ABSTRACT OF THE DISSERTATION

Countermeasures Against Vesicant-Induced Epithelial De-adhesion in the Cornea

By

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Sulfur mustard is a chemical weapon and vesicant (blister-inducer) that causes severe effects to the cornea. These injuries are characteristically delayed in healing and may be recurrent over time. The experiments in this dissertation were designed to test the efficacy of countermeasures targeting metalloproteinases, thought to be over-activated in the cornea following vesicant exposure. An ex vivo culture model was developed using dissected corneas from young adult rabbit eyes. Each cornea was exposed drop wise to 20 nmol 2-chloroethyl ethyl sulfide (CEES, half mustard) or 100 nmol nitrogen mustard (NM). These are less potent and less toxic analogs of sulfur mustard, and induce mild or moderate injury, respectively.

Twenty-four hours after NM exposure there are observable separations between the epithelium and the stroma, termed microbullae. I hypothesize that the microbullae are caused by activation of the enzyme ADAM17 (a disintegrin and a metalloprotease 17), causing cleavage of the transmembranous anchoring protein, collagen XVII. Four ADAM17 inhibitors were compared to evaluate the effect of attenuating activity of this enzyme. The countermeasures effectively improved the appearance of the epithelial-
stromal junction as seen by the preservation of epithelial-stromal attachments and
improved histology as well as and decreasing ADAM17 activity.

After vesicant exposures there was also upregulation of MMP-9, the protease
responsible for the necessary matrix degradation after wounding that leads to healing.
The MMP-9 levels after vesicant exposure are enhanced abnormally. The MMP
inhibitors, doxycycline and minocycline, were employed as effective countermeasures to
inhibit the prolonged upregulation of MMP-9. To determine whether activation of
ADAM17 and MMP-9 are due to ERK signaling, the inhibitor PD98059 was assessed.
When used immediately after exposure, the compound was able to inhibit the ability of
ERK to phosphorylate the cytoplasmic domain of ADAM17, thereby inhibiting collagen
XVII cleavage. MMP-9 upregulation after 24 hrs was also inhibited as a downstream
affecter of the ERK pathway when PD98059 was used. These experiments identify which
countermeasures are the best candidates to test in vivo in rabbits exposed to sulfur
mustard, and explore a mechanism of how mustards affect the extracellular matrix of the
epithelial-stromal junction.
Dedication

To my friend and colleague Lakshmi Raman. I will never forget the support and laughter she gave me. Our time together was too short. I wish I could share this with her, but I will cherish the memories and take them with me.

To my parents and brother: Stephanie, Al and Jeff DeSantis, who have supported everything I’ve done since I was born. I can’t begin to express enough joy for being part of this family. Also, my Nanny Louise who always told me I was beautiful, and told everyone how proud she was of me for “cutting up eyeballs”.

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Our laboratories comply with all laws, regulations, guidelines and polices pertaining to the Office of Laboratory Animal Welfare (OLAW). At Rutgers University, OLAW Assurance #A3262-01 is approved through 3/31/2014. The Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accreditation has been maintained since 7/8/94. Battelle Biomedical Research Center is OLAW approved with Assurance #A3034-01 through 9/30/13, and their AAALAC accreditation has been maintained since 1/31/1978. As Association for Research in Vision and Ophthalmology scientists, we comply with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and all applicable local laws (see http://www.arvo.org/eweb/dynamicpage.aspx?site=arvo2&webcode=AnimalsResearch).
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LIST OF ABBREVIATIONS

