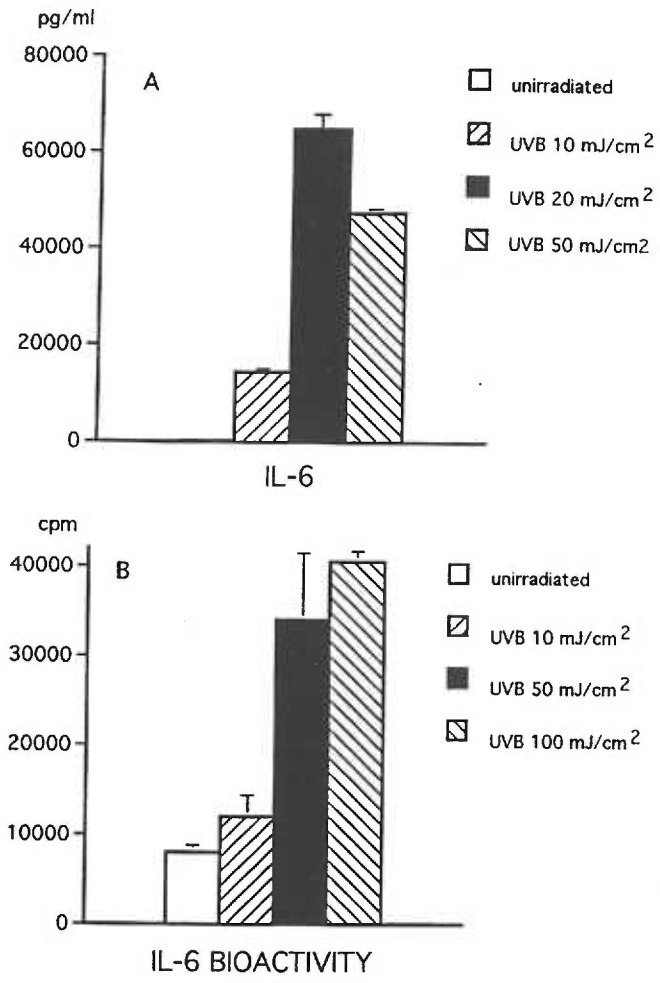

FIGURE 1.4 **(A)** Induction of interleukin-6 in human corneal stromal cells. Nearly confluent cultures of early passage human corneal stromal cells were irradiated with 0, 10, 20, and 50 mJ/cm^2 UVB (Westinghouse FS-40 bulbs), and secreted interleukin-6 was measured by enzyme-linked immunosorbent assay in supernatants collected 24 hours after irradiation. Standard deviations of values for triplicate samples are indicated by error bars. Nonirradiated cells produced 165 pg/ml interleukin-6. These results are representative of experiments conducted three times. **(B)** Ultraviolet induction of interleukin-6 bioactivity in human corneal stromal cells . Nearly confluent cultures of early passage human corneal stromal cells were irradiated with 0, 10, 50, or 100 mJ/cm^2 UVB Westinghouse FS-40 bulbs), and secreted interleukin-6 activity was measured in supernatants collected 24 hours after irradiation by the B9 interleukin-6-specific proliferation bioassay. Data are shown at a supernatant dilution of 1:64, with standard deviations for values of triplicate samples indicated by error bars. These results are representative of experiments conducted three times.

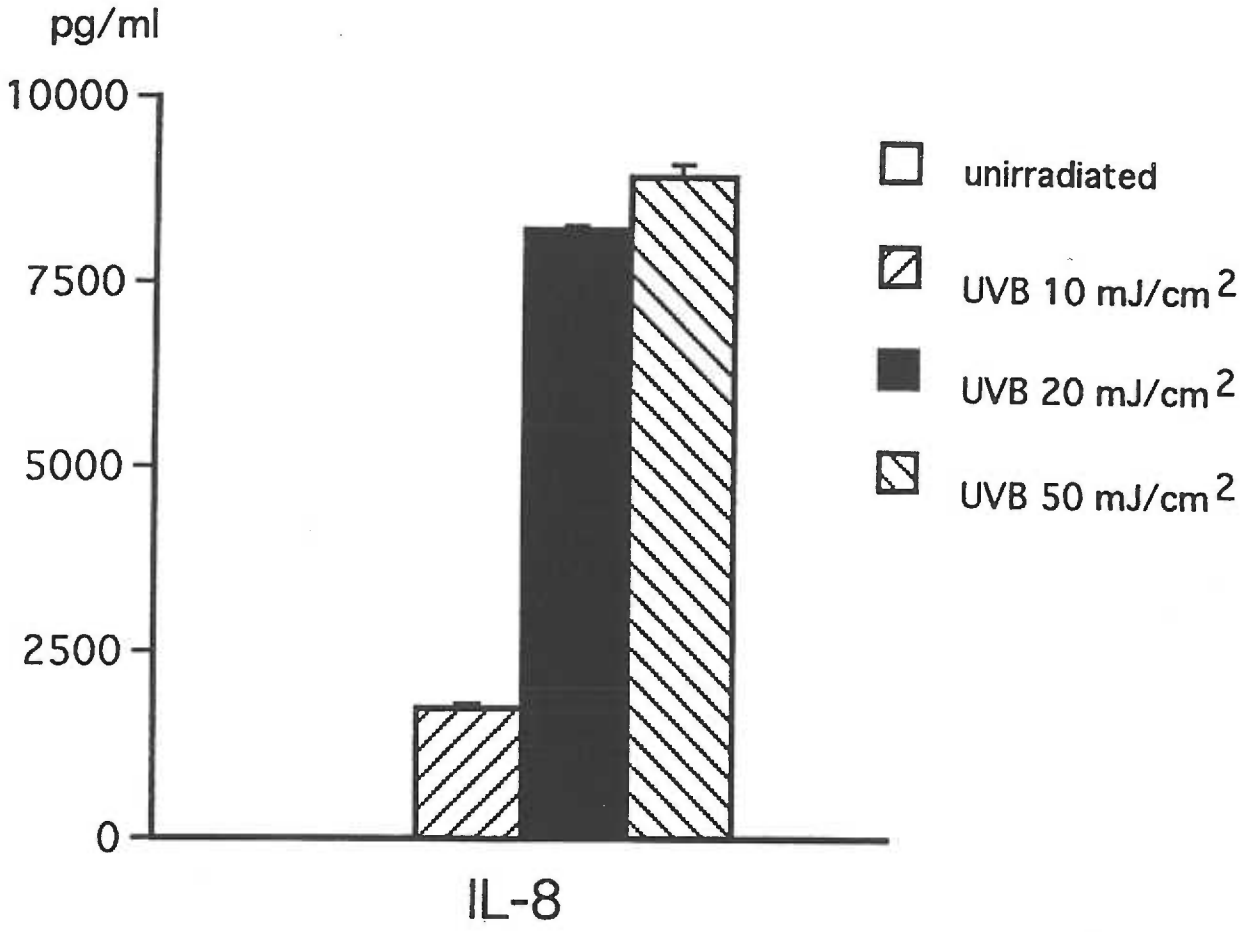

FIGURE 1.5 Ultraviolet induction of secreted interleukin-8 inhuman corneal stromal cells. Subconfluent cultures of early passage human corneal stromal cells were irradiated with 0, 10, 20, and 50 mJ/cm^2 UVB (Westinghouse FS-40 bulbs), and secreted interleukin-8 was measured by enzyme-linked immunosorbent assay in supernatants collected 24 hours after irradiation. Standard deviations of values for triplicate samples are indicated by error bars. Nonirradiated cells produced no detectable interleukin-8. These results are representative of experiments conducted three times.

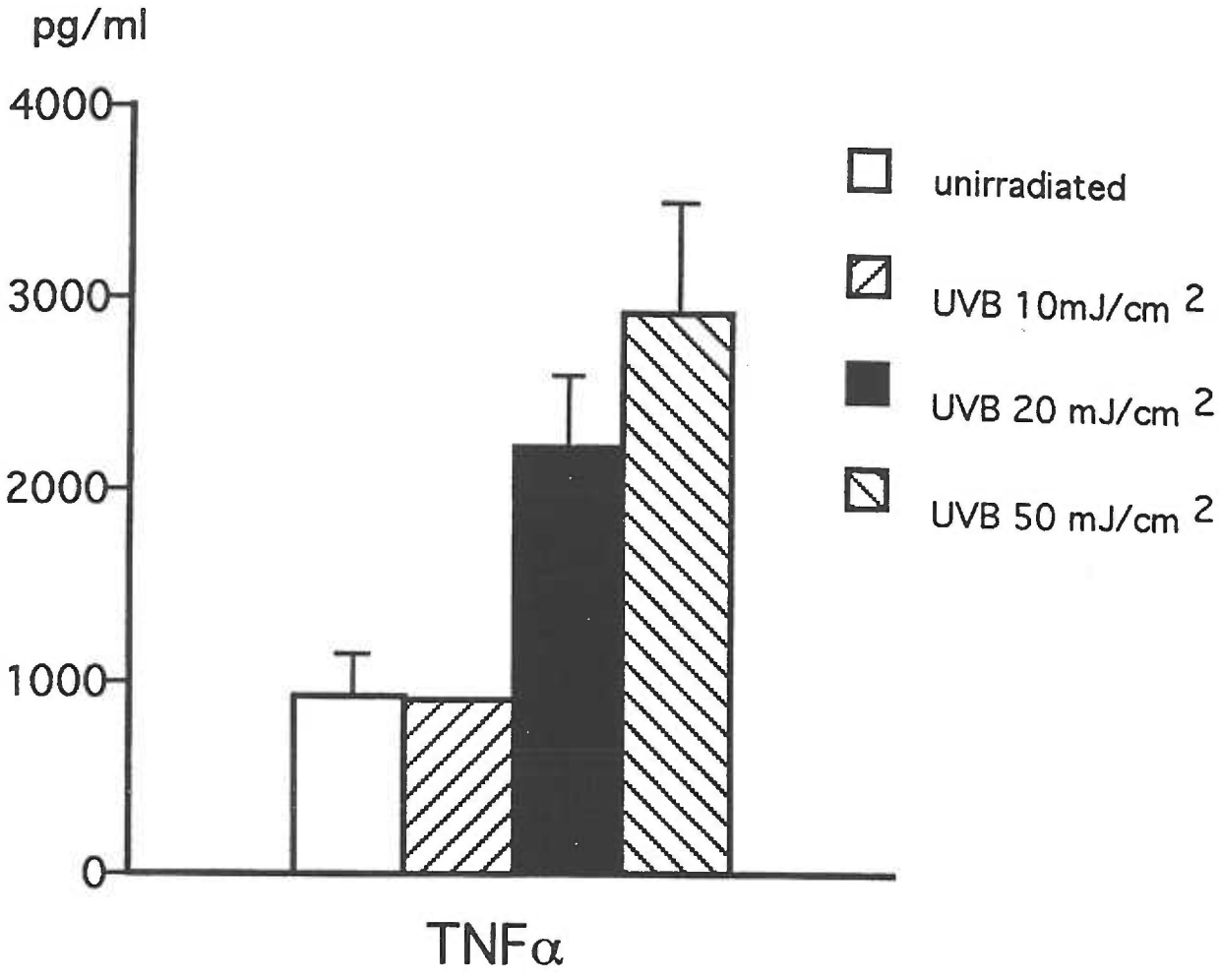

FIGURE 1.6 Ultraviolet induction of tumor necrosis factorα in human corneal stromal cells. Subconfluent cultures of early passage human corneal stromal cells were irradiated with 0, 10, 20, and 50 mJ/cm^2 UVB (Westinghouse FS-40 bulbs), and tumor necrosis factor α was measured by enzyme-linked immunosorbent assay in cell lysates collected 24 hours after irradiation. Standard deviations of values for triplicate samples are indicated by error bars. These results are representative of experiments conducted three times.

