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In Vivo Imaging of Corneal Inflammation: New Tools for Clinical Practice and Research

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Abstract

Purpose—Infectious and inflammatory corneal diseases are a major cause of blindness. To date, assessment of corneal inflammation, has only been possible by slit-lamp biomicroscopy. The purpose of this study is to review the current state of imaging technologies enabling in vivo imaging of inflammation in the cornea.

Methods—Literature review of peer-reviewed articles on in vivo imaging modalities.

Results—Current means of diagnosis and treatment follow-up for immune and infectious keratitis are limited to slit-lamp biomicroscopy. Several modalities are currently emerging, allowing for in vivo imaging of corneal inflammation, including in vivo confocal microscopy, anterior segment optical coherence tomography, and intravital multiphoton microscopy.

Conclusion—Several in vivo imaging technologies are currently evolving, allowing for objective assessment of corneal inflammation and treatment response.

Keywords

cornea; inflammation; in vivo imaging; confocal microscopy; OCT; multi-photon microscopy

INTRODUCTION

Corneal disease is one of the leading causes of blindness worldwide.¹ It accounts for up to 90% of the cases of vision loss in some underdeveloped countries.² Contrary to the rest of the world, where trachoma, ophthalmia neonatorum, and onchocerciasis are common, developed countries face corneal blindness due to infectious agents like bacteria, viruses, protozoa and fungi on the one hand, and noninfectious conditions like immune or autoimmune conditions and trauma on the other hand. Inflammation of the cornea, with or without violation of the epithelium, constitutes keratitis and is present in all of the abovementioned conditions, leading to significant morbidity.

Patients with keratitis typically present with a painful red eye, photophobia, tearing, decreased visual acuity, and foreign body sensation. Risk factors associated with bacterial keratitis are contact lens wear, ocular trauma, previous ocular surgery, corneal sutures, topical steroids, diabetes mellitus, lid malalignment, and ocular surface diseases.³ A prompt

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and accurate diagnosis of corneal inflammation is a milestone for successful treatment and follow-up.⁴ The severity of disease can range from a mild, self-limiting disease to a vision-threatening condition. The ophthalmologist faces the challenge of differentiating among multiple clinical entities, and clinical suspicion will usually guide the initial treatment. Patient history and slit-lamp examination are often the only tools available for diagnosis and monitoring of treatment.

Recently, a number of new imaging technologies have emerged in research and clinical ophthalmology. Optical Coherence Tomography (OCT), now widely used in retinal and glaucoma imaging,⁵ has recently become available for with the anterior segment use with excellent resolution. Further, several generations of In Vivo Confocal Microscopes (IVCM) are being employed both in clinical corneal imaging as well as in research.⁶ Moreover, two-photon or multi-photon microscopy (MPM), which allows three-dimensional tissue imaging over time, is an important assets in investigational imaging.⁷ Here, we will discuss the potential use and limitations of each of these technologies for imaging corneal inflammation in vivo.

MATERIALS AND METHODS

Systematic literature review was preformed in Medline databases in December 2009 in order to review the types of corneal inflammation and to describe the current state of imaging techniques for the cornea. The individual or combined searched terms included: “cornea”; “inflammation”; “corneal inflammation”; “keratitis”; “infectious keratitis”; “confocal microscopy”; “optical coherence microscopy”; “two-photon microscopy”; and “multi-photon microscopy.”

RESULTS

Viral Keratitis

Viral keratitis is more commonly associated with the herpesviridae (Herpes Simplex Virus-1 [HSV-1], Herpes Simplex Virus-2 [HSV-2], Varicella Zoster Virus [VZV], Epstein Barr Virus [EBV], Cytomegalovirus [CMV]), as well as adenoviruses. Herpetic keratitis is the leading infectious cause of corneal blindness in the United States with around 500,000 cases reported annually.⁸ HSV has the ability to infect most human cell types, causing lytic infections of epithelial cells and fibroblasts, as well as latent infections in neurons. Viral cultures and polymerase chain reaction (PCR) can be used for diagnostic purposes.

All members of the herpes virus family are characterized by the ability to causing recurrent infections and inflammation, which in turn is a main cause of corneal scarring. Each new attack is typically associated with epithelial or stromal keratitis, and increases the possibility of scarring and non-reversible loss of vision.⁹⁻¹¹ Generally, steroids are used in the clinic to suppress corneal inflammation. However, the role of steroids in herpetic keratitis is controversial, as steroids not only suppress the immune response to the infectious agent, but they also inhibit the formation of collagen and mucopolysaccharides, which are fundamental for the integrity of the tissue. Further, steroids can lead to reactivation of HSV keratitis and delayed resolution. Being able to detect inflammation early (before visible on slit-lamp), and to objectively quantify inflammation, could aid in the treatment and management of this potentially blinding disease.