ADAM  a disintegrin and metalloprotease
BMZ   basement membrane zone
Ca^{2+}  divalent calcium ion
CEES  2-chloroethyl ethyl sulfide
DMEM  Dulbecco’s modified eagle medium
DOX   doxycycline
EGFR  epidermal growth factor receptor
ERK   extracellular signal related kinase
GPCR  G protein coupled receptor
H&E   hematoxylin & eosin
HG    hydrogel
IOP   intraocular pressure
MAPK  mitogen activated protein kinase
MEK   mitogen activated protein kinase/ extracellular signal related kinase kinase (MAPK/ERK kinase)
MMP   matrix metalloproteinase
NM    nitrogen mustard
nmol  nanomoles
PBS   phosphate buffered saline
PBST  phosphate buffered saline + tween
PD    MEK inhibitor PD98059
PK    penetrating keratoplasty
<table>
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<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>SM</td>
<td>sulfur mustard</td>
</tr>
<tr>
<td>T735</td>
<td>threonine position 735</td>
</tr>
<tr>
<td>TACE</td>
<td>TNFα converting enzyme</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>transforming growth factor β1</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>divalent zinc ion</td>
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CHAPTER I: GENERAL INTRODUCTION

1.1 The importance of a clear cornea

Sight is among the primary senses that people do not take for granted. When we first meet someone and when we talk to someone, it is polite to look them in the eye. When we see something frightening or shocking, we reflexively cover our eyes to protect ourselves from what we are seeing. Loss of vision is a devastating handicap that robs us of the ability to drive a car, distinguish the look on someone’s face, or to look at old photos. A 2002 Prevent Blindness America study found that blindness ranked third (after cancer and heart disease) as people’s major fear (Leonard, 2002). A 2006 Eli Lilly study showed that diabetics are more concerned about losing their sight than dying. Blindness is associated with feelings of frustration, loss of independence, depression, loss of self-confidence, and strained relationships according to the release (MacGregor, 2006).

The eye is not perfectly round in shape. The cornea is the most anterior portion of the eye. It is steeply curved to allow bending and focusing of light that enters the eye. Figure 1.1 shows the structure of the cornea and its relation to the eye. The cornea provides 75% of our focus, while the lens (posterior to the cornea) is responsible for the fine adjustments. Although only about 1 mm thick in humans, the cornea is a strong structure containing three main layers. The anterior layer, the epithelium, regenerates rapidly after trauma. Trauma to the middle layer, the stroma, will usually result in scarring because of slower cell renewal. If the scarring occurs in line with the pupil, loss of vision can occur. The posterior layer of the cornea, the endothelium, provides a pathway for nutrient uptake and waste removal via simple facilitated diffusion and
secondary active transport (Goldberg et al., 2008). The endothelium keeps the cornea relatively hydrated (Klintworth, 1977). Damage to the endothelium will result in edema, which causes corneal clouding, and impaired vision (Goldberg et al., 2008) due to fluid being able to gain access to the stroma and swelling it, disrupting the lattice of the stromal collagen fibrils. The fibril structure gives the cornea its transparency. Fluid leaking into the cornea is counterbalanced by the endothelium, which generates fluid secretion through ion transport and aquaporin channels that counterbalance the continuous leaking of fluid into the corneal stroma (Bonanno, 2003; Verkman, 2005).

The cornea and the lens must remain clear to allow the retina to process what we are seeing, and direct the information to the brain. Corneal transparency is maintained by the geometric array of collagen fibers in the stroma with bands of fibrils at 90° angles to each other. The arrangement of fibrils allows passage of light while minimizing back scatter. The stroma is theoretically similar to the dermal layer in the skin, except for the composition of collagens used in the fibrils and their strictly maintained and highly organized architecture. In the cornea, the epithelial-scleral junction is similar in components to the epidermal-dermal junction in skin. In skin sulfur mustard causes a disruption at the lamina lucida of the basement membrane (Z. Zhang et al., 1995). Stromal fibrils are heterotypic complexes of type I & type V collagen arranged in parallel layers with respect to one another. Within each layer are specialized proteoglycans with unique water-holding properties (Fini et al., 2005). Corneal stromal collagens undergo unusually slow rates of turnover and the fibroblasts of the stroma (keratocytes) replicate at extremely slow rates, unlike the epithelium. However, a cornea totally denuded of epithelium may heal within three days. Stromal injuries can persist for more than 5 years
(Ljubimov et al., 1996; Maguen et al., 2008). A clear cornea suggests that the stroma has not been damaged. An opaque cornea signifies significant stromal damage or endothelial dysfunction, and may require a corneal transplant.