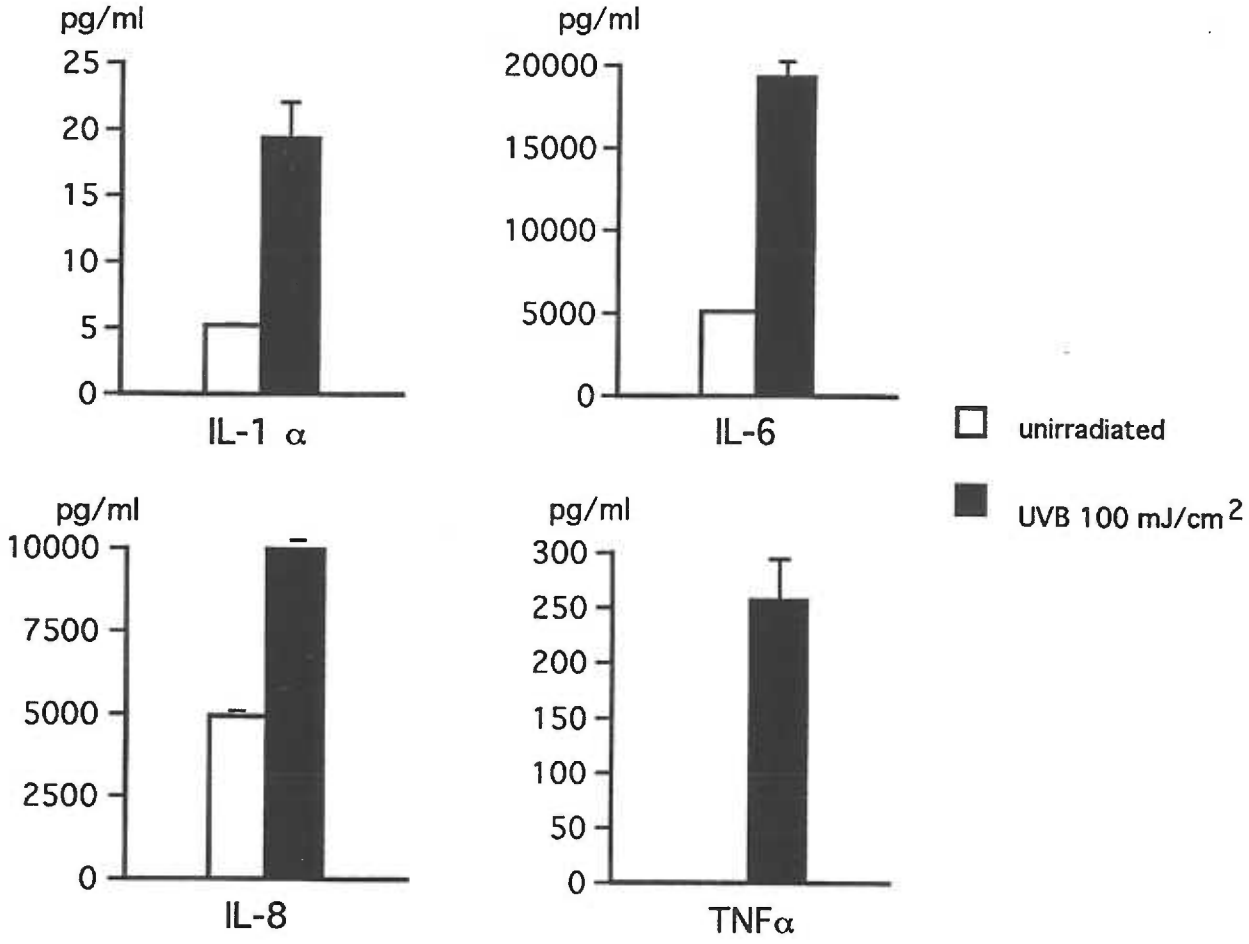

FIGURE 1.7 Ultraviolet induction of secreted cytokines by whole human cornea. Pairs of whole human corneas were irradiated with 0 or 100 mJ/cm^2 UVB (Westinghouse FS-40 bulbs), and cytokines (interleukin-lα, interleukin-6, interleukin-8, and tumor necrosis factor α) that were secreted into organ culture supernatant were measured by enzyme-linked immunosorbent assay 24 hours after irradiation. Standard deviations of values for triplicate samples are indicated by error bars. Nonirradiated cells produced no detectable tumor necrosis factor α.-These results are representative of experiments conducted three times

CHAPTER 2

IL-1ra production in the cornea.

Introduction

Cytokines have been implicated as important mediators of inflammation and tissue damage in the cornea.[21, 22, 184, 185, 197, 199] It has been demonstrated by our group and others that corneal cells are capable of producing a number of inflammatory cytokines including the potent immunomodulating cytokine interleukin-1.[24, 30, 109, 187-189, 192, 200] Increased tissue IL-1 production has been detected in many inflammatory disease states.[9, 201] In the eye, intravitreal injection of IL-1 produces an acute anterior uveitis in animal models [21, 22, 197] and release of IL-1 from implanted intracorneal pellets results in corneal neovascularization.[23] Corneal inflammation and neovascularization have been reported in transgenic mice in which high intraocular constitutive production of IL-1 was generated by using a crystalline gene promotor construct.[26, 202] Furthermore, the addition of IL-1 to cultured corneal cells augments the production of interleukin 8 and the expression of intracellular adhesion molecule-1 (ICAM-1), which could initiate the inflammatory cascade in the cornea.[31, 203]

Factors that inhibit the effect of IL-1 on various target cells may play an important role in modulating corneal inflammation. The IL-1 receptor antagonist is a recently characterized molecule that acts as a pure antagonist of biologically active IL-1.[71, 75] IL-1ra blocks cellular responses to IL-1 by binding almost irreversibly to the IL-1 receptor without triggering signal transduction.[76, 204]

Alternatively spliced products of the IL-lra gene result in three forms of IL-1ra, designated secreted IL-lra (sIL-lra) and intracellular IL-lra (icIL-lra), which are produced differentially in various cell types and may have distinct biologic roles in modulating IL-l-induced responses.[72] For example monocytes, macrophages, neutrophils, and hepatocytes have been reported to secrete sIL-lra, whereas icIL-lra is produced by fibroblasts, macrophages, and epithelial cells.[72] In addition, a second smaller form of icIL-lra (16 kD) has been described recently which appears to have a similar function to that of the larger, 18-kD, form of icIL-lra.[121, 205] As an antagonist of IL-1, IL-lra has been demonstrated to inhibit IL-1 inducible events both *in vitro* and *in vivo*.[80, 206-210] We have demonstrated that the administration of IL-lra effectively blocks the onset of IL-l-induced uveitis in a rabbit model.[211] However, little is known regarding the production and regulation of IL-1ra in the eye. In this investigation, we examine the production of sIL-lra and icIL-lra in human corneal cells and whole cornea. The production of IL-lra by the cornea may have important consequences for modulating IL-1-mediated inflammation and tissue repair in the anterior segment of the eye.

Results

Expression of IL-lra mRNA in human corneal epithelial and stromal cells. IL-lra mRNA expression was measured in cultured human corneal epithelial cells and corneal stromal fibroblasts by Northern blot analysis (Fig. 2.1).

As indicated in this figure, human corneal epithelial cells constitutively express IL-lra mRNA (1.8 kb), while only low levels of IL-lra mRNA are detected in unstimulated human corneal stromal fibroblasts. After the addition of recombinant human IL-lα (20 U/ml) for 6 hours to the cultured epithelial and stromal cells, there are significant levels of IL-lra mRNA detected in both corneal epithelial cells and corneal stromal cells. Equivalent loading is confirmed by hybridization to the ^{32}P-labeled cDNA probe for the cyclophilin gene. It should be noted that high levels of IL-lra mRNA are detected in the IL-l-treated human corneal epithelial cells in spite of relatively less mRNA loaded in this lane. Thus, both corneal stromal cells and epithelial cells are capable of expressing IL-1ra mRNA.

Production of IL-lra protein by human corneal epithelial and stromal cells. The production of IL-lra peptide by corneal epithelial cells and stromal fibroblasts was determined by immunohistochemistry (Fig. 2.2). In this representative figure, monolayers of both corneal epithelial and stromal cells stain positively for IL-lra using a primary anti-human IL-lra antibody (Fig. 2.2, A and C). The specificity of this result is confirmed by the absence of staining when pre-immune IgG is used as the primary antibody (Fig. 2.2, B and D). These control slides were stained with methylene blue for visualization of cellular morphology. Thus, both human corneal epithelial and stromal cells produce

IL-lra protein *in vitro*.

Immunolocalization of IL-l ra in whole human cornea. The production of corneal IL-lra protein was then immunolocalized in freshly isolated whole human cornea (Fig. 2.3). Immunohistochemical staining of this tissue demonstrates prominent staining for IL-lra in the epithelial layers (EP) of the cornea (Fig. 2.3 A) especially in the more differentiated suprabasal cells. Likewise, there is definite staining of individual stromal cells (S) in the corneal stroma indicating that these cells also produce IL-lra (Fig. 2.3 A, arrow). In contrast, no IL-lra is detected in the endothelial layer (EN) of the cornea (Fig. 2.3 B). Staining specificity is confirmed by the use of preimmune mouse IgG as a control antibody (Fig. 2.3 C). These studies thus localize IL-lra production to both corneal epithelium and stroma but not to the corneal endothelial layer.

Differential production of icIL-lra and sIL-lra mRNA by human corneal epithelial and stromal cells. Two forms of IL-lra have been identified: icIL-lra and sIL-lra. Using specific-IL-lra oligonucleotide primers, the expression of both icIL-lra and sIL-lra mRNA was measured in corneal cells. These two forms of IL-lra are differentially produced by various cell types and may have distinct intracellular and extracellular biologic roles in mediating IL-1 inflammatory responses. After RT-PCR amplification, IL-lra mRNA is detected in both corneal epithelial cells and stromal fibroblasts (Fig. 2.4). Both corneal

cell types express the 266-bp PCR product for icIL-lra (Fig. 2.4, lanes 3-6). Unexpectedly, corneal epithelial cells also express sIL-lra mRNA (201 bp product) both constitutively and after the addition of IL-1α to the cultured cells (Fig. 2.4, lanes 3 and 4). To our knowledge, this is the first example of a true epithelial cell expressing the secreted form of IL-lra. In contrast, the stromal fibroblasts do not express sIL-lra mRNA (Fig. 4, lanes 5 and 6). Neither icIL-lra mRNA nor sIL-lra mRNA is detected by RT-PCR in the corneal endothelial cells (data not shown). This is in agreement with the immunohistochemistry studies which failed to demonstrate staining of the endothelial layer with an IL-lra antibody. Synovial fibroblast mRNA derived from a patient with rheumatoid arthritis serves as a positive control for icIL-lra and sIL-lra (Fig. 2.4, lane 1) and human dermal microvascular endothelial cell mRNA serves as a negative control (no IL-lra expression) for these studies (Fig. 2.4, lane 2). Since these RT-PCR studies are not quantitative, no conclusion can be drawn regarding the lack of induction of IL-lra by IL-1 in this figure (Fig. 2.4, lanes 3 and 5) compared with the Northern blot in Figure 2.1. Thus, these studies demonstrate the differential expression of icIL-lra and sIL-lra mRNA in corneal epithelial and stromal cells as well as the unique production of sIL-lra mRNA by corneal epithelial cells

Differential production of IL-l ra protein by human corneal epithelial and stromal cells. IL-lra in corneal cell lysates and supernatants was also

examined by ELISA in triplicate using cells from three different corneas. Significant amounts of IL-lra are detected in supernatants and cell lysates from unstimulated epithelial cells which increased following the addition of IL-lα (20 U/ml) to the cultured cells (Fig. 2.5). Likewise, cell lysates from corneal stromal fibroblasts (unstimulated and stimulated with IL-lα) contain quantities of IL-lra similar to those detected in the epithelial cell lysates (Fig. 2.5). In contrast to corneal epithelial cells, no IL-lra is detected in stromal cell supernatants. Thus these studies are consistent with our previous PCR studies in Figure 2.4 which indicate that human corneal epithelial cells are capable of producing both icIL-lra and sIL-lra whereas stromal cells apparently produce only icIL-lra.