Bacterial Keratitis

Bacterial keratitis is usually caused by gram-positive cocci, among which *Staphylococcus sp.* are the most prevalent, gram-positive bacilli (*Propionibacterium acnes*), and gram-negative bacilli such as *Pseudomonas aeruginosa* and *Serratia*.¹¹ *N. gonorrhea* and *C.*

trachomatis should also be considered in the sexually active patient. Contact lens use is a major risk factor for bacterial keratitis (particularly for *Pseudomonas*), in addition to trauma and history of intraocular surgery. Corneal damage in *Pseudomonas* keratitis is caused both by the organism itself, as well as by host lysosomal enzymes and oxidative substances secreted by neutrophils, keratocytes, and epithelial cells during the inflammatory response.¹²

The hallmark of suspected bacterial keratitis includes ulceration of the epithelium with focal or diffuse suppurative stromal inflammation, affecting corneal transparency.¹⁰ In *Pseudomonas* keratitis specifically, the pattern is more distinctive; loss of corneal transparency with peripheral epithelial edema is accompanied by a “ground glass” stromal appearance, potentially leading to deep stromal abscesses. Corneal scrapings for smears and cultures are taken, if appropriate, and the patient may be admitted for treatment with fortified topical antibiotics.^{10,13,14} The extent of inflammation and objective response to treatment may be difficult to measure initially, as limited information can be obtained by slit-lamp examination due to the potential corneal opacification.

Fungal Keratitis

Fungal keratitis constitutes 6–20% of the total cases of keratitis in the United States.¹⁰ Fungal infections of cornea are caused by filamentous fungi (e.g. *Aspergillus* sp., *Fusarium* sp.) and yeasts (*Candida* sp.), and are also associated with predisposing factors, among which contact lens use and trauma with vegetative matter play a major role. Gram and Giemsa stain, KOH prep, cultures, and PCR are techniques used to confirm the diagnosis, while treatment follows with topical and systemic antifungals.^{11, 15} Recently, a role for IVCN has emerged in rapid diagnosis of fungal keratitis, as only one-fourth of cultures becomes positive after two weeks. Fungal keratitis poses a therapeutic challenge for the clinician due to the limitation of the available antifungal agents and the extent to which these agents can penetrate in the tissue. Further, fungal keratitis is associated with significant ocular inflammation. In contrast to bacterial keratitis, steroids limit the success of medical therapy in fungal keratitis, as they decrease clearance of the pathogen and may lead to perforation.¹⁶ Therefore, being able to objectively quantify inflammation and monitor its extent would potentially aid in guiding proper medical and surgical therapy.

Acanthamoeba Keratitis

A rare but potentially devastating corneal infection, most commonly associated with soft contact lens wear, is *Acanthamoeba* keratitis. While corneal cultures and smears remain the gold standard in the diagnosis of infectious keratitis, they can take weeks and have a very low yield (between 0 to 68%). Further, in advanced cases, microorganisms are located deep in the cornea, and corneal scrapings are not sufficient, requiring corneal biopsy. The use of IVCN is currently emerging as a rapid, non-invasive technique for diagnosis of *Acanthamoeba* keratitis through visualization of *Acanthamoeba* cysts, with a high sensitivity and specificity. Similar to fungal keratitis, steroid use in this disease limits the success of medical therapy, as it decreases clearance of the pathogen. Again, being able to objectively quantify inflammation and monitor its extent would aid in guiding medical and surgical therapy.

Other Causes of Corneal Inflammation

Immune and autoimmune diseases may also cause keratitis as part of their systemic manifestations. Rheumatoid arthritis, for example, commonly causes marginal ulceration and thinning, or central corneal melts. Systemic lupus erythematosus also has corneal symptomatology, mostly limited to manifestations in the epithelium and dry eye syndrome.^{10, 17} In addition, rosacea, a chronic and idiopathic inflammatory disorder of the skin, affects the eye in 3–58% of cases.^{18, 19} The mechanism involved resembles type IV

hypersensitivity reaction. Conjunctival samples of patients have shown infiltration of T cells, phagocytic cells, and antigen presenting cells (APCs).^{10, 20}

Peripheral ulcerative keratitis (PUK) comprises a group of corneal inflammatory disorders that result in peripheral corneal thinning. The exact mechanism remains to be elucidated, but evidence suggests the involvement of humoral and cell-mediated autoimmune processes.^{21, 22} Moreover, photokeratitis, caused by exposure to ultraviolet light, or keratitis due to chemical burns are additional causes of noninfectious keratitis. Clinically, there is corneal opacification and a punctuated staining pattern,²³ but the level of inflammation is not always apparent by slit-lamp biomicroscopy.