Drug diffusion into the eyes from the systemic circulation is slow and inefficient. The blood-ocular barriers (the blood–aqueous and the blood–retina barrier), protect the eye but also prevent drug distribution to the anterior and posterior chambers, limiting bioavailability. Most drugs applied to the eye surface as solutions have ocular bioavailability in the range of about 10% with most of the drug being cleared by local systemic absorption (Goldberg et al., 2008). Solutions are in contact with the eye surface for a very short period of time because of punctual draining, and the tear film quickly washes them away. The contact time, local drug concentration, and duration of action, can be prolonged by designing topical formulations with higher viscosities. The ideal drug delivery system for corneal wound repair should be non-toxic, transparent, easy to administer, have no effect on ocular structural integrity, provide a microbial barrier, and decrease the time of wound healing (Anumolu et al., 2010).

1.2 How the cornea heals

The cornea is immune privileged. The ocular immune privilege is a product of anatomical, physiological and immunoregulatory processes that inhibit both the adaptive and innate immune systems. When the overlying epithelium is damaged or infected, corneal stromal cells undergo apoptosis as part of the healing process. Epithelial cells provide keratocytes with a wide variety of anti-inflammatory and immunosuppressive molecules including Fas ligand (FasL or CD95L) for deletion of infiltrating inflammatory
cells, transforming growth factor-β (TGF-β) for inhibition of T cell proliferation, macrophage migration inhibitory factor for inhibition of natural killer cells, α-melanocyte-stimulating hormone for inhibition of delayed-type hypersensitivity (DTH), and complement regulatory proteins (Niederkorn, 2003).

In severe corneal injuries, TGF-β results in stromal fibrosis, and the fibrotic repair tissue fills and seals wounds, causing excessive scarring and contracture in the cornea. This process does not restore normal function as contraction alters shape and interferes with the focusing light on the retina (Fini et al., 2005). In skin, platelets released from the vasculature are trapped within the matrix producing a scab. Upon injury of the avascular cornea, cleavage of fibrinogen to fibrin occurs, and is deposited on the wounded surface. Failure to remove the fibrin by plasminogen results in corneal opacity, scarring, and neovascularization (Ayala, 2007; Drew et al., 2000; Kao et al., 1998).

The cornea is able to respond to injuries in other ways. After a chemical injury, corneal ulceration occurs as a result of a series of biochemical and physiological events. These include local ascorbic acid and fibroblast deficiencies, neutrophil infiltration and the release of enzymes. Both acid and alkali cause immediate corneal damage as a result of tissue interaction with the hydrogen or hydroxyl ions. Although alkali injuries tend to be more severe, many of the same pathologic processes occur in acidic ocular injuries. Collagen fibrils shorten, which leads to a rapid increase in intraocular pressure (IOP) that may be sustained for several hours. Coagulation of the corneal epithelial surface creates a barrier for further penetration of chemicals. Mild chemical burns induce erosion of the epithelium and faint corneal haziness, but often no ischemic necrosis of the conjunctiva or sclera. In mild and moderate burns, the limbal stem cells are lost, and the scleral
epithelium begins to re-epithelialize the ocular surface. This coverage is followed by corneal neovascularization. Although neovascularization can cause significant or even total loss of vision, its occurrence is desirable in these burns because the vessels help stabilize the anterior eye and prevent the development of ulceration and perforation. In more severe burns, a localized loss of fibroblasts occurs, leading to a defect in the production of new collagen and glycosaminoglycans (Burns et al., 1989).

1.3 Dangers of Sulfur Mustard Attacks

The reality of chemical and biological terrorism in our society emerged with the 9/11 terrorist attacks and the anthrax being delivered to targets by US mail. The threat of civilians becoming exposed to terrorist agents in trains and subways is unfortunately expected. Sulfur mustard (aka 2, 2-dichlorodiethyl sulfide, HD, SM or mustard gas) is a potent vesicant (blistering agent) and terrorist agent used in warfare since World War I. Sulfur mustard chemical threats are likely because it is relatively easy to manufacture from common materials. Trains, subway cars, and other confined areas are vulnerable to mustard gas attack. Terrorist attacks on civilians in Iran in the 1980s have increased our awareness of warfare agents and the need to develop countermeasures to protect civilians, as well as military personnel.

