Published in final edited form as:

Expert Rev Ophthalmol. 2015 ; 10(4): 383–392. doi:10.1586/17469899.2015.1049157.

Sensory nerve regeneration after epithelium wounding in normal and diabetic cornea

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Summary

The cornea is the most densely innervated mammalian tissue. The sensory nerves are responsible for sensations of dryness, temperature, touch, and pain, and play important roles in the blink reflex, wound healing, and tear production. Many ocular and systemic diseases can adversely affect corneal sensory nerve and consequently impair their function. One of such systemic diseases is diabetes mellitus (DM) which causes sensory degeneration, neurotrophic keratopathy (DNK), and delayed wound healing. In this review, we summarize recent discoveries revealing mechanisms underlying the pathogenesis of DNK and the impairment of sensory nerve regeneration in post wound diabetic corneas in using animal model of human diabetes. Because it is generally believed that common mechanisms are operative in the pathogenesis of diabetic peripheral neuropathy in different tissues, the findings in the corneas have implications in other tissues such as the skin, which often leads to foot ulceration and amputation in diabetic patients.

Diabetes mellitus (DM) is a major disease worldwide, and its prevalence has risen significantly in the past several decades. Two types of diabetes, Type 1 and 2, both manifest with hyperglycemia but have distinctive pathophysiological mechanisms. Type 1 diabetes (T1D) arises following autoimmune destruction of the insulin-producing pancreatic beta cells, is often diagnosed in childhood and adolescence, and requires exogenous insulin treatment. The cause of the autoimmune process is unknown, and is thought to be triggered by one or more environmental agents in genetically susceptible individuals. [1, 2] In Type 2 diabetes (T2D), individuals exhibit insulin resistance and a resulting relative insulin deficiency to varying degrees, and exogenous insulin treatment may or may not be required. Its pathogenesis is multifactorial, involving both genetic and acquired factors which cause overproduction and inefficient utilization of glucose. T2D is often diagnosed in the adult population, although its incidence in the youth has been rising, alongside increasing obesity in this group. T2D often occurs concurrently with hyperlipidemia and hypertension, creating a constellation of signs and symptoms known as metabolic syndrome, where increases in

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Financial and competing interests disclosure

F Yu received grant NIH/NEI RO1 EY010869 Research to Prevent Blindness to Kresge Eye Institute. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

free fatty acid levels, inflammation, and oxidative stress have been implicated in its pathogenesis. [3, 4]

Ocular complications from DM have become a leading cause of blindness in the world. [5] In addition to abnormalities of the retina (diabetic retinopathy) and the lens (cataract), ocular surface disorders are also relatively common in DM patients, including impaired corneal sensation, reduced tear secretion, conjunctival squamous metaplasia, goblet cell loss, and keratopathy. [6–10] Diabetic keratopathy is estimated to occur in more than 70% of diabetic patients [9–11] and increases corneal susceptibility to trauma with epithelial erosions and ulcerations. [12–14] These complications pose significant difficulties in the management of diabetic patients. [15–17]

The cornea is a transparent tissue consisting of three cellular layers: the epithelium, stroma and endothelium. Many major hyperglycemia-induced pathological changes in the cornea occur in the stratified epithelia, including alterations in the epithelial basement membrane such as thickening [13, 18, 19], decreased number of hemidesmosomes [20] and deposition of advanced glycation end products. [13, 21] In a T1D rat model, there are increased intracellular levels of tonofilaments and glycogen, thickenings and infoldings of subepithelial basement membrane and basal cell degeneration. [22] In diabetic patients, corneal thickness is increased while there is a thinning of the epithelium [18], which is consistent with findings of lower basal epithelial cell density in T2D patients. [23] This is confirmed by observations that hyperglycemia also directly and significantly alters their structure and function of corneal epithelial cells, resulting in basal cell degeneration [14, 22, 24], decreased [25, 26] or increased [27] cell proliferation, superficial punctate keratitis [28], breakdown of barrier function [29, 30], fragility [31], and abnormal deposition of laminin and type IV collagen. [32] Given the significant impact on diabetes on the corneal epithelium, diabetic keratopathy is also termed diabetic corneal epitheliopathy. [33, 34] Clinically, the cornea appears normal in patients with diabetic keratopathy in the absence of corneal injury. However, trauma and ocular surgeries such as vitrectomy for vitreous hemorrhage may require the removal of epithelial cells, putting the fragile structure of the corneal epithelium at risk. While a normal healthy cornea can repair injury to its epithelium, in diabetic patients, there is a considerable delay in corneal re-epithelialization after injury. The impairment of corneal epithelial wound healing could result in several types of epithelial disorders, such as persistent epithelial defects and recurrent erosion in postoperative patients. [9, 35–38] Furthermore, delayed healing of the epithelial defect(s) eventually lead to complications that result in loss of vision.