Dry eye syndrome (DES) is also a common cause of corneal inflammation, albeit less severe. Although inflammation is now known to be an important factor in the pathophysiology of DES, the role of inflammation in DES had been controversial for many years, partly because it was not detectable on slit-lamp examination. Proinflammatory cytokines (interleukin [IL]-1, IL-6, IL-8, tumor necrosis factor [TNF]- α) and chemokines have been detected both on the corneal surface and the tear film in recent years.²⁴ DES has been shown to be a CD4+ T cell-mediated disease, associated with a delayed-type hypersensitivity reaction (type IV hypersensitivity). A potential crucial step in understanding the pathogenesis of this disease is to elucidate the role of bone marrow-derived corneal APCs in T cell activation. APCs include epithelial and stromal dendritic cells (DCs), which increase during corneal inflammation and acquire an activated phenotype.^{25, 26} However, to study the role of immunity and inflammation in patients, tools are needed to visualize immune cells in vivo. Visualizing subclinical inflammation would allow early diagnosis and treatment in patients with DES.

New Technologies in Clinical Ophthalmology

In 2006, Keay et al. demonstrated that the current standards of treating microbial keratitis lead to increased morbidity, both in terms of visual outcome as well as in terms of direct cost to the health care system.³ Ophthalmologists have always relied mostly on slit-lamp biomicroscopic changes of the cornea to reach diagnostic conclusions and provide the appropriate treatment. Subjective slit-lamp observation remains the backbone of clinical examination of the eye, but objective tools and technologies are needed in order to offer earlier and more comprehensive understanding of alterations occurring at a cellular level.

In Vivo Confocal Microscopy—The term “confocal” derives from the fact that both the condenser, shedding light on the area under study, and the objective lens, allowing for observation, focus exactly on the same point. Although the confocal microscopy was patented in 1957 by Minsky, the first in vivo images of the cornea were published in 1990.^{6, 27}

Three types of confocal microscopes are available. The tandem scanning confocal microscopy (TSCM) sheds light of high intensity through a pinhole, and a camera generates a two-dimensional image. However, the TSCM is not being produced any longer. In contrast, scanning and examination times are greatly reduced, contrast is greater, and the stroma can be imaged, with the newer slit scanning confocal microscope (SSCM), thanks to its ability to scan, in parallel, many points along the axis of the slit. The newest addition is the laser scanning confocal microscope (LSCM), which uses a coherent light source with the laser beam scanning over the back of the microscope objective by scanning mirrors. The LSCM is commercially available as the Heidelberg Retina Tomograph (HRT)3 in conjunction with the Rostock Cornea Module (RCM), and has a class I laser that poses no risk of ocular injury. However, the LSCM poses a theoretical risk of epithelial injury as well as the possibility of producing artifacts due to its applanating effect. The resolution

varies between the different confocal microscopes, with 1 $\mu\text{m}/\text{pixel}$ in the newer LSCM HRT 3/RCM. The acquisition time is 30 images/sec in the LSCM HRT3/RCM and 25 images/sec in SSCM. IVCN can be used for imaging all corneal layers and structures: epithelium, Bowman's layer, sub-basal nerve plexus, stromal keratocytes, Descemet's membrane, endothelial cells, and immune cells.^{6, 28–30}

In vivo studies have demonstrated that IVCN can be used to assess the density and distribution of Langerhans Cells (LCs), a subtype of APCs in the corneal epithelium, providing a deeper comprehension of corneal epithelial immunology.^{31–33} Increased numbers of LCs or DCs have been reported in cases of bacterial³⁴ and herpetic keratitis,³¹ in dry eye patients³⁵, as well as following contact lens wear³⁶. IVCN has recently been employed to study immune cell migration to ocular tissues and to assess the extent of inflammation (Figure 1).^{37–40} These studies relied on the shape and morphology of the structures to interpret IVCN images, where neutrophils, dendritic cells, and lymphocytes can, so far, be differentiated.