The production of IL-lra peptides in corneal epithelial and stromal cells was further examined by cellular metabolic labeling studies with ^{35}S followed by immunoprecipitation with an IL- lra specific antisera. These studies indicate that both corneal epithelial cells and stromal cells produce both a 16- and 18-kD form of IL-lra (Fig. 2.6, lanes l and 2) which is consistent with previously reported sizes of icIL-lra(l) and icIL-lra(2).[121, 205] Additionally, in the epithelial cells, a 22-kD band is detected which is identical to the previously reported[72] glycosylated secreted form of IL-lra (Fig. 2.6, lane l). As a positive control, immunoprecipitation studies were carried out with the U937 monocytic cell line which has been reported to produce both the intracellular and secreted forms of

IL-lra.[167] As indicated, bands of 16, 18, and 22 kD are clearly produced by this cell line (Fig. 2.6, lane 3). As a negative control, the G361 human melanoma cell line which does not produce IL-lra was utilized (Fig. 2.6, lane 4). These studies further support the unique observation that corneal epithelial cells are capable of producing both secreted and intracellular IL-lra (type 1 and type 2) whereas stromal cells apparently produce only icIL-lra (type 1 and type 2).

Discussion

IL-1 is a potent immunomodulating cytokine with pleiotrophic effects on a variety of tissues.[9, 201] The role of IL-lα and IL-1β as well as other inflammatory cytokines in mediating inflammation and tissue damage in the eye has been evaluated with *in vivo* models of ocular disease.[21-23, 26, 197, 202] Studies of isolated corneal cells also support the role of IL- 1 as a potential mediator of inflammation.[27, 31, 101, 203, 212] The relevance of IL-1 in corneal inflammation is further supported by observations that corneal cells themselves can produce this cytokine in response to various stimuli.[109, 110, 188, 189, 192, 200, 213]

In this report, we have demonstrated for the first time that IL-lra mRNA and protein are produced in human corneal cells and whole human cornea. As determined by Northern blot analysis, human corneal epithelial cells constitutively produce IL-lra with increased production after the addition of IL-lα whereas IL-lra mRNA is expressed in corneal stromal fibroblasts at significant levels only

after IL-1α induction. IL-lra protein production is likewise demonstrated in both cultured epithelial and stromal cells by immunohistochemistry. Similarly, immunohistochemical analysis of whole human cornea reveals prominent staining for IL-lra in the epithelial layer and staining of individual stromal cells within the stromal layer of the cornea. The epithelial layer stained more intensely in the more differentiated suprabasal layer which is consistent with previous reports in which increased IL-lra was detected in more differentiated epidermal cells.[121] The ability of corneal epithelial and stromal cells to produce IL-lra is consistent with the reported production of IL-lra by epithelial cells and fibroblasts from other tissues.[78, 121, 214-216] The relatively large amounts of IL-lra produced by corneal epithelial cells may indicate that this cytokine antagonist plays an important role in IL-1-induced inflammatory responses in the cornea. In contrast, the endothelial layer demonstrates no immunostaining for IL-lra or IL-lra mRNA which is consistent with prior observations that endothelial cells from other tissues produce no IL- lra.[214]

Corneal epithelial and stromal cells were further evaluated to determine which type of IL-lra peptide is produced by these cells. The form designated sIL-lra is a glycosylated 22-26-kD peptide that was first identified as a product of monocytes and later of neutrophils.[5, 214, 217] Low levels of sIL-lra have also been reported in dermal and synovial fibroblasts by some investigators while other

studies demonstrate no sIL-lra production by these cell types.[121, 214-216] A second form of IL- lra designated intracellular IL- lra (icIL- lra) is a nonglycosylated 18-kD molecule that resides in a cell-associated compartment.[214] More recently, a smaller 16-kD icIL-lra(2) peptide has also been described.[121, 205] This intracellular peptide appears to function in a similar fashion as the larger 18kD icIL-lra(1).[121, 205] Dermal and synovial fibroblasts have been reported to produce significantly more icIL-lra than sIL-lra suggesting that icIL-lra is the major form of this protein synthesized by these cells.[215, 216] Other studies were unable to detect icIL-lra in endometrial or dermal fibroblasts.[121, 214] Epithelial cells such as keratinocytes have also been reported to produce icIL-lra.[121, 214] Additional epithelial cells found to produce icIL-lra include retinal pigment epithelial cells, nasal epithelium, and bronchial epithelium.[214] In contrast, sIL-lra has not been previously reported to be produced by any epithelial cell type.

The differential production of sIL-lra and icIL-lra in corneal epithelial and stromal cells reported in our study suggests that corneal cells exhibit a unique pattern of IL-lra cell distribution. We found that human corneal epithelial cells produce both icIL-lra and sIL-lra, based on ELISA, RT-PCR, and immunoprecipitation. In contrast, human corneal stromal fibroblasts produce only icIL-lra and no sIL-lra. Additionally, both corneal epithelial and stromal cells produce the recently described 16-kD icIL-lra (2). The icIL-lra peptides may act

either extracellularly when released from injured cells or may act intracellularly by binding to IL-1 receptors prior to external expression.[121] It was unexpected to find that corneal epithelial cells are capable of producing sIL-lra. The relevance of this novel observation for corneal biology is unclear but the production of the secreted form of this peptide, in contrast to icIL-lra, has the advantage of allowing this peptide to be available (secreted) and induced without requiring cell damage or death. This could be particularly important for the integrity and function of the cornea where inflammation, tissue damage, and scarring can result in significant loss of visual acuity. This unique situation contrasts with the epidermis in which keratinocytes are capable of producing icIL-lra but not sIL-lra.[121]

Hammerberg et al.[218] demonstrated that icIL-lra proteins are detected in the cytosol of normal epidermis and that this was significantly increased in psoriatic epidermis. Although in this report PCR studies detected transcripts of both icIL-lra and sIL-lra mRNA in the epidermis, it was indicated that this was likely due to the inclusion of cells such as Langerhans' cells in the epidermal biopsies. No detectable sIL- lra peptide was found in either normal or psoriatic epidermis by these investigators. Thus our study indicates that the human cornea uniquely produces IL-lra. Further evaluation of the biologic behavior of corneal IL-lra could lead to novel strategies to modulate inflammatory reactions in the cornea.

Acknowledgments

This work was supported by National Institutes of Health grant ROlEY09218 and National Eye Institute Training grant T32 EY07123. Dr. Rosenbaum is a Senior Scholar supported by Research to Prevent Blindness, New York City.

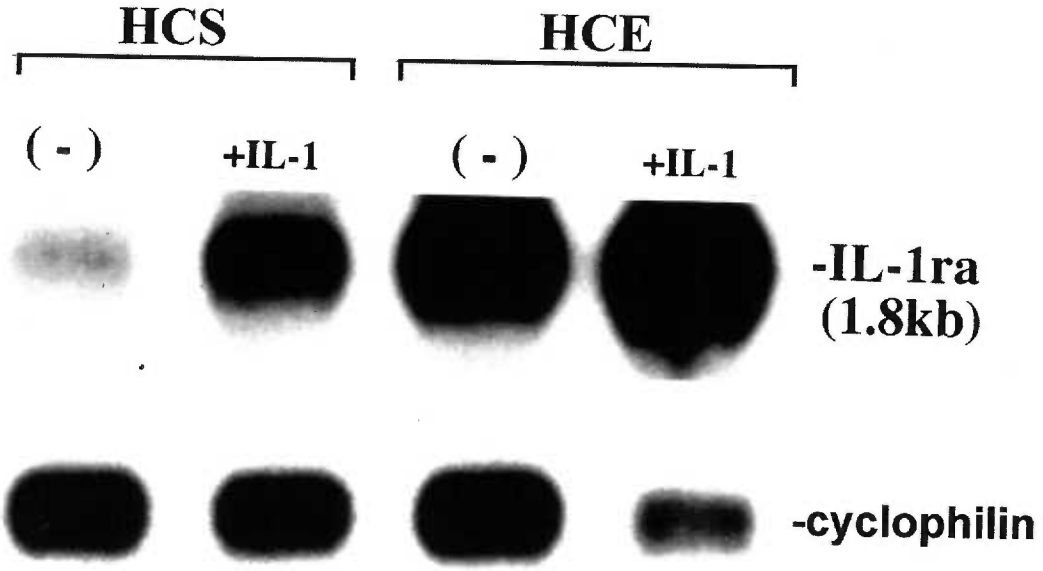

Figure 2.1 IL- lra mRNA expression by human corneal epithelial cells and human corneal stromal fibroblasts. The expression of IL-lra mRNA was determined in human corneal epithelial cells (HCE) and human corneal stromal fibroblasts (HCS) by Northern blot analysis using a ^{32}P-labeled cDNA probe specific for human IL-lra. Each lane contains 5 µg poly (A) + RNA from either unstimulated cells (-) or cells stimulated with IL-lα (20 U/ml) for 6 hr. Equivalent loading of RNA was verified by hybridizing each blot with a ^{32}P-labeled cDNA probe for cyclophilin. Production of ^{32}P-labeled probes and hybridization by J. Brown

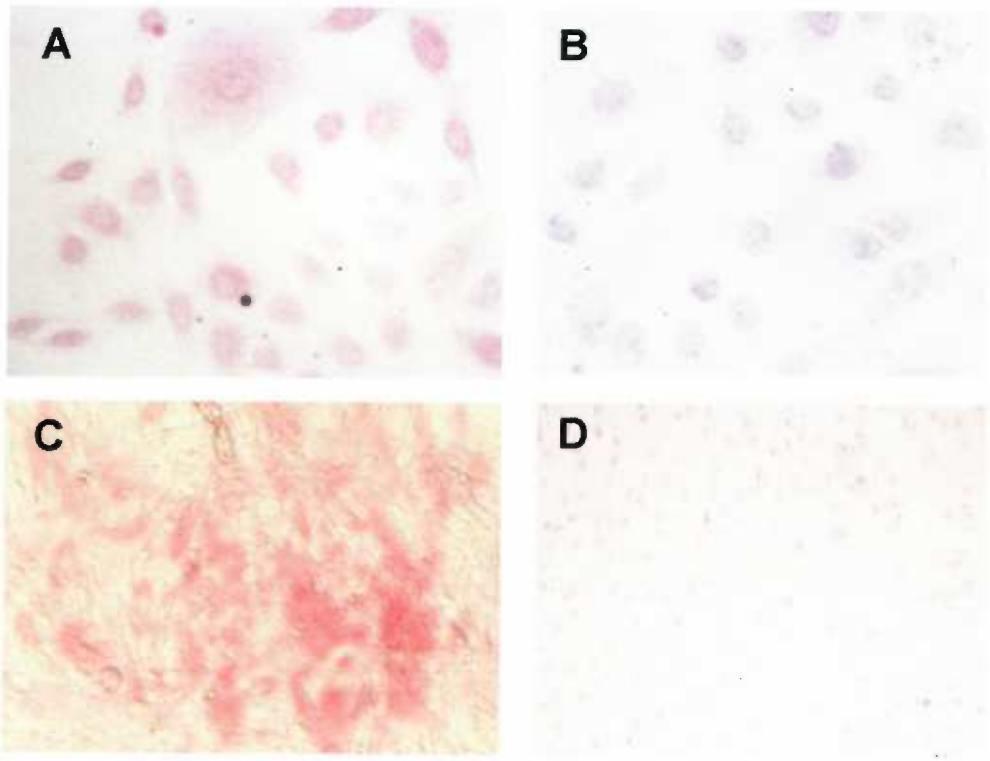

Figure 2.2 Immunodetection of IL-1ra in cultured human corneal epithelial cells and corneal stromal fibroblasts. Monolayers of human corneal epithelial cells (**A**) and human corneal stromal fibroblasts (**C**) were stained with a monoclonal murine anti-IL-1ra antibody followed by secondary alkaline phosphatase conjugated goat anti-mouse IgG. Negative controls included human corneal epithelial cells (**B**) and corneal stromal fibroblasts (**D**) stained with preimmune mouse IgG as the primary antibody followed by secondary alkaline phosphatase conjugated goat anti-mouse IgG and then counterstained with methylene blue to allow the cellular morphology to be visualized.