Studies revealing differences between corneal epithelium changes in type 1 and type 2 diabetes are lacking. Decreased basal cell density has been noted in type 2 diabetic patients [23, 39, 40] and mice [41], as well as in type 1 diabetes. [42] However, examination of diabetic corneas with confocal microscopy did not reveal differences in epithelial density between type 1 and type 2 diabetes. [43] Additionally, thinning of the epithelium was noted in both type 2 [41] and type 1 [42] diabetes. In a study on human type 1 patients, it was observed that while those with neuropathy had thinning of the epithelium, those in the earlier stages of the disease without neuropathy had epithelial thickening. [18]. However, it is likely that this thickening is due to edema resulting from dysfunction of the corneal

endothelium. [44, 45] Therefore, there is generally little data to support any differences in the corneal epithelium between type I and type II diabetic patients.

While the cornea is an avascular tissue, it is the most densely innervated and sensitive tissue in the human body (approximately 7,000 nociceptors per mm²). [46] The sensory nerves are responsible for sensations of dryness, temperature, touch, and pain, and play important roles in the blink reflex, wound healing, and tear production. [28, 47–52] Corneal nerves are largely derived from the ophthalmic division of the trigeminal nerve. Dozens of major stromal nerve bundles enter the cornea from the limbus and penetrate into the mid-stroma. Each stromal bundle gives rise through repetitive branching, to a moderately dense midstromal plexus, as well as a dense subepithelial plexus (SEP). The SEP becomes less dense and has a patchier distribution as it reaches the central cornea. Branches from the SEP in turn penetrate Bowman's membrane, more so peripherally than centrally, to innervate the corneal epithelium and form an extensive and complex subbasal nerve plexus (SBP) with a distinctive whorl-like pattern. SBP nerves then give rise to intraepithelial nerve terminals that innervate all epithelial layers. [46, 53] Corneal nerves originate from large myelinated A δ and small unmyelinated C fibers. A δ fibers respond primarily to mechanical and heat stimuli via mechanosensory and mechanoheat receptors, respectively, while C fibers respond to cold stimuli via cold receptors. Both fiber types have polymodal receptors that respond to mechanical, thermal and chemical stimuli. [54–60] As they enter the stroma, the nerve fascicles lose their perineurium and myelin sheaths approximately 1 mm from their origin in the limbus, in order to maintain transparency of the central cornea. [58, 61] As they progress anteriorly into the cornea, the nerves are only surrounded by a Schwann cell sheath, which is lost when the nerves penetrate Bowman's membrane. [57] A δ fibers run parallel to the corneal surface within the basal cell layer, while C fibers run perpendicular to the surface, turning towards the corneal surface.

Corneal innervation and sensitivity are adversely affected in many systemic conditions including diabetes, and such impairment may lead to neurotrophic keratopathy, ulceration and even perforation. [62–65] Morphological changes in diabetic animal corneas have been well documented and include irregular nerve fiber beading, decreases in nerve plexus density, thinner fibers, and ultrastructural changes including swelling of axons, irregular distribution of fibrils, mitochondrial degeneration and Schwann cell basal lamina abnormalities. [66–68] A recent study confirms that although the main stromal nerve trunks are similar in diabetic and normal human donor corneas, there was a significant decrease in central epithelial nerve density in diabetics. [69] Interestingly, it was also observed that there were abundant nerve fiber loops in the stroma of diabetic human corneas and these loops seemed to form from resistance presented by the basement membrane, blocking their entry into the epithelium. [69] Independently of diabetes, it appears that obesity produces similar corneal nerve defects as those in diabetes. Both diet-induced obese rats [70] and mice [71] have shown a decrease in nerve fiber length, density, and corneal sensitivity that mirror the changes observed in the corresponding diabetes models.