Anterior Segment Optical Coherence Tomography (AS-OCT)—The OCT was introduced in 1991.⁴¹ It was initially applied to retinal imaging in 1993,⁵ and more recently to anterior segment imaging.^{42, 43} While the IVCN provides data on a cellular level, the AS-OCT provides cross-sectional anatomical images. Clinical and research publications are evolving very rapidly over the last years, demonstrating the interest and potential of this technology.⁴⁴ Numerous AS-OCT devices are now commercially available, with a high resolution of 5–20 μm , making it an excellent technique for imaging the anterior segment. Currently available AS-OCT devices have different laser wavelengths. AS-OCTs with shorter wavelengths provide higher resolution, while AS-OCTs with longer wavelengths provide higher tissue penetration.

The time domain OCT (TD-OCT, Visante AS-OCT, Carl Zeiss, Meditec Inc., Dublin, CA), for example, has the ability of scanning 400 A-Scans/sec with a longer wavelength allowing for deeper tissue penetration. The Fourier-Domain OCT RTVue on the other hand (Optovue Inc., Fremont, CA) is an ultra high-speed OCT, capable of performing 26,000 A-Scans/sec and can be used for both anterior and posterior segment imaging. It applies a shorter wavelength with a very high resolution of 5 μm . It is much faster, since it scans the tissue in parallel, rather than serially, and the high resolution offers detailed images of the area under image.

To date, the AS-OCT has been used for imaging studies of physiological anatomical structures and for diagnosing pathological situations. It has been shown to have excellent repeatability in pachymetric mapping of the cornea,⁴⁵ in measurement of the depth of anterior chamber depth,⁴⁶ as well as the angle of the anterior chamber.⁴⁷ Further, AS-OCT has been used to measure the tear meniscus,⁴⁸ in anterior segment tumor imaging to differentiate cystic from solid lesions,⁴⁹ to assess the penetration of tumors,⁵⁰ and to monitor post-surgical patients.^{51, 52}

In a recent case series, the imaging capabilities of the AS-OCT in microbial keratitis were investigated, demonstrating the use of this modality to extract useful conclusions concerning the extent of microbial keratitis and treatment response.⁵³ Corneal thickness increased at the initial stages of microbial keratitis, and decreased with the resolution of the inflammation. Further, infiltrate size and depth can be measured and quantitatively monitored following treatment, demonstrating an initial increase in size, followed by decrease in size with resolution.⁵³ Despite the feasibility of detecting and monitoring corneal inflammation with new generation AS-OCTs (Figure 2), no large systematic studies have been performed to date. The application of AS-OCT in diagnosis and treatment of keratitis could aid the

clinician by providing data that cannot be obtained through slit lamp examination alone, particularly to determine the progress of the disease after initiation of treatment.

New In Vivo Imaging Technologies in Animal Research: Multi-Photon Microscopy

The invention of two-photon or multi-photon microscopy (MPM), and its application in science in 1994,⁵⁴ has been a breakthrough in investigational in vivo imaging, leading to increased interest in visualization of cell migration, interaction, and function over time. This modality offers various advantages over conventional confocal microscopy; first, the long wavelength of the infrared light used allows for more in-depth penetration of the organic matrix to up to 400 microns. Second, less phototoxic phenomena occur thanks to the sensitive detectors that acquire image from very weak light signals and to the ability to excite a small 1 μm focal point, protecting the surrounding tissue from fluorophore bleaching. These traits allow imaging of live tissues with high resolution over extended periods of time. Moreover, there is no need for cellular and tissue staining or slicing, as the whole imaging process can be performed in vivo with the aid of genetically engineered mice that have fluorescent cell populations. In the cornea, more specifically, the interaction of infrared light with collagen elicits second harmonic generation of UV-photons that can be registered. This can be recorded separately and serves to delineate the corneal stromal collagen from surrounding tissue.⁵⁵

MPM has not been used in the clinical practice yet. However, several research groups are currently investigating its potential applications in steady state and inflammation. In a skin model, MPM documented successfully the parallel influx and efflux of Langerhans cells in steady state,⁵⁶ providing evidence for the turnover rates and migration of this APC population. MPM studies of gut DCs have further shown how luminal dendrites interact with non-invasive *Salmonella*.⁵⁷ Further, studies in the lymph node demonstrated immotile naïve DCs that had the ability to interact with T cells by extending their dendrites. However, when DCs were activated during inflammation, they became motile, allowing for easier interactions with T cells.⁵⁸