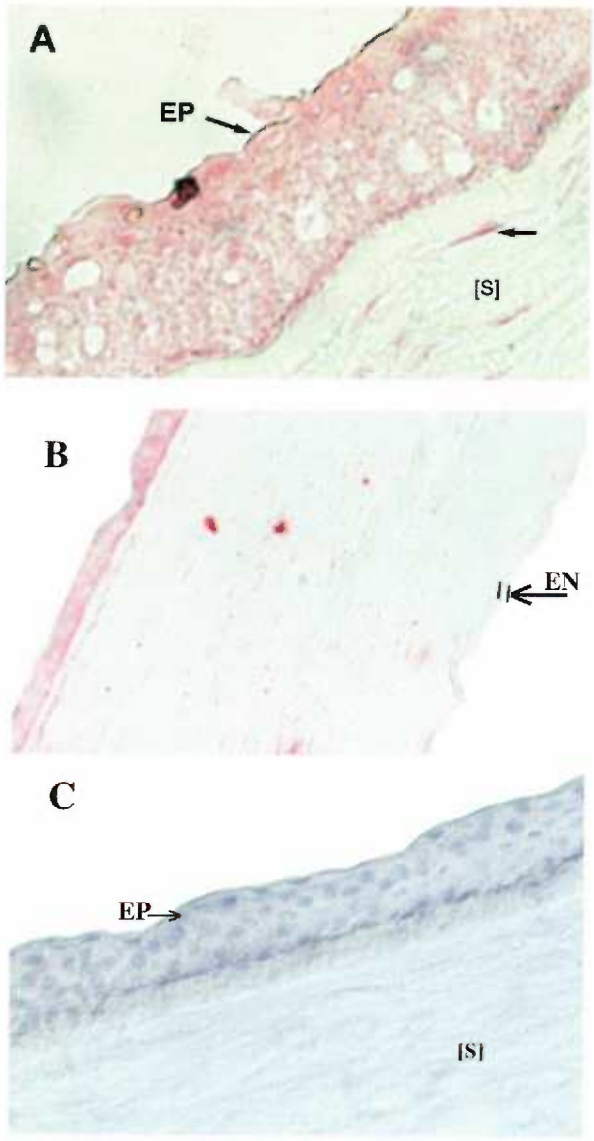

Figure 2.3 Immunolocalization of IL-1ra in whole human cornea. Immunohistochemistry was performed on human cornea sections using a murine monoclonal anti-IL-1ra antibody followed by alkaline phosphatase conjugated goat anti-mouse IgG as the secondary antibody. The epithelial (EP) and stromal (S) layers are shown in **(A)** (x400) with individual stromal cells indicated by an arrow. The endothelial (EN) layer is indicated by the arrow in **(B)** (x200). Staining specificity was evaluated by the use of preimmune mouse IgG as a control antibody in **(C)** (x400).

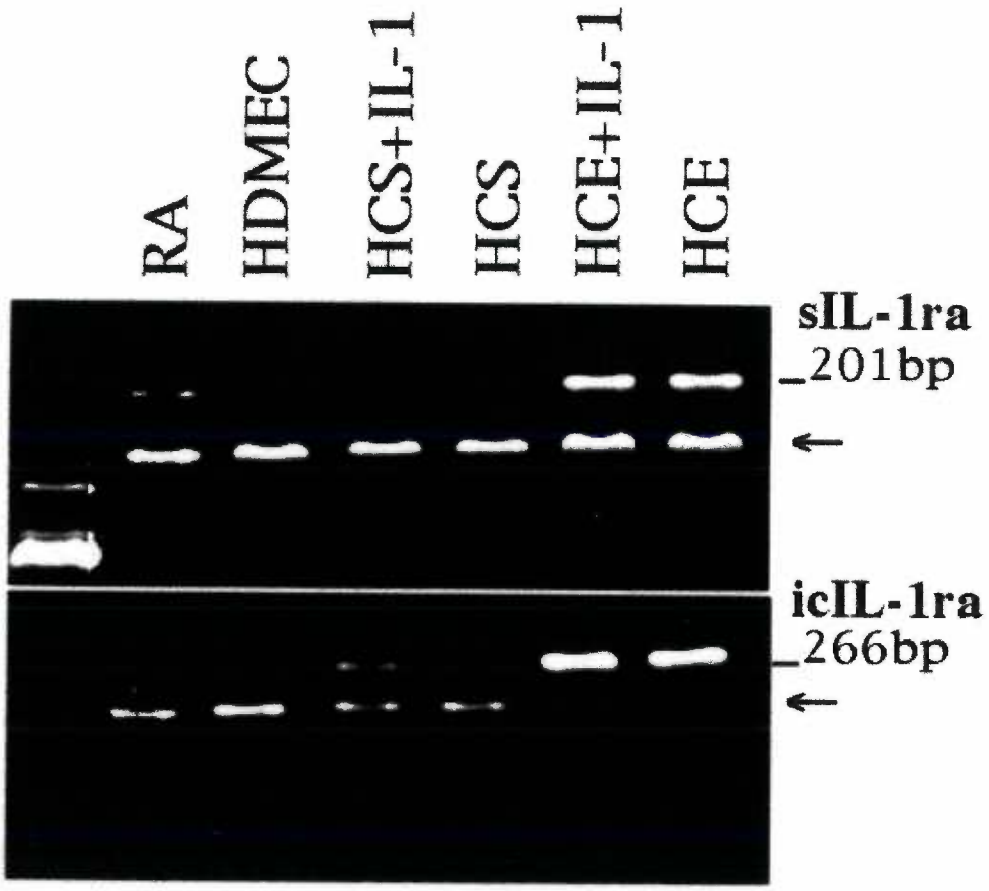

Figure 2.4 Differential expression of icIL-lra mRNA and sIL-lra mRNA by human corneal epithelial cells and corneal stromal fibroblasts. Reverse transcribed poly (A)+ mRNA from human corneal epithelial cells (HCE) and human corneal stromal fibroblasts (HCS) was amplified by PCR using primers specific for icIL-lra and sIL-lra. IL-lra mRNA was measured in both uninduced cells and cells treated with IL-1α (20 U/ml) for 6 hr. Rheumatoid arthritis synovial fibroblast mRNA (RA) served as a positive control and human microvascular endothelial cell mRNA (HDMEC) served as a negative control for both icIL-lra (266 bp) and sIL-lra (201 bp). Cyclophilin gene specific primers (arrows) were used as loading and amplification controls. RT-PCR done by X.Huang, primers designed by S. R. Planck, PhD.

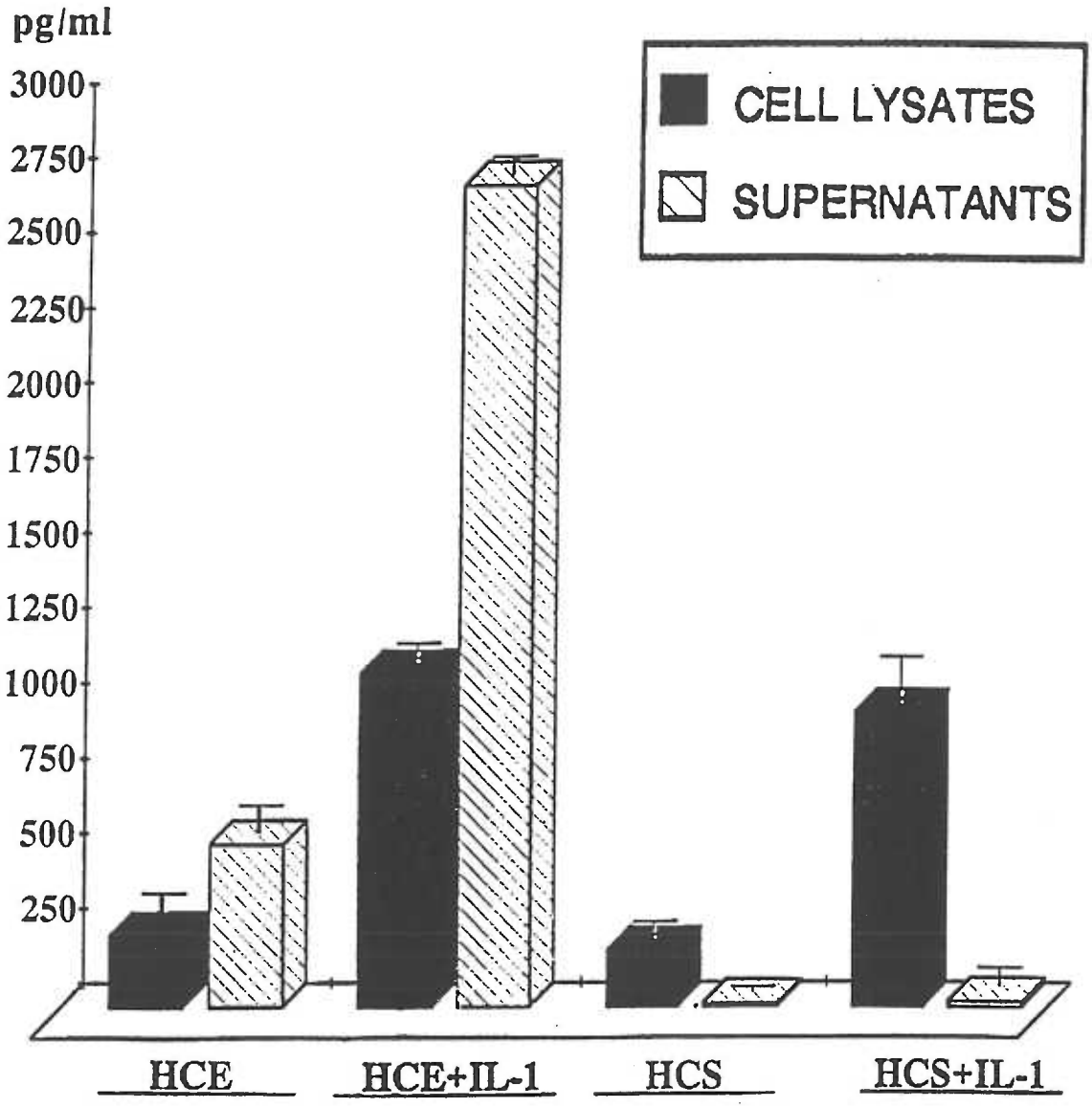

Figure 2.5 Differential production of IL-lra peptide by human corneal epithelial and stromal cells. IL-lra production in human corneal epithelial cells (HCE) and corneal stromal fibroblasts (HCS) was evaluated in unstimulated cultured cells or cells pretreated with IL-lα (20 U/ml) for 48 hr. Both cell associated IL-lra (lysates) and secreted IL-lra in culture supernatants were measured by ELISA with SD indicated by error bars.

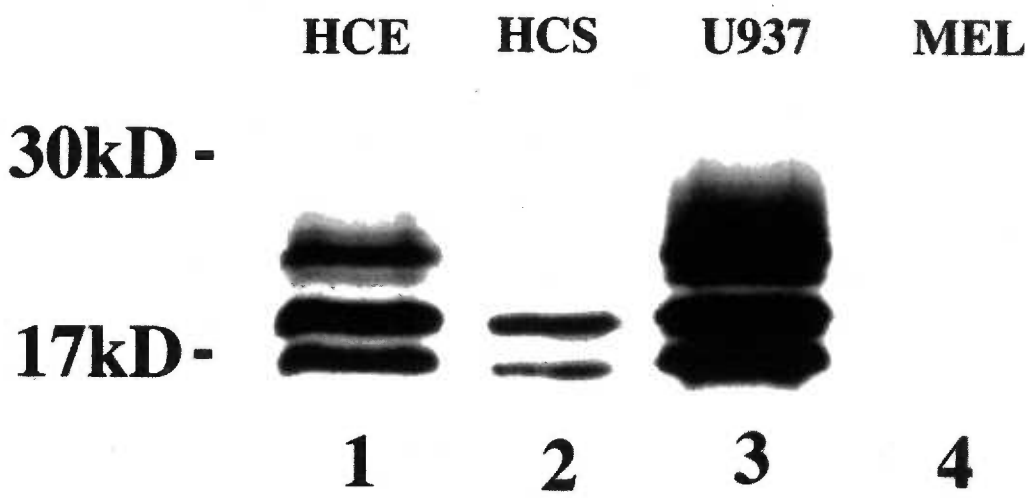

Figure 2.6 Identification of IL-lra peptide produced by corneal epithelial cells and corneal stromal fibroblasts. ^{35}S-labeled corneal epithelial cells (HCE) and corneal stromal fibroblasts (HCS) were immunoprecipitated using a rabbit polyclonal anti-IL-lra antibody. The U937 monocytic cell line served as a positive control (U937) and the G361 human melanoma cell line (MEL) served as a negative control for the expression of the IL-lra peptides.