Sulfur mustard (SM) targets the eyes, lungs and skin, but the eyes are the most sensitive organ to the agent. As in skin, the basal epithelial cells, basement membrane zone, and the anterior stroma become damaged. In skin, however, sulfur mustard causes a separation of the cell layers at the basement membrane zone, which becomes filled with
fluid and forms a blister. The cornea does not form blisters, but instead forms microbullae (micro blisters).

Chemical warfare has been present since the Chinese began using arsenical smoke as a weapon in 1000 BC. Like arsenic, sulfur mustard is presently a widely used terrorist warfare agent. It was one of the major agents used during World War I because it is relatively easy to produce, causes devastating effects, and large stockpiles are still present in various countries. SM is a yellow, oily liquid. While the distilled form has no odor, the technical grade smells like garlic or mustard (Balali-Mood et al., 2005). SM is not soluble in water, but has good solubility to alcohols or organic solvents. Its slow hydrolysis means it is highly persistent and stable in rainy conditions. It is heavier than air and tends to concentrate in low-lying areas for up to 5 days (Kehe et al., 2005). Its half-life in the body is about 3.5 hours, dermatotoxicity increases with increasing temperatures, and it can be carried long distances by wind.

Sulfur mustard was first synthesized around 1822. It is easily made from products typically used to make plastics. In 1860 it was noted to have vesicant properties, and SM was termed a chemical warfare agent shortly thereafter. It is made from the addition of sulfur dichloride to ethylene for form 2-chloroethylsulphenyl chloride, which is then added to a second molecule of ethylene (Balali-Mood et al., 2005). It was first used on July 12, 1917 in a field near Ypres, Belgium during WWI. More than 1 million mustard shells were fired at the Germans over the next 10 days. Approximately 1,200,000 soldiers were exposed to mustard gas during that war and about 400,000 soldiers required prolonged medical observation.
Studies of Iranian soldiers and other victims of sulfur mustard poisoning during the Iran-Iraq war of the 1980s have shown that effects have a short delayed onset of 2 - 24 hrs and can reoccur over many years. Erythema, edema, photophobia, visual deterioration and ocular ulceration (Genersch et al., 2000; Javadi et al., 2005; Kehe et al., 2005) can occur with a latency period between outbreaks. The delayed outbreaks are possibly due to loss of limbal stem cells, and failure of new cells to re-epithelialize. Between 75-90% of SM-exposed victims experienced ocular injuries. Eyes have a much lower threshold dose (12 µg x min/l) compared to skin (200 µg x min/l) (Kadar et al., 2001; Papirmeister et al., 1985; Petrali et al., 2000; Petrali et al., 1997; Pleyer et al., 1999). Corneas do not form blisters because following corneal swelling (increased thickness and hydration), endothelial fluid expulsion re-establishes normal corneal hydration. In other words, secretion of fluid will exceed the leak into the cornea (Bonanno, 2003).

Animal studies have helped to elucidate additional facts about exposure. In mice, acute clinical responses to ocular sulfur mustard occur 2 – 4 hrs after exposure, and consists of photophobia, eyelid swelling, conjunctivitis, corneal edema, cellular infiltration (Amir et al., 2000) and eventual loss of the epithelial layer around 24 hr post exposure. The initial sensation in humans is reported as vapor entering the eye without immediate pain. Effects can vary from mild to severe, and are related to the dose and duration of exposure.