Clinically, diabetic patients often have decreased corneal sensitivity as measured with an esthesiometer. [18, 72] Recent advances in technology have allowed for rapid, non-invasive, and high-quality visualizations of the corneal structure using in vivo corneal confocal

microscopy (IVCCM). IVCCM has been used as an excellent clinical tool for assessing corneal nerve abnormalities, demonstrating reduced nerve fiber length, nerve fiber density, nerve branch density and increased tortuosity in diabetes. [40, 73–78] Nerve fiber alterations of the SBP and loss of corneal sensation not only progress in parallel with diabetic retinopathy [40, 76], but have been shown to precede the development of retinopathy in diabetics. [72] Interestingly, IVCCM appears to allow for the early detection of the initial stages of neuropathy, demonstrating diabetic nerve fiber damage earlier than corneal sensation testing and vibration perception assessment in the lower limb. [18, 79]

Diabetic peripheral neuropathy (DPN) is one of the most common and debilitating complications of DM, with more than half of all diabetic patients developing altered sensation as a result of damage to peripheral sensory neurons. [80–82] There are two phases of diabetic polyneuropathy, beginning with an early reversible metabolic phase that is progressively overshadowed by structural degenerative changes, eventually resulting in nerve fiber loss. This late structural phase of DPN appears to affect type 1 diabetics more severely. Progressive axonal atrophy and loss is therefore expressed to a larger extent in type 1 diabetes. [83]

Diabetic neurotrophic keratopathy is a degenerative corneal disease induced by an impairment of the trigeminal nerve and considered to be a component of diabetic polyneuropathy, as well as a major cause of cornea morbidity in diabetic patients. [15, 84–87] Accumulating evidence has revealed a strong correlation between corneal nerve abnormalities and DPN. For instance, several studies showed that corneal sensitivity threshold was significantly correlated with a majority of established measures of DPN [74, 75, 77, 88–90] and corneal nerve fiber structural morphology, as assessed by IVCCM, correlated well with functional measures of systemic small nerve fiber injury such as cooling detection thresholds, axon reflex-mediated neurogenic vasodilatation in response to cutaneous heating by laser Doppler imaging flare technique, and heart rate variability. [91] In addition, nerve fiber alterations of SBP progress in parallel with DPN. [76] Among many available parameters measurable by IVCCM, corneal nerve fiber length shows the strongest associations with other diagnostic tests of neuropathy, as well as with established risk factors for neuropathy. [77, 89, 90] IVCCM accurately defines the extent of corneal nerve damage and repair, acts as a surrogate measure of somatic neuropathy in diabetic patients, and represents a new tool in the diagnosis, clinical evaluation, and follow-up of DPN. [73, 75] To this end, ongoing studies are investigating the use of automated corneal nerve quantification and show reproducible promising results. [92] In fact, corneal re-innervation assessed by IVCCM may be the earliest sign of neuronal improvement in diabetics based on two recent transplant studies. In type 1 diabetic patients undergoing curative pancreatic transplantation, small fiber repair can be detected via IVCCM within 6 months after transplantation, despite marked nerve fiber damage prior to transplantation. [93] Moreover, type 1 diabetic patients undergoing simultaneous pancreas-kidney transplantation, demonstrated significant improvements in corneal nerve fiber density, branch density, and length via IVCCM after 12 months while there was no significant changes in neurologic impairment, neurophysiology, corneal sensitivity, or intraepidermal nerve fiber density. [94]