CHAPTER 3

A model of photokeratitis in an IL-1ra mouse.

Introduction

The causative relationship between corneal UV exposure and photokeratitis has been well established. A recognized characteristic of this disease is the delayed onset of patho-physiologic changes, after either clinical or experimental UV exposure. Data presented previously in this dissertation have established that the cornea responds to UV radiation by the production of inflammatory cytokines, particularly interleukin-1 (IL-1). The increase in corneal IL-1 preceding the onset of keratitis is compatible with the hypothesis that IL-1 plays a role in the inflammatory aspects of this disease process. I further hypothesized that inhibiting IL-1 activities might reduce the inflammatory responses of the cornea to UV irradiation. I tested this hypothesis by inducing UVB photokeratitis in transgenic mice that over-express the endogenous IL-1 inhibitor, IL-1ra.

IL-1 has been shown to increase the production of chemotactic cytokines (chemokines) in a variety of cell types and tissues. Data presented previously in this dissertation show that UV exposure of human cornea resulted in the production of IL-8. The time course of IL-8 mRNA up-regulation suggested that this chemokine was a secondary response to UV-induced IL-1 production. IL-1 up-regulation of IL-8 production in human cornea and human corneal cells has also been found by other investigators.[31] The release of this chemokine has been linked to leukocyte recruitment and neovascularization in the cornea. [183] While a

murine homologue of IL-8 has not been found, some investigators propose that three murine chemokines; KC, macrophage inflammatory protein-2 (MIP-2), and granulocyte chemotatic protein-2 (GCP-2), fill similar roles in the murine immune system. A murine homologue of one of the human IL-8 receptors, CXC chemokine receptor 2 (CXCR2) has been found, and mice with deletions in this receptor show deficiencies in neutrophil recruitment in some models of inflammation.[219] Infection of the cornea with Herpes simplex virus is followed by recruitment of neutrophils into the corneal stroma, [151] and this virus is known to up-regulate IL-1 production in the cornea soon after infection.[213] In a study of bacterial keratitis, the numbers of neutrophils infiltrating the cornea following Pseudomonas infection correlated with the levels of corneal IL-1.[220] The corneal edema and transient corneal inflammation seen in photokeratitis could also be due to the release of interleukin-1 in the corneal stroma. Exposure of corneal cells to IL-1 triggers the expression of intracellular adhesion molecule-1 (ICAM-1), an adhesion molecule that interacts with surface receptors on immune cells, stimulating the inflammatory process.[221] IL-1 also upregulates ICAM-1, and other adhesion molecules, on peripheral corneal blood vessels resulting in increased vessel adhesiveness for circulating leukocytes. This adhesiveness facilitates leukocyte attachment to vessel walls and diapedesis.[203] Factors released from leukocytes infiltrating into the corneal stroma can contribute to corneal edema.[152]

Treatment with exogenous IL-1ra has been shown to reduce inflammation and tissue damage in experimental models of brain ischemia[222], immune complex-induced lung injury[223] and reactive arthritis.[224] Other investigators have found that addition of exogenous IL-1ra can reduce neovascularization and neutrophil recruitment into the anterior segment of the eye.[86, 225] Studies using over-expression of IL-1ra have shown reduction of tissue damage and inflammation in several different experimental disease models.[83, 84, 226, 227]

Because IL-1ra has been shown to reduce inflammation in the eye and other tissues, I examined whether increasing IL-1ra could reduce the corneal inflammation that follows UV irradiation. In this study the corneas of transgenic mice that over-express IL-1ra and nontransgenic littermates were given a single dose of UVB irradiation, and the inflammatory response was measured histologically. The model of photokeratitis used in these studies is one of a limited inflammatory insult, showing only low levels of neutrophil infiltration. UV radiation damage is restricted to the epithelium and anterior stromal layers of the central cornea. The vascularized peripheral cornea and conjunctival tissues were shielded from the UV radiation to limit the initial production of inflammatory mediators to the cells of the central cornea. The local production of inflammatory cytokines in this model allows examination of how paracrine signaling affects leukocyte recruitment in the cornea.

Results

Effect of IL-1ra transgene on UVB-induced corneal stromal inflammation.

Because IL-1ra has been shown to reduce leukocyte recruitment in other experimental disease models [83, 84, 226, 227], I chose to look at corneal neutrophil recruitment in transgenic mice that over express IL-1ra (Gift of D. Hirsh, Columbia University). These IL-1ra transgenic mice possess an additional six copies of the IL-1ra gene controlled by the endogenous promotor. When compared with normal littermates, the IL-1ra transgenic mice showed a 10-fold increase in serum IL-1ra levels in response to LPS challenge.[158] The transgene construct does not contain the alternative exons used in intracellular IL-1ra mRNA, so only secreted IL-1ra is produced. Transgene positive mice appear normal and can be distinguished from nontransgenic littermates only by genotyping.

IL-1ra transgenic and nontransgenic littermates were examined histologically to quantify the stromal inflammation following UVB exposure (50 mJ/cm^2). Representative sets of serial sections spaced every 100 μm across the cornea were histochemically stained to reveal neutrophils. The number of neutrophils infiltrating the corneal stroma was ascertained. The mean (± SD) of neutrophil numbers from the five most inflamed nonsequential sections was plotted as the measure of corneal inflammation (Fig. 3.1). Nontransgenic mice

showed a group mean of 24.8 neutrophils per section with a standard error of 2.32. The transgenic mice showed a group mean of 8.5 neutrophils per section with a standard error of 1.02. A substantial reduction in neutrophil numbers (65%) was seen in the UVB-irradiated corneas of the IL-1ra transgenic mice when compared with the irradiated corneas of nontransgenic littermates. Although the degree of inflammation was low, the difference between the transgenic and nontransgenic groups was statistically significant (t-test, $p=0.001$). The counts of all sections were conducted in a masked manner, prior to genotyping of the mice.

None of the UVB-exposed animals had any evidence of infiltration of neutrophils into the anterior chamber of the eye. This lack of neutrophils suggests that the more posterior layers of the cornea and structures posterior to the cornea (iris, ciliary body, and lens) had not sustained any significant damage from the UVB treatment.

To determine the kinetics of neutrophil recruitment into the cornea following UVB irradiation, groups of mice (4 transgenic and 4 nontransgenic, each timepoint) were sacrificed at twelve hour intervals from 12 to 72 hours after irradiation. The examination of these mice showed that the neutrophil infiltration occurred in stages. A characteristic buildup of neutrophils in the areas of the limbal vasculature occurred prior to their migrating out into the stroma and to the UVB-exposed central cornea. The influx of neutrophils coincided with the loss of

epithelial integrity and exposure of the stromal surface. The majority of neutrophils remained in the anterior half of the stroma near the epithelial layer, rather than spreading uniformly throughout the stroma. No evidence of neutrophils entering the stroma from the corneal surface was observed. No neutrophils were observed adhering to or migrating through the epithelial layer. However, any neutrophils present on the immediate surface of the eye may have been lost during processing. The initial inflammatory infiltrate consisted of neutrophils. No positive immunostaining for macrophages was seen in the stroma in any of the timepoints up to and including 72 hours.

The progress of epithelial loss and healing was monitored by the technique of fluorescein staining.[228] No differences in epithelial healing rates were seen between transgenic and nontransgenic mice. No major histologic differences were seen in the degree of epithelial healing at the 72 hour endpoint. All mice had fully re-epithelialized the wound with only a central ridge of hyper-proliferative epithelium remaining at 72 hours, and most of the wound had reestablished the basal cell/wing cell epithelial stratification.

Immunohistochemical staining for adhesion molecules and cytokines.

To determine if neutrophil numbers were reduced in transgenic corneas because of an absence of adhesion molecules, UVB-irradiated corneas were

examined for the presence of ICAM-1 and P-selectin. Immunohistochemical staining of frozen sections detected the presence of ICAM-1 and P-selectin at 24 and 48 hours post-UVB exposure (50 mJ/cm^2). Positive staining for ICAM-1 was seen in stromal fibroblasts and corneal endothelium, with the strongest staining in the peripheral cornea and limbus (Fig. 3.2, A and B). Positive staining for P-selectin was seen only in blood vessels of the limbus and conjunctiva (Fig. 3.2, C and D). No differences were seen in the levels of ICAM-1 or P-selectin staining between nontransgenic (Fig. 3.2 A and C) and transgenic corneas (Fig. 3.2 B and D). Non-specific staining was observed in the superficial layers of the corneal epithelium in all sections including preimmune IgG and secondary antibody-only controls. Additional staining with polyclonal antibodies against murine IL-1α, IL-1β, TNFα, and KC was attempted on both the paraffin and frozen sections. These antibodies either showed no specific staining or had such high background staining so as to be uninterpretable. Several different techniques of antigen recovery were attempted on the paraffin embedded materials (e.g., protease digestion and microwave heating) and these still failed to give specific staining. Additional irradiated eyes were tested after manual paraffin embedding with reduced temperatures and milder fixation procedures in an effort to preserve antigenicity but yielded no improvement in staining specificity.

RT-PCR analysis of cultured stromal fibroblasts.

In order to clarify which element of the inflammatory process might account for the reduction in neutrophil recruitment in the corneas of the transgenic mice, cultured corneal cells were examined for differences in the expression of mRNA for neutrophil-attracting chemokines. RT-PCR analysis of MCFB was done because it offered the ability to test homogenous cultures of a single cell type from transgenic and nontransgenic mice with identical stimulation.

Initial RT-PCR analysis of mRNA responses to stimulation of nontransgenic MCFB with UVB irradiation showed that the nontransgenic fibroblasts upregulate mRNA for the chemokines, KC and MIP-2, after UV exposure (Fig. 3.3, lane 6) with barely detectable messages for these chemokines in unstimulated controls (Fig. 3.3, lane 4). The transgenic fibroblasts do not upregulate mRNA for the chemokines KC and MIP-2 following UV exposure (Fig. 3.3, lane 3) and no detectable message for these chemokines is seen in the unstimulated controls (Fig. 3. 4, lane 1). Both transgenic and nontransgenic MCFB show strong levels of mRNA for both of these chemokines in response to high levels of IL-1 stimulation (Fig. 3.3, lanes 2 and 5). Both transgenic and nontransgenic MCFB also show constitutive expression of another chemokine mRNA, GCP-2 (Fig. 3.3, bottom row). This initial experiment was repeated with similar results.

The ability of exogenous IL-1ra treatment to inhibit UV-induction of chemokine mRNA was tested by treating UV irradiated nontransgenic MCFB with recombinant human IL-1ra. Human and murine IL-1ra have a high degree of homology (77%)[229] and studies have shown that human IL-1ra is effective in blocking IL-1 responses in murine cells. I found that pre-treating the nontransgenic cell line with 1 μg/ml of recombinant human IL-1ra for 2 hours prior to UVB exposure and for the four hours after exposure blocked the induction of KC mRNA as was detectable by RT-PCR (Fig. 3.4 lane 3). However, later RT-PCR experiments with a slightly different protocol on nontransgenic MCFB showed a different pattern of mRNA expression for the chemokines KC and MIP-2 (Fig. 3.5). The source of the variability is unclear, but it prevents making any conclusions regarding the KC and MIP-2 mRNA during UVB-response. However, the RT-PCR studies consistently showed increases in mRNA for KC, MIP-2, and GCP-2, following IL-1 stimulation (100 pg/ml) of MCFB cells.