Most SM-exposed victims eventually have complete resolution, but severe lesions can reoccur with delayed onset and ongoing chronic inflammation. Mustard gas has a direct inflammatory effect by inducing the release of inflammatory mediators, and these
inflammatory/ immune reactions are in part responsible for the chronic or delayed course (Kadar et al., 2001). Patients from the Iran/Iraq war presented recurrent lesions in the cornea sometimes 8 to 25 years later. These recurrent effects years after initial exposure are referred to as “delayed mustard gas keratopathy” (Pleyer et al., 1999). A common phenotype of delayed mustard gas keratopathy is a hazy central cornea, corneal calcification, scarring and opacification (Richter et al., 2006). Opacification of the cornea is seen predominantly in the lower and central portions. Protection by the eyelid perhaps spares the upper part of the cornea. Treatments are palliative and are aimed at reducing ocular irritation and improving vision. Ocular lubricants, therapeutic contact lenses and penetrating keratoplasty have been attempted (Pleyer et al., 1999). However, there is no real therapy that reduces the effects of mustards.

Penetrating keratoplasty, or corneal transplant with a matched donor cornea seems to be the only effective treatment to avoid recurrences after sulfur mustard exposure, and these generally fail if neovascularization has already occurred. Patients experience pain with mustard gas injury since the cornea has such a high density of nerves, and transplant is capable of immediate alleviation of pain and improved vision (Richter et al., 2006). On the other hand, there is a risk of transplant rejection from the mustard-induced chronic inflammation with cellular infiltration, vascularization and damage to the limbus. Transplantation is more successful if the limbal stem cells are present (Genersch et al., 2000; Javadi et al., 2005). It is speculated that lack of stem cells could be responsible for the latent injury seen in mustard gas keratopathy. Figure 1.2 shows calcification from delayed mustard gas keratopathy and subsequent clearing after penetrating keratectomy.
In the absence of a donor cornea, autorotation keratoplasty and denervating the cornea are some other, but less successful, options. In autorotation keratoplasty, the necrotic and scarring lesions are moved to the center of the cornea to stabilize the periphery for subsequent penetrating keratoplasty. The idea behind denervation is that pain due to necrosis and damaged epithelium irritating the adjacent nerves will be ended by their removal (Richter et al., 2006).

Sulfur mustard analogs, 2-chloroethyl ethyl sulfide (CEES, half mustard) and nitrogen mustard (NM, mechlorethamine), are less potent alternatives, used in laboratory settings that are not approved to use SM (Figure 1.3). Closely related chemically and toxicologically to SM, nitrogen mustard is currently a used as a chemotherapeutic agent (known as Mustargen) because of its cytotoxicity (Balali-Mood et al., 2005). Sulfur mustard and nitrogen mustard are bifunctional (2 arm) alkylating agents, which attack nucleophilic sites in the extracellular space, on membranes and within cells (Kadar et al., 2001). We used CEES and NM to pre-screen effectiveness of countermeasures before moving forward with in vivo rabbit studies that were performed at Battelle Biomedical Research Center.

1.4 Experimental model

The ex vivo organ culture model is the most effective way to simulate the live cornea because all cell layers remain present, intact and differentiated. The ex vivo culture model was originally adapted from Foreman et al. (1996). The cultures were set up by dissecting the cornea from young adult rabbit eyes (PelFreez) with 2-3 mm of scleral rim still attached around the outside of the cornea. Placing the cornea epithelial-
side down, the endothelial side was filled with agar. Once the agar solidified, the cornea was flipped over and placed in a culture dish epithelial-side up. Medium was added only up to the scleral rim to keep the epithelial cells air-lifted and differentiated (Figure 1.4). After an overnight incubation at 37°C, 5% CO₂ the cornea was exposed drop wise to 20 nmol CEES to induce mild injury or 100 nmol NM to induce moderate to severe injury. Two hours post exposure the contaminated medium was removed from the dishes and replaced with fresh medium, or medium plus a counteragent. The counteragent applications were repeated a total of four times in 24 hours, beginning at 2 hours post exposure.