In the cornea, Ward et al. studied naïve corneal LCs in explanted corneas by MPM, and demonstrated “dendritic surveillance extension and retraction habitude- dSEARCH.”⁵⁹ When corneas were inflamed and explanted, this movement was enhanced, while rapid lateral movement was also shown.⁵⁹ Hamrah et al. recently demonstrated the feasibility of in vivo MPM for corneal imaging in steady state and inflammation (Figure 3), demonstrating migratory kinetics of DCs and monocytes in the central and peripheral cornea.⁶⁰ Further, Cursiefen et al. have recently reported the interaction of immune cells and lymphatic vessels in vivo by MPM.⁶¹

DISCUSSION

Late diagnosis of corneal inflammation has been shown to lead to increased morbidity, cost, and poor visual outcome.³ Still, to date, clinicians rely on subjective evaluation of the cornea by slit-lamp biomicroscopy, an instrument that was developed over a century ago in the 1890s. Herein, we described the applications of new and exciting technologies for high-resolution in vivo imaging of the cornea, to visualize corneal inflammation and immunity in context of disease.

The ability to image corneal inflammation and immune cell populations in vivo could soon be translated into clinical practice. First, the morphology of cells that appear in the inflamed eye can provide important clues that could help differentiate among different underlying conditions. In addition, objective quantification of inflammatory cells or infiltrates could be used as an index of severity to guide treatment levels. Advantages of imaging corneal

inflammation as an indicator for diagnosis and monitoring treatment include speed, ease of use, objectivity, and its noninvasive nature.

Currently, IVCN has the widest spectrum of clinical applications, including early diagnosis of *Acanthamoeba* and fungal keratitis, with a high sensitivity and specificity. Immune cells are known to migrate in a particular order to the site of inflammation. The determination of immune cell type and quantification by IVCN could allow classification of state of disease, as well as determination of treatment response in any type of keratitis, beyond identification of pathogens.^{31–34} More specifically, careful analysis of the IVCN images of immune cells, studying size, shape, morphology, presence or absence of dendrites, could provide the clinician with valuable information about the chronicity of disease and the response to treatment. Finally, as recently described by Liang et al.,³⁸ IVCN has been shown to promptly indicate the toxic effects of new drugs on the ocular surface in rabbits, emphasizing the need for future prospective human studies.

Anterior segment OCT, on the other hand, can detect fine changes in corneal microarchitecture during inflammation. The resolution AS-OCT currently seems inadequate to depict and identify the infectious agent itself. However, in microbial keratitis, where edema and infiltrates are present, close monitoring of corneal and infiltrate thickness and distribution could guide diagnosis and could be useful for monitoring response to the disease, as minimal changes could easily be detected. However, differentiating corneal infiltrate from scarring is currently difficult, due to similar optical properties. Recently, Konstantopoulos et al. demonstrated that infiltrate thickness, corneal thinning, and endothelial inflammatory plaque can be measured with the AS-OCT, and that response to therapy can be monitored in cases of bacterial keratitis.⁵³ Moreover, in patients with PUK, where peripheral thinning is notable, anatomical changes of the cornea can be measured and monitored with AS-OCT.

Finally, MPM offers numerous advantages over other imaging technologies, offering the advantage to study inflammation and immune cells over time. Prolonged imaging times on various subsets of immune cells can give us answers to fundamental questions about ocular immunology and immune response, and broaden our understanding on the mechanisms involved in infectious, immune, alloimmune and autoimmune keratitis. Currently, its properties render it impractical for use with human subjects; the amount of time required for proper imaging is long and therefore the desirable fixation time seems unrealistic for the human eye. If these obstacles are surmounted, a new promising technology could be introduced to clinical research with MPM.

In conclusion, while the addition of these new technologies is exciting, they require a considerable amount of translational and clinical research, prior to being further integrated in clinical ophthalmology. Nevertheless, a fundamental transition is currently taking place that will likely decrease morbidity, improve the effectiveness of treatment, and set new standards for patient care.

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FIGURE 1.

(A, B) In vivo confocal image of a patient with acute fungal keratitis, demonstrating a significant influx of leukocytes. (C) In vivo confocal image of a patient with chronic *Acanthamoeba* keratitis, demonstrating a significant increase in dendritic Langerhans cells in the corneal epithelium, with extensive dendritic processes. (D) Corneal nerves and immune cells depicted with in vivo confocal image in a patient with viral keratitis.

FIGURE 2. Slit lamp (left) and OCT image (right) of a patient with fungal keratitis, demonstrating inflammation in the anterior stroma.

FIGURE 3.

Two Photon Microscopy picture of the cornea in a Major Histocompatibility Class II (MHCII)-GFP mouse. MHC II-positive antigen presenting cells appear green and stromal collagen appears in blue (second harmonic generation). Peripheral (lower right) and central (upper left) cornea are visualized.