Determination of KC levels by ELISA.

Because the RT-PCR data was inconclusive for increases in the level of KC mRNA following UVB irradiation, the question of how UVB exposure effects KC levels was addressed by measuring KC protein production. To test whether UVB irradiation stimulated increases of KC production in nontransgenic MCFB,

the levels of KC protein in tissue culture supernatants of UVB-irradiated and nonirradiated nontransgenic MCFB were determined by KC-specific ELISA. Also, the effect of exogenous IL-1ra treatment on KC production was tested in additional irradiated and nonirradiated MCFB cultures. UVB-irradiated (15 mJ/cm^2) cultures displayed a 6-fold increase in KC levels in their supernatants compared to nonirradiated controls (Fig. 3.6, columns 1 and 2). The addition of IL-1ra (1 μg/ml) blocked any UVB-stimulated increase in KC levels (Fig. 3.6, columns 1 and 3). The addition of high dose of IL-1α (100 pg/ml) gave almost a 19-fold increase in KC levels (Fig. 3.6, columns 1 and 4). This experiment was repeated and gave similar results. The finding that cultured murine corneal fibroblasts produce KC has not been previously reported, although there have been reports of the detection, by RT-PCR, of KC expression in whole cornea.[230]

To test whether IL-1ra was capable of suppressing KC production in normal MCFB established from mice of a different genetic background, I repeated the earlier experiments on corneal fibroblasts with a C57BL6/SV129 F1 background. The IL-1ra transgenic and nontransgenic mice have a CBA/C57BL6 background. As seen in the previous experiments, UVB-irradiated (15 mJ/cm^2) cultures displayed an increase in KC levels in their supernatants compared to nonirradiated controls (Fig. 3.7, columns 1 and 3), although the increase was lower (2.3-fold). KC levels in UVB-irradiated cultures treated with IL-1ra (1

μg/ml) again showed no increase in KC levels (Fig. 3.7, columns 1 and 4). These data show that exogenous IL-1ra is able to block UVB-induced KC production in corneal cells derived from different genetic backgrounds.

The viability of IL-1ra treated and untreated cultures was measured to test whether recombinant IL-1ra treatment had inhibited KC production through a cytotoxic effect. After culture supernatants were removed, the untreated control and IL-1ra treated control cells were stained with the vital dye, Trypan Blue. Viable and nonviable cells were counted for each plate in 10 random fields of an inverted tissue culture microscope at 100X. No decrease in viability (97%) or cell number was seen in the IL-1ra treated cultures compared to the untreated control (data not shown). Monolayers were lysed after supernatant collection and total protein or total DNA concentrations were compared to assure equal cell numbers between cultures.

Discussion

I have examined the effect of UV irradiation on the production of immune mediators in cells of the cornea because this environmental agent can lead to significant ocular inflammation and pathologic changes. A possible mechanism by which UVB can incite pathologic changes in the cornea is to increase levels of inflammatory cytokines and chemokines. Inflammation contributes to the

elimination or control of infectious agents. However, chronic or overzealous inflammatory responses can result in injury to surrounding healthy tissue.

The results presented here indicate that increased expression of IL-1ra can significantly decrease the level of corneal inflammation after UVB exposure. Transgenic mice over-expressing IL-1ra showed a significant decrease in the number of neutrophils recruited into the cornea following UVB irradiation.

That the reduction in neutrophil recruitment was due to the over-expression of IL-1ra was supported by several elements of this work. First, examination of cultured corneal fibroblasts showed increases in the level of KC production in response to UVB irradiation. Second, the treatment of cultured fibroblasts with exogenous IL-1ra blocked the UVB-induced up-regulation of KC production. Thirdly, all cultured stromal fibroblasts examined by RT-PCR showed increases in mRNA levels for the three chemokines, KC, MIP-2, and GCP-2, following IL-1 stimulation. The protein level of the neutrophil attracting chemokine, KC, was shown to increase in response to IL-1 stimulation. Measurement of the protein levels of the two other chemokines, MIP-2 and GCP-2, examined by RT-PCR await the availability of antibodies suitable for immunohistochemistry and ELISA.

The current experiments do not determine the source or level of increased corneal IL-1ra. Whether increased IL-1ra from the transgene is produced locally

by stromal fibroblasts, corneal epithelial cells, and/or results from increased IL-1ra in the serum or aqueous humor is unresolved. *In situ* hybridization and more quantitative mRNA assays could provide the necessary data to resolve these questions.

These data suggest a possible mechanism for the *in vivo* decreases in neutrophil infiltration in IL-1ra transgenic mice, that of IL-1ra reducing the level of a stromal chemoattractants produced in response to UV injury. The evidence from our earlier studies demonstrate the ability of UVB to induce IL-1 production in corneal cells. The current study shows that IL-1 can induce chemokine production. Inhibition of UVB-induced chemokine production by IL-1ra supports the hypothesis that IL-1 is a mediator of this UVB effect. The reduction of corneal chemokine levels may cause a decrease in neutrophil migration into the central cornea, and thereby, reduce inflammation. This mechanism is also supported by reports from other laboratories that treatment with IL-1ra can reduce corneal inflammation *in vivo* following transplantation or corneal suturing.[85, 225]

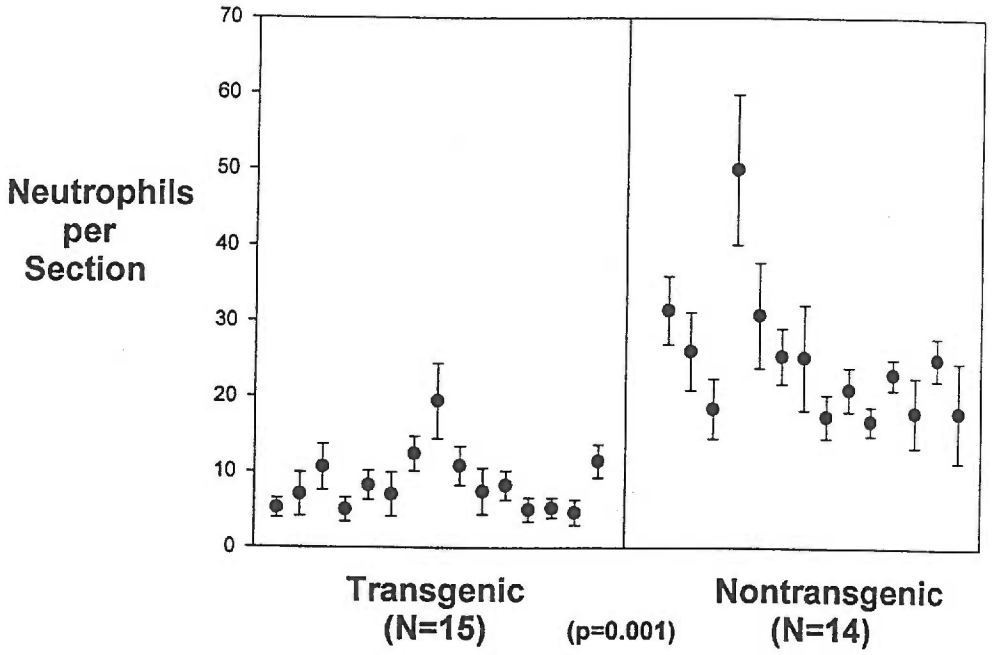

Figure 3.1 Quantitation of corneal neutrophil infiltration in UVB-irradiated IL-1ra transgenic and nontransgenic littermates. Serial 5 μm sections from paraffin-embedded UVB-irradiated (50 mJ/cm^2) eyes were stained for the presence of neutrophil esterase. Each point represents the mean (±standard deviation) of the number of positively stained cells in the corneal stroma from 5 sections from an individual mouse. Number of neutrophils in the stroma was used as a measure of corneal inflammation and differences between the two groups were statistically significant (t-test, p=0.001).

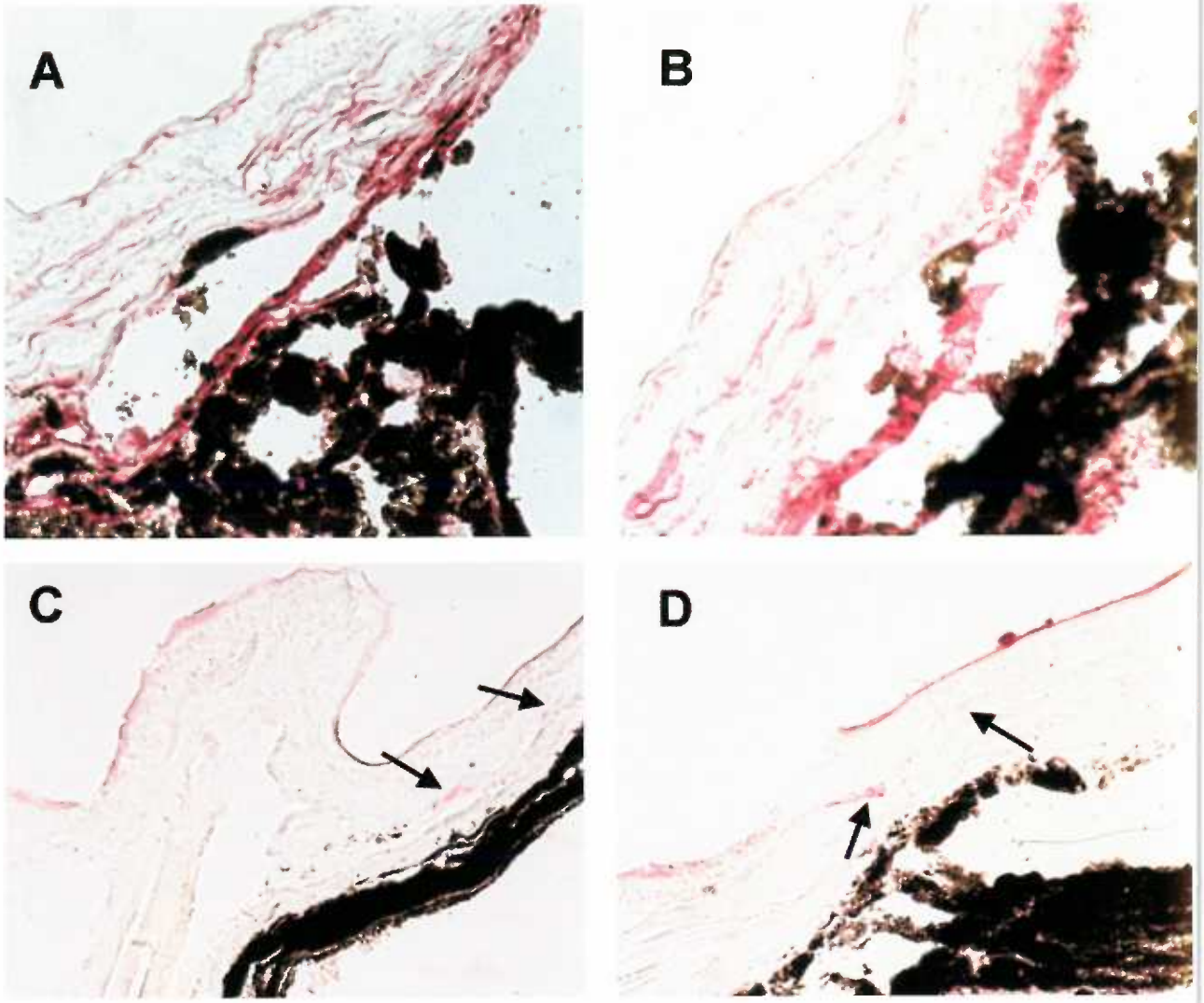

Figure 3.2 Immunolocalization of ICAM-1 and P-selectin. Immunohistochemistry was performed on frozen sections of UVB-irradiated nontransgenic and transgenic mouse corneas with rat monoclonal antibodies against mouse ICAM-1 and P-selectin. ICAM-1 was seen in the corneal stroma, endothelium, limbus, and ciliary body in both nontransgenic (A) and transgenic mice (B). P-selectin was seen only in the blood vessels (arrows) of the limbus and conjunctiva in both nontransgenic (C) and transgenic mice (D). All corneas shown were 24 hours post-irradiation, all photographs are 400X.