Epithelial cell culture alone was not sufficient. Epithelial cell culture fails to mimic corneal wound healing since there is only a single layer of cells, epithelial migration is absent, and the role of keratocytes in the stroma is not considered. Our model needed to contain stromal and basement membrane components in addition to epithelial cells. Submerged organ culture models can cause a reduction in epithelial cell layers, epithelial and stromal edema and endothelial and keratocytes deterioration. The air interface organ culture technique is considered to be the most appropriate model for the maintenance of the cornea, permitting long-term culture with maintenance of epithelial integrity. It has already been tested for endothelial and stromal cell viability (Foreman et al., 1996). The appearance of a cloudy cornea signifies damage induced by NM (Figure 1.5). This effect could require a corneal transplant if it occurred in vivo. In vivo studies are expensive and require elaborate maintenance procedures, and are more proficient when candidate drugs have already been identified.
The rabbit cornea culture model’s observed ex vivo effects from CEES and NM have been compared with the clinical effects from SM reported in animal models (Anumolu et al., 2010; Gordon et al., 2010; Kadar et al., 2009; Kadar et al., 2001; Richter et al., 2006). Histological examination revealed that each vesicant caused the same injury, only to different degrees. In general, a very thin irregular epithelium was observed with focal areas of absence (Figure 1.6). The results demonstrated that CEES and NM could be used to study sulfur mustard injury, and could be used to identify and validate possible counteragents to ameliorate these effects. Our goal was to screen a variety of drugs, and proceed with the most effective counteragents to in vivo rabbit eyes exposed to sulfur mustard. This is critical because there are currently no FDA approved treatments for mustard compound exposures to the cornea.

1.5 Matrix Metalloproteinases as targets for therapy

By determining the cellular targets of injury caused by sulfur mustard, we can begin to pinpoint countermeasures. One attractive candidate target is the family of matrix metalloproteinases (MMPs). MMPs are responsible for degrading the extracellular matrix after wounding to allow provisional matrix deposition that supports cell to migration and repair the region (C. Chang et al., 2001). MMP activity can re-establish conditions that permit reconstruction of the parallel layers of collagen across the injured region (Fini et al., 2005), restoring corneal clarity.

MMPs are synthesized as “pre-proMMP”s containing an N-terminal signal “pre-domain” region that supports protein secretion, after which, the enzyme exists as the inactive “proMMP”. The enzyme becomes active upon cleavage of the “pro-peptide”
domain by other MMPs or proteases. This is referred to as the “cysteine switch” since the pro-domain contains a conserved cysteine sequence (Nagase et al., 1999; Puerta et al., 2004). The catalytic domain is composed of $\beta$-sheets, $\alpha$-helices and bridging loop structures. The active site contains two Zn$^{2+}$ ions. One serves a structural role, and the other is the site of peptide hydrolysis (Babine et al., 1997; Kim et al., 2005; Puerta et al., 2004; Whittaker et al., 1999).

Non-injured, or non-stimulated cells, produce no or low amounts of MMPs, except for MMP-2, which is constitutively expressed. MMP-9 becomes activated by growth factors that induce cellular responses by activating a variety of intracellular signal cascades and pro-inflammatory cytokines, transforming growth factor-$\beta$1 (TGF-$\beta$1), interleukin-1 (IL-1) and tumor necrosis factor $\alpha$ (TNF$\alpha$), after injury (Hanemaaijer et al., 1998). MMP-9 may be responsible for extracellular matrix remodeling in sterile corneal ulceration, dry eye-associated disease (Fini et al., 1998; Fini et al., 1996; Matsubara et al., 1991) and other corneal diseases.

MMP-9 (aka gelatinase B) is particularly important for remodeling and degradation of basement membrane zone components (Giasson et al., 1994a, 1994b; Ollivier et al., 2007). Many corneal disorders may occur when there is an imbalance between proteinases and proteinase inhibitors in favor of the proteinases, causing pathologic degradation of stromal collagen and proteoglycans in the cornea. When the epithelium is damaged, the corneal stromal cells undergo apoptosis or transition to a repair phenotype (Fini et al., 2005; West-Mays et al., 2006). As keratocytes renew slowly, any undesirable changes to the tissue are limited (Puerta et al., 2004; Vu et al., 1998).
In skin, sulfur mustard causes progressive upregulation of MMP-9 through the 7th day post-SM exposure (Shakarjian et al., 2006). Similar effects have been noted in animal studies of the cornea (Kadar et al., 2009). Since MMPs degrade components of the extracellular matrix, uncontrolled expression can result in tissue injury (Birkedal-Hansen et al., 1993; Lukashev et al., 1998; Wilson et al., 2004). These observations led us to use MMP-9 inhibitors to attenuate the amount of damage caused by over activated MMPs induced by CEES and NM.