Delayed corneal epithelial wound healing is also common in diabetic corneas and may compromise vision. Particularly for diabetic retinopathy patients undergoing vitrectomy, the removal of corneal epithelium during the procedure results in a considerable delay in reepithelialization.[9, 36, 38, 95] Delayed epithelial healing predisposes patients to sight-threatening complications, such as stromal opacification, surface irregularity, and microbial keratitis. [35] Using type I and II diabetic animal models, we and others have demonstrated significant delay in corneal epithelial wound healing and have highlighted several signaling pathways that are affected in diabetes as possible underlying mechanisms. [30, 96–101] Although defects in corneal innervation in diabetic eyes are considered an important contributing factor for diabetic keratopathy and delayed epithelial wound healing, it appears that epithelial cells are directly affected by diabetes. Both cultured diabetic human corneas, due to metabolic memory effects [102–104], and porcine corneas cultured in high glucose conditions (25 mM versus 5 mM) [34] have similarly affected epithelial wound closure responses, suggesting that epithelial cells, either from the stem cells in the limbus, or in the cornea itself, are adversely affected by diabetes directly. Indeed, gene therapy for cultured diabetic human corneas normalizes expression of diabetes-suppressed genes such as HGF, as well as microRNAs such as miR-146a, which accelerates delayed wound healing, further confirming a direct pathophysiological role of the epithelium in diabetic keratopathy. [105, 106]

The role of corneal innervation in epithelial wound healing is an area of active research. Corneal nerve fibers exert important trophic influences on the corneal epithelium and contribute to the maintenance of a healthy ocular surface. [46] Corneal denervation results in significant changes in the corneal epithelium including cell swelling, loss of microvilli, sloughing of epithelial cells into the tear film, decrease in basal cell mitogenesis, epithelial thinning, and an increase in epithelial permeability. [107–109]. Corneal denervation either mechanically or chemically also significantly impairs corneal epithelial wound healing. [107, 110–112]. These findings highlight the influence of corneal innervation on epithelial homeostasis and repair. The interaction between corneal epithelium and nerves appears to be a reciprocal process based on the mutual release of soluble trophic factors. For instance, in vitro co-culture shows that trigeminal neurons release neurotransmitters and peptides to stimulate epithelial growth, proliferation, differentiation, and collagen production [112–114]. Corneal epithelial cells on the other hand secrete growth factors to promote neurite extension and survival, [115–117] including NGF [118], VEGF-a [119], CNTF [120, 121] and EGF [34]. Our recent study using a whole genome cDNA array revealed that while NGF, CNTF, VEGF-a, IL-6 (a neurotrophic cytokine) were all up-regulated in response to wounding in normoglycemia corneas, the upregulated expression of NGF, CNTF and IL-6 were significantly suppressed in diabetic rat corneal epithelial cells. NGF may also be produced by myeloid cells to promote trigeminal ganglion neurite growth, which may have further implications for corneal nerve regeneration. [122] HGF is another growth factor produced by epithelial cells and shows altered production in diabetes. [102] Gene therapy restoring HGF expression in human corneas accelerated epithelial wound healing and reversed alterations of epithelial stem cell marker patterns in cultured human diabetic corneas. [104, 123]

It is generally believed that sensory nerves release neurotrophic factors, primarily Substance P (SP) and CGRP to nourish the epithelium. Early studies have suggested that SP, in the presence of IGF-1, plays a major role in corneal epithelium homeostasis and wound healing. [124–127] SP and its receptors are abundant in the cornea and promote epithelial migration and proliferation. [128–131] Nakamura et al. demonstrated synergistic effects of SP and other growth factors (IGF-1 or EGF) on promoting epithelial wound healing. [99, 132–136] In fact, several clinical trials using topical SP showed promising results in treating persistent epithelial defects and neurotrophic keratopathy. [137–139] More recently, SP was also found to promote diabetic corneal epithelial wound healing through molecular mechanisms mediated by the neurokinin-1 receptor in type I (STZ-induced) mice. [140] Calcitonin gene-related peptide (CGRP) co-localizes with SP in corneal nerve fibers. The corneal epithelium is densely innervated by CGRP-positive nerves [141] and expresses CGRP receptors. [142] CGRP is elevated after laser keratectomy and is found to stimulate epithelial migration and differentiation, but not proliferation. [112, 143] An early study showed that while SP stimulated corneal epithelial proliferation, CGRP inhibited the mitotic activity of corneal cells, an effect that could be reversed by simultaneous administration of SP. [47] In an ex vivo wound healing study, CGRP was found to increase the epithelial wound healing rate by 25%. [143] Corneal innervation may also be required for the maintenance of limbal stem cells and therefore indirectly affect epithelial homeostasis and wound healing. [144]