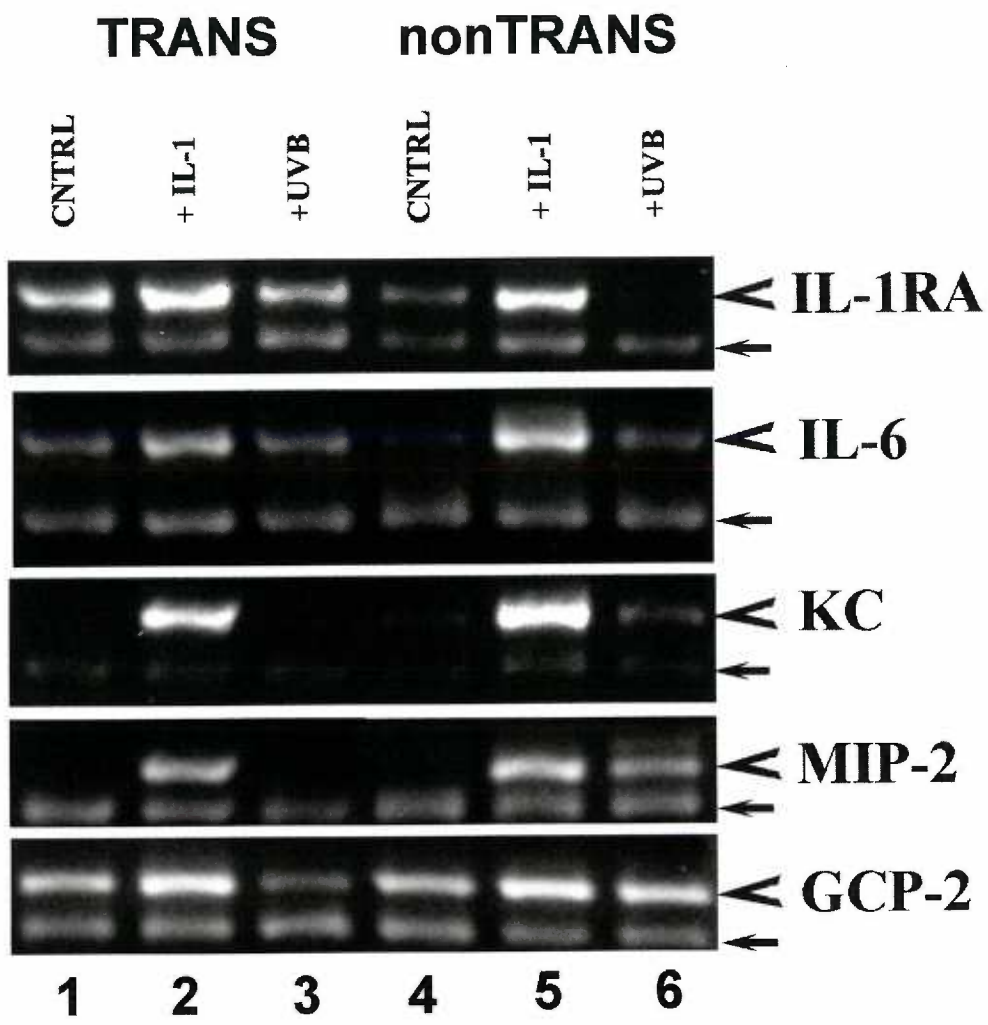

Figure 3.3 RT-PCR analysis of MCFB responses to UV irradiation. Total RNA from UVB-irradiated (15 mJ/cm^2) transgenic (first 3 lanes) and nontransgenic (last 3 lanes) MCFB was analyzed by RT-PCR for the presence of chemokine mRNA. Detectable mRNA for KC and MIP-2 was seen in nontransgenic MCFB after irradiation (4 hours) but absent in transgenic MCFB. No difference in the detection of another chemokine (GCP-2 mRNA) was seen. IL-1 stimulation (100 pg/ml) served as positive control for stimulation of chemokine mRNA. Large arrowheads point to bands for specified mRNA, GAPDH (small arrow) served as positive control for RT-PCR reaction.

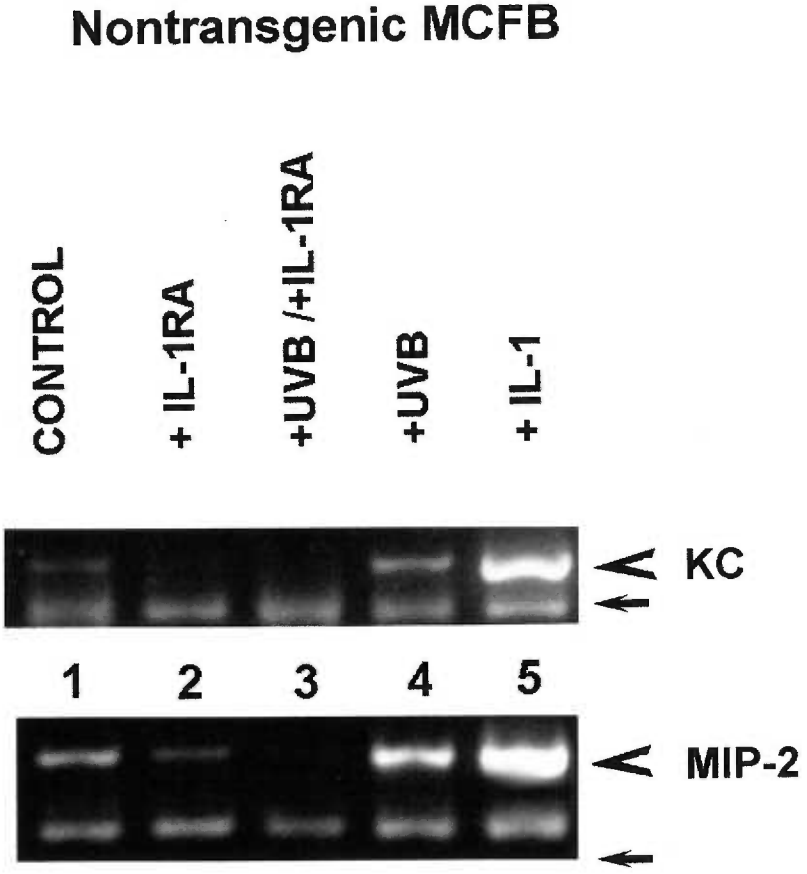

Figure 3.4 RT-PCR analysis of KC and MIP-2 mRNA in UVB-irradiated nontransgenic MCFB with and without IL-1ra treatment. Total RNA from UVB-irradiated (15 mJ/cm^2) nontransgenic MCFB was analyzed by RT-PCR for the presence of KC and MIP-2 mRNA. Detectable mRNA for KC was seen in IL-1ra untreated nontransgenic MCFB after irradiation (4 hours) but absent in MCFB treated with 1μg/ml of recombinant human IL-1ra. Detectable mRNA for MIP-2 was seen in IL-1ra untreated nontransgenic MCFB after irradiation (4 hours) but absent in MCFB treated with 1μg/ml of recombinant human IL-1ra. IL-1 stimulation (100 pg/ml) served as positive control for stimulation of KC mRNA. GAPDH served as positive control for RT-PCR reaction (small arrow).

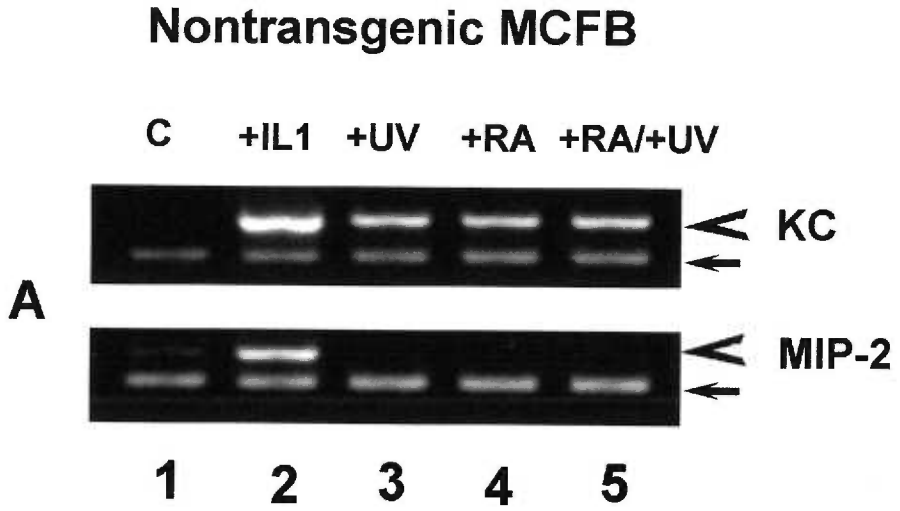

Figure 3.5 RT-PCR analysis of MCFB responses to UVB-irradiated, with and without IL-1ra treatment. Total RNA from UVB-irradiated (15 mJ/cm^2) nontransgenic MCFB was analyzed by RT-PCR for the presence of KC and MIP-2 mRNA. Detectable mRNA for KC was seen in all lanes except control. Detectable mRNA for MIP-2 was seen only in IL-1 treated postive control. GAPDH (small arrow) served as positive control for RT-PCR reaction. RT-PCR done by X. Huang.

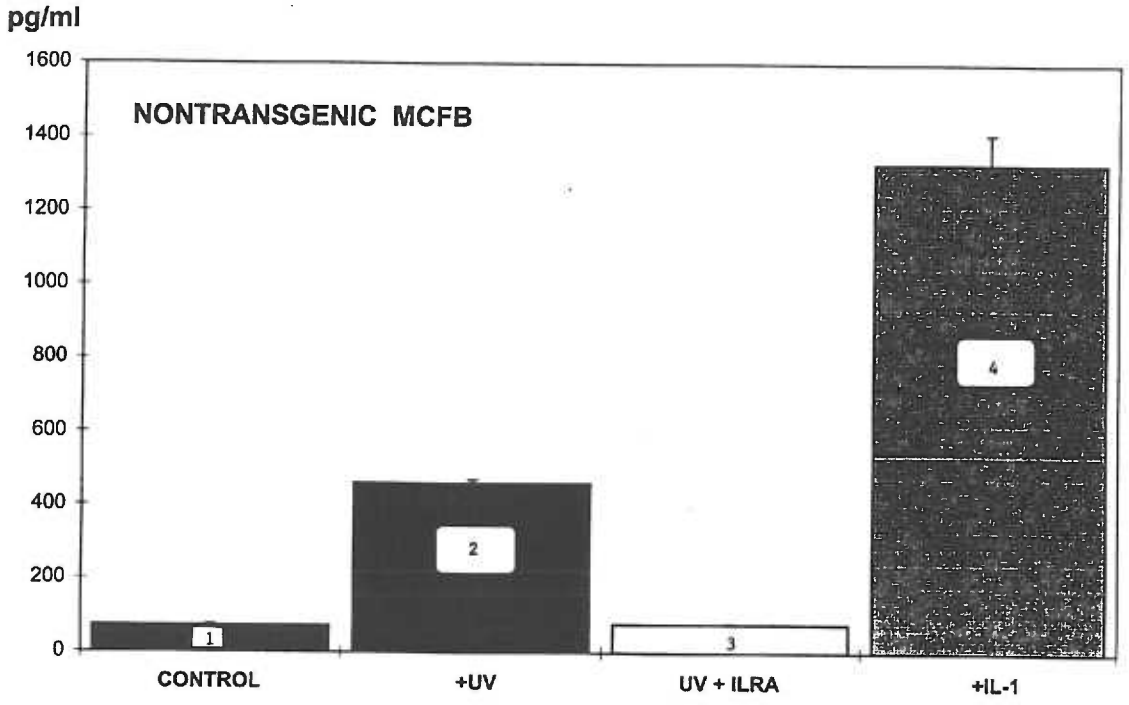

Figure 3.6 Determination of KC production in nontransgenic MCFB monolayers following UVB irradiation and IL-1ra treatment. Levels of KC protein in nontransgenic MCFB tissue culture supernatants were determined by ELISA. UVB irradiation of nontransgenic MCFB monolayers increased secreted KC levels in 24 hour tissue culture supernatants by 6-fold over KC protein levels in nonirradiated control cultures. Treatment of monolayers with 1μg/ml of recombinant human IL-1ra immediately after UVB irradiation blocked any increase in the levels of KC secretion. Data represents triplicate samples, error bars = standard deviation.