Tetracyclines are known MMP inhibitors. They have been used systemically and locally for the treatment of various diseases. Tetracyclines exert biological functions in addition to their antimicrobial properties, by inhibiting the catalytic activities of collagenases and gelatinases (Golub et al., 1991) preventing tissue degradation.

Doxycycline is a tetracycline derivative used off-label for ocular surface diseases such as infection, rosacea and corneal ulceration (Akpek et al., 1997; Gooz et al., 2006). Its use is topical since oral administration does not result in a therapeutic dose reaching the cornea. However, it is not FDA approved for ocular use. It has been found to be an effective MMP-9 inhibitor in human endothelial cells (Hanemaaijer et al., 1998), skin keratinocytes (Uitto et al., 1994) and prostate cancer cells (Lokeshwar, 1999). Minocycline is commonly prescribed for bacterial infections and skin irritations such as acne, but has not yet been studied as an ophthalmic drug. Minocycline has a shorter systemic half-life than doxycycline when ingested orally (FDA website).

There is some speculation as to the mechanism of MMP-9 upregulation by mustard injury. TGF-β1 can stimulate MMP-9 expression (Kim et al., 2005). Activated cellular responses by TGF-β receptors are transduced through intracellular signaling
pathways during inflammation and wound healing. After ligand binding, the type II TGF-β receptor phosphorylates the type I TGF-β receptor. The activation of the type I receptor triggers the phosphorylation of cytoplasmic proteins. Mitogen activated protein kinases (MAPK) are serine-threonine protein kinases that are activated by diverse stimuli ranging from cytokines, growth factors and cellular stress. MAPK are typically organized in a three-kinase module: a MAPKK kinase which phosphorylates a MAPK kinase which phosphorylates a MAPK. Kim et al. (2005) found that doxycycline can inhibit TGF-β1 induced MMP-9 activity, possibly through the Smad or extracellular signal related kinase (ERK) signaling pathway in human corneal epithelial cells.

ERK has been shown to be involved in corneal epithelial wound healing (Glading et al., 2000; Klemke et al., 1997). ERK is activated by the Raf/MEK/ERK phosphorylation cascade. Western blots from primary human corneal epithelial cells indicate that 10 ng/ml TGF-β1 was able to induce phosphorylation of ERK1/2 within 15 minutes, peaking at 60 minutes after exposure. The inhibitory action of doxycycline on pERK1/2 was comparable with that of the ERK inhibitor, PD98059 (Kim et al., 2005).

The MMP-9 gene contains activator protein-1 (AP-1) binding sites in its promoter region (Fini et al., 1998; Vu et al., 1998). AP-1 transcriptional activity is typically regulated by MAPK. Therefore, upstream triggers of MMP-9 most likely involve MAP kinases (Fini et al., 2005). Doxycycline may also function as a MAPK inhibitor, attenuating the activation of MMP-9 by TGF-β1. These findings support the use of doxycycline for ocular surface diseases in which TGF-β1 may play a role in pathogenesis.
1.6 **ADAM17 induces shedding of collagen XVII**

The basement membrane zone (BMZ) is defined as the basal surface of the basal epithelial cells, the basement membrane, and the upper-most region of the stroma. Collagenous transmembrane proteins in the basal surface of basal epithelial cells function as both surface receptors and matrix molecules. They are homotrimers of α-chains which contain an N-terminal intracellular domain, a hydrophobic transmembrane stretch and a large extracellular C-termini containing triple helical domains (Franzke et al., 2003).