Neurotrophins are another family of proteins thought to mediate nerve-epithelial interactions that maintain homeostasis and repair of the cornea. Their roles in cell function and survival were first described in the nervous system but have now been shown to be important in epithelial cells as well. [46] All four members of this family, nerve growth factor (NGF), neurotrophin 3 (NT-3), neurotrophin 4/5 (NT-4), and brain-derived neurotrophic factor (BDNF), as well as their corresponding Trk receptors are expressed in the human cornea. [145, 146] NGF has been shown to improve non-healing epithelial defects, as well as to stimulate epithelial proliferation and migration, possibly through ERK 1 activation. [146–150] Additional neurotrophic factors are also under active investigation. Mice with wounded corneas treated with ciliary neurotrophic factor (CNTF)-containing eye drops showed an increased nerve fiber density 8 weeks after wounding, as opposed to those receiving sham treatment. [121] Glial cell-derived neurotrophic factor (GDNF) was found to stimulate the migration of cultured human corneal epithelial cells. [151]

Ongoing research has also highlighted new players in this reciprocal dialogue and interaction between corneal nerves and epithelium. A recent study confirmed the interdependency and mutual support between corneal nerves and epithelium, emphasizing the importance of SP and BMP7 signaling. [114] Another has identified a possible therapeutic effect of Vitamin B12, demonstrating its effect on accelerating not only reepithelization but also corneal re-innervation after mechanical injury. [152] In addition, vascular endothelial growth factor has also been found to promote anatomical and functional recovery of injured peripheral nerves in the avascular cornea. [153, 154] Pigment epithelial-derived factor plus DHA promotes regeneration of calcitonin gene-related peptide-positive corneal nerves, accelerating wound healing and return of corneal sensitivity. [155] Endogenous opioids play important roles in neuromodulation and tissue growth and repair. They, and their receptors, have been shown to be present in human corneal epithelium and

serve to regulate cell proliferation, migration, and epithelial wound healing. [156–158] Interestingly, it appears that naltrexone, an opioid antagonist, facilitates reepithelialization in both diabetic rats [159], rabbits [160], and organ cultured human corneas. [158]

Aldose reductase (AR) is the rate-limiting enzyme in the polyol pathway in which glucose is converted to sorbitol. An increase of its activity has been suggested as one of the underlying mechanisms of diabetic pathophysiology. Treatment with AR inhibitors has been shown to reduce diabetic corneal changes. [161] AR inhibitors such as CP-45,634 and CT-112 can facilitate reepithelialization in diabetic and galactosemic rats. [162, 163] Moreover, CT-112 has been shown to be effective in reversing abnormal morphological characteristics of corneal epithelial cells, restoring corneal sensitivity, and treating corneal epitheliopathy in diabetic patients. [164, 165] It is interesting to note that topical AR inhibitors can partially reduce the endothelial morphologic changes observed in diabetic rats. [166]

Recent studies have shown that corneal stem/progenitor cells are also altered in diabetes. Ueno et al demonstrated a reduced expression of stem cell markers in T2D mouse corneas and their restoration with IGF-1 treatment. [167] Ljubimov's group, using organ-cultured human corneas from autopsies, has demonstrated that corneal, particularly limbal stem cell gene therapy has beneficial effects on diabetic corneal wound healing and stem cell marker expression, indicative of a possible new treatment regimen. [105, 106, 168] In fact, autologous transplantation of limbal stem cells has been used successfully to treat certain limbal stem cell deficiencies. [169] Zickri et al showed that hemopoietic stem cell therapy ameliorates microscopic and cellular changes in the diabetic rat corneal epithelium. [170] Traditional neurotrophic factors such as CNTF have also been shown to promote the activation of corneal stem/progenitor cells, along with enhanced wound healing and nerve regeneration in diabetic mouse corneas. [120] Systemically speaking, Sarkar et al reported that fluorescent bone marrow cells of the *thy1*-YFP mouse share surface markers with myeloid-derived suppressor cells, which suppress T-cell proliferation in the allogenic mixed lymphocyte reaction in mouse corneas, and secrete nerve growth factor and promote neuroregeneration in vitro. [122]