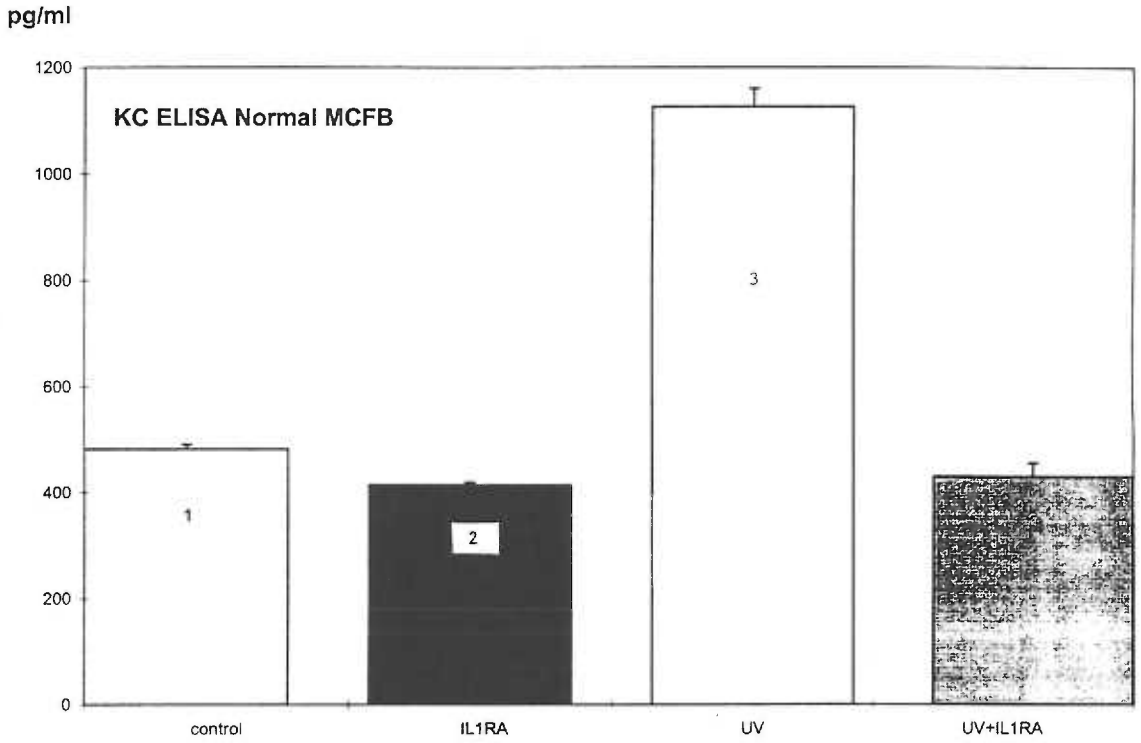

Figure 3.7 Determination of KC production in normal MCFB monolayers following UVB irradiation and IL-1ra treatment. Determination of levels of KC production in normal C57Bl6/Sv129 MCFB tissue culture supernatants was determined by ELISA. UVB irradiation of normal MCFB monolayers increased secreted KC levels in 24 hour tissue culture supernatants by 2.3-fold over nonirradiated controls. Treatment of monolayers with 1 μg/ml of recombinant human IL-1ra immediately after UVB irradiation blocked any increase in the levels of KC secretion. Data represents triplicate samples, error bars = standard deviation.

A model of UVB Photokeratitis

In this model of corneal inflammation paracrine signaling between the different corneal cell types plays a significant role in the recruitment of neutrophils into the corneal stroma. The interactions between the various cells occur during different phases of the corneal response to UVB injury. The normal uninjured cornea has an intact stratified epithelium and a network of interconnected stromal fibroblasts (Diagram 2A).

The initial response of stromal fibroblasts to UVB exposure is the breaking of the intercellular connections between neighboring cells. One result of the loss of intercellular contacts is an increased expression of ICAM-1 on stromal fibroblasts (Diagram 2B). Increases in the rate of epithelial cell shedding deplete the corneal surface of superficial epithelial cells. The exposed epithelial wing cells release IL-1 into the tears stimulating an inflammatory response in the peripheral corneal tissues. Higher levels of adhesion molecules on the surface of endothelial cells in the peripheral cornea allow circulating neutrophils to adhere to the vessel walls and begin extravasating into the limbal and conjunctival tissues.

The apoptotic death of epithelial cells causes the release of large amounts of IL-1 and the loss of epithelial integrity (Diagram 2C). Without the epithelial barrier, released IL-1 can readily diffuse into the corneal stroma. Stromal fibroblasts in the injury area respond to the IL-1 by upregulating the production of

chemokines such as KC and MIP-2. The presence of the chemokine gradient stimulates neutrophil migration from the peripheral cornea into the corneal stroma.

In the IL-1ra transgenic cornea (Diagram 2D), the increased levels of stromal IL-1ra make the stromal fibroblasts less responsive to the IL-1 diffusing into the stroma from the injured epithelial cells. This reduces the level of chemokine production by transgenic stromal fibroblasts weakening the chemokine gradient in the corneal stroma. Because stimulation by chemokines is a major impetus to neutrophil migration and the primary cue guiding migrating neutrophils, fewer neutrophils leave the vascular peripheral cornea and migrate into the stroma toward the injured central cornea.

The ability of increased IL-1ra to reduce corneal inflammation suggests the biologic function of corneal IL-1ra is to attenuate IL-1 responsiveness in the cornea.

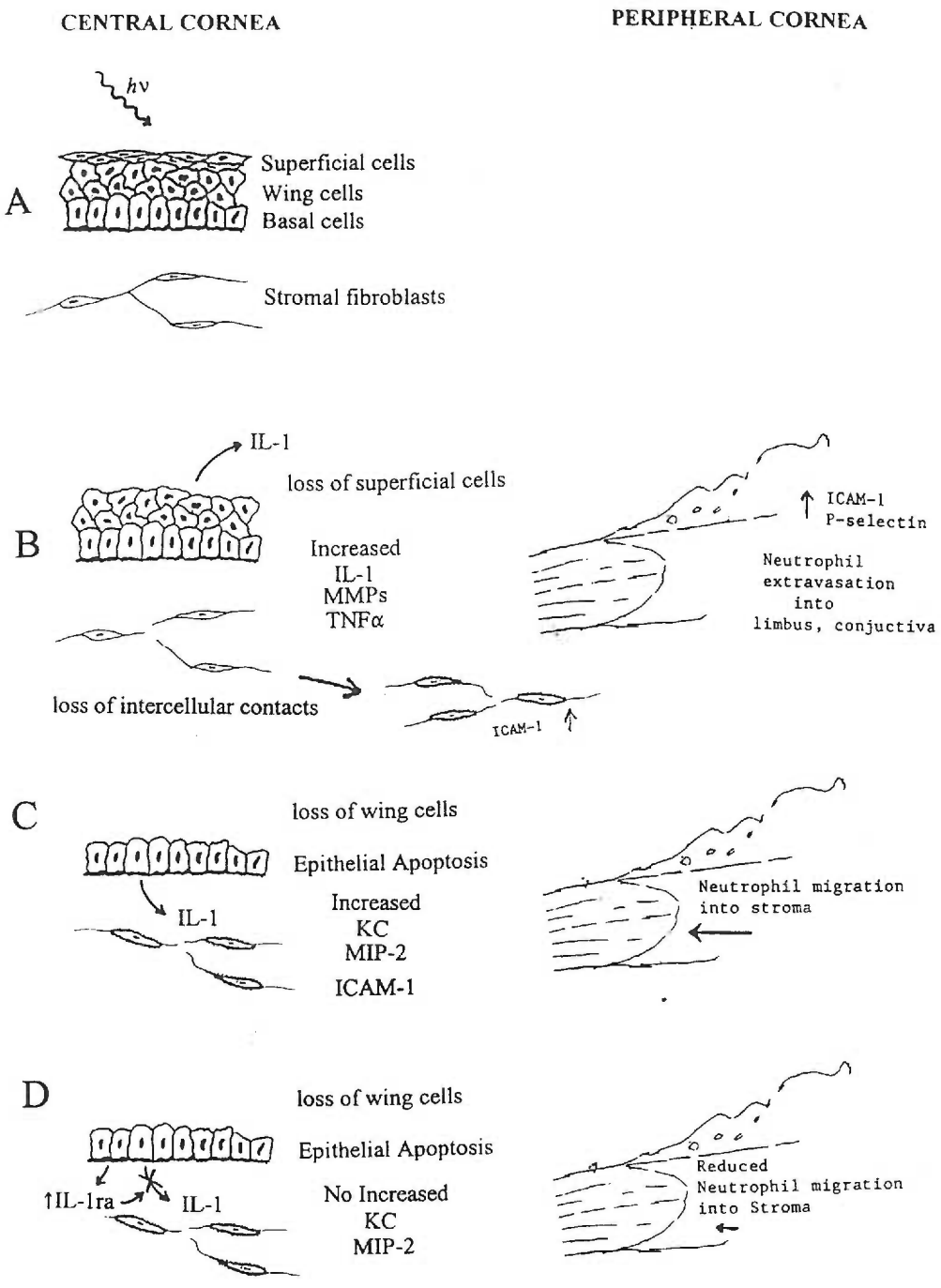

Diagram 2. UVB Photokeratitis

CONCLUSIONS

Conclusions

These studies address several facets of corneal inflammation. The data presented here strongly support the hypothesis that corneal cells respond to UVB irradiation with the production of inflammatory cytokines, particularly IL-1. These studies also support the secondary hypothesis that the cornea possesses at least one specific mechanism of counteracting the inflammatory properties of IL-1, that of IL-1ra production. These studies have shown that human corneal epithelial cells have a unique pattern of IL-1ra production, the production of secreted and intracellular IL-1ra. Epithelial cells from other organs investigated do not produce secreted IL-1ra. The full significance of this ability to secrete IL-1ra has not been elucidated, and it is not known whether the corneal epithelial cells of other animals also have this ability.

Whereas the work presented here does not directly prove that cytokines are the primary mediators of photokeratitis, the data are consistent with this hypothesis and offer support for it. The studies of human corneal fibroblasts have shown these cells produce the chemokine, IL-8, in response to UVB irradiation or exogenous IL-1. The RT-PCR analysis of murine corneal fibroblasts displayed increased mRNA levels for the neutrophil-attracting chemokines, KC, MIP-2, and GCP-2, after stimulation with IL-1. Also, we have shown that these cells increase production of KC protein in response to UVB or IL-1. This suggests that the

production of neutrophil-attracting chemokines can occur in the cornea after exposure to UVB radiation. The presence of a chemotactic gradient would stimulate neutrophil migration into the cornea. The inhibition of a UVB-stimulated increase in chemokine production by treatment with IL-1ra supports the hypothesis that IL-1 is a mediator of this effect. The IL-1ra inhibition of increased chemokine production provides a possible mechanism for the reduction of inflammation seen in the transgenic mice.

The work presented here gives insights into mechanisms of inflammation initiation and control in the cornea. The corneal production of IL-1 in response to UVB reveals how a corneal response to an environmental insult can result in corneal inflammation. The findings on corneal IL-1ra show a mechanism for reducing the consequences of local IL-1 release.

These studies have relevance to the condition of photokeratitis and other immune-mediated corneal diseases such as Herpes keratitis. Answers to preventing corneal damage from excessive inflammation may be found in understanding how inflammation is successfully resolved in conditions like photokeratitis. Understanding the endogenous mechanisms controlling corneal inflammation offers the potential of supplementing or manipulating these mechanisms as new therapies for inflammatory corneal disease.

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