Despite the wealth of knowledge on the interplay between corneal nerves and epithelium, their interaction in diabetes and diabetic neurotrophic keratopathy remains elusive. Our laboratory [30, 97, 98, 171] and others [68, 70, 172] have demonstrated a significant delay in corneal epithelial wound healing, corneal re-innervation and nerve regeneration after epithelial injury in type I and II diabetic rats. More recently, we showed that dendritic cells (DCs), a type of antigen presenting cell, migrate alongside the healing corneal epithelial cells after epithelial debridement and that local depletion of dendritic cells resulted in a significant delay in epithelial wound closure, suggesting a functional necessity of dendritic and epithelial cells at mucosal surfaces for maintaining corneal homeostasis and for tissue repair. [173] Moreover, we found that DCs mediated epithelium-innervation and guided sensory nerve regeneration after epithelial injury. In diabetic corneas, DC infiltration and migration were greatly suppressed, along with associated defects in sensory nerve regeneration, indicating that DCs and neurons were functionally interdependent and morphologically interconnected (unpublished data). Leppin et al also reported co-localization of DC and nerve endings but found that there is an increased corneal DC density

in both type I and II diabetic mice. They argued that hyperglycemia leads to corneal DC infiltration and the direct contact between DCs and the SBP can be assumed to be a trigger of nerve fiber damage and thus a contributing factor to polyneuropathy in diabetic corneas. [174] Similarly in diabetic patients, Langerhans cell density is noted to be increased with IVCCM, particularly in the earlier phases of corneal nerve damage. [175] These newer and ongoing studies, albeit with significant discrepancies, illustrate an emerging trend in viewing the corneal epithelium not as a simple layered structure but as a platform of complex cell interaction and interplay where the epithelial, neuronal and immune systems converse and influence one another.

In summary, it is now well established that corneal innervation and nerve regeneration after injury are significantly impaired in diabetes and may represent an earlier manifestation of diabetic neuropathy. Compromised epithelial wound healing is another morbidity common in diabetic corneas. Accumulating evidence support the notion that corneal nerves and epithelium actively engage and influence each other. This interdependency appears to be one of the initial casualties affected by the damage induced by diabetes. Understanding the underlying mechanisms not only allows further contributions to potential new treatments for diabetic and neurotrophic keratopathy, but may also provide early diagnostic cues for treating the devastating effects of DPN.

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Five-year view

A standardized assessment of diabetic peripheral neuropathy, based on confocal microscopy and non-contact corneal esthesiometry, will be established for accurate detection, characterization, and quantification of diabetic peripheral neuropathy, important for defining at-risk patients, anticipating deterioration, monitoring progression, and assessing new therapies.

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Key issues

- Diabetes mellitus is a rapidly growing major health problem in US and worldwide.
- 70% of diabetic patients have complications in the cornea, clinically termed diabetic keratopathy.
- One of underlying cause of diabetic keratopathy is the loss of neurotrophic support, resulted from degeneration of sensory nerves, termed diabetic neurotrophic keratopathy (DNK).
- The cornea is mostly heavily innervated tissues in our body. Degeneration of corneal sensory nerve can be detected as early stage of diabetes.
- Corneal nerve damage is directly related to the severity of somatic neuropathy in diabetic patients, i.e. DNK is a component of diabetic peripheral neuropathy.
- Diabetic peripheral neuropathy is an underlying cause of chronic foot ulcers; which can lead to limb amputation.
- Currently, the only method of directly examining peripheral nerves is to conduct skin punch or sural/peroneal nerve biopsies, which are uncomfortable and invasive.
- A battery of non-invasive including corneal confocal microscopy and non-contact corneal esthesiometry, and tear measurement has developed and routinely used clinically to assessing DNK.
- Assessing this damage noninvasively in the cornea has been proposed as an effective method for the early detection, diagnosis, staging severity, and monitoring progression of diabetic peripheral neuropathy.

The subbasal sensory nerve plexus

Figure 1.

Defects of corneal innervations in 1-month-old GK rats. Subbasal plexus of the central cornea of Wistar and type 2 diabetes Goto-Kakizaki (GK) rat was visualized with Whole-mount immunostaining of β -tubulin and representative images are shown.