The molecular structure of basement membranes is consists mainly of type IV collagen laminin networks and proteoglycan networks. Laminin-332 (previously known as laminin-5) binds to the hemidesmosomal components α6β4 integrin and collagen XVII, ensuring *in vivo* maintenance of epithelial adhesion to the basement membrane. Mutations in the genes encoding laminin-332 chains, α6β4 and collagen XVII result in hereditary blistering diseases called epidermolysis bullosa (Darling et al., 1997). Laminin-332 is the major constituent of the anchoring filaments and is a heterotrimer composed of α3, β3, and γ2 chains.

Collagen VII is the major component of anchoring fibrils in the stroma. The fibrils are bound to the distal regions of laminin-332, indirectly connecting the collagen with the hemidesmosomal portion of the anchoring complex. (Jones et al., 1998; Nievers et al., 1999). The role of the anchoring fibrils is vital for the function of the anchoring complex. Anchoring fibrils extend from the basement membrane to structures called anchoring plaques in the connective tissue matrix or loop back to the basement membrane, connecting it to the underlying connective tissue. The anchoring fibrils bind to, or wrap around collagen fibrils in the stroma, making a series of interactions that
make epithelial detachment difficult. A schematic of these interactions is shown in figure 1.7.

Collagen XVII (BP180, BPAG2) was the first transmembranous collagen shown to be shed from the cell surface. Collagen XVII mediates adhesion of epithelial cells to the underlying basement membrane of cornea or epidermis. It is closely associated with α6β4 integrin in hemidesmosomes (Borradori et al., 1999; Franzke et al., 2002). During cell migration and in wound healing, the epithelial and stromal connection must be severed. Collagen XVII molecules are cleaved in order to free cells so they may migrate and close wounds. Provisional matrix molecules make new basement membrane for cells to migrate upon. At a certain stage in healing, cleavage of collagen XVII prevents reestablishment of the epithelial-scleral junction.

Collagen XVII is a trimer of three 180 kDa chains (Franzke et al., 2002). In humans, collagen XVII molecules are cleaved from their full length to a 120 kDa extracellular fragment at the noncollagenous NC16A domain (Borradori et al., 1996; Burgeson et al., 1997; Hirako & Owaribe, 1998; Hirako, Usukura, et al., 1998; Jones et al., 1994). The intracellular domain of collagen XVII interacts with the β4-integrin subunit and is essential for incorporation of the molecule into the hemidesmosome. The extracellular domain containing NC16A binds to the α6-integrin subunit (Borradori et al., 1999). The extracellular linker domain, NC16A, between the plasma membrane and the collagenous Col15 domain, is functionally important for both ectodomain shedding and proper folding of the triple helical structure of collagen XVII (Franzke et al., 2009).

As noted, the ectodomain of collagen XVII is proteolytically shed from the cell surface, releasing a shorter form of the collagen into the extracellular matrix. This
process is catalyzed by a group of enzymes collectively referred to as *sheddases* (Black et al., 1998; Franzke et al., 2003). Maintenance of epithelial-stromal (or epidermal-dermal) adhesion through multiple protein-protein interactions is a pivotal role for collagen XVII, and is demonstrated by skin diseases in which the lack of protein or loss of function of collagen XVII leads to skin blistering. One such disease is generalized atrophic benign epidermolysis bullosa (GABEB) (Floeth et al., 1998; Gatalica et al., 1997; McGrath et al., 1995; Schumann et al., 2000; Schumann et al., 1997).

The ADAM (a disintegrin and metalloproteinase) family of proteins are secreted and transmembranous proteins with important roles in regulating cell phenotype via their effects on cell adhesion, migration, proteolysis and signaling (Edwards et al., 2008). ADAM17 (aka TNFα converting enzyme, TACE) is a member of this family. Although matrix metalloproteinases (MMPs) and ADAMs both belong to the metzincin (zinc containing) family of metalloproteinas, they have structural differences in the amino acids surrounding their active site, as well as considerable overall structural differences. MMPs are associated with degradation of the extracellular matrix, while ADAMs are generally cell-associated or transmembranous and the substrates are more varied (Hooper, 1994; Newton et al., 2001). The activation pathway is predominant for the activation of several MMPs and appears to be mediated by furin and other pro-protein convertases. Pro-domain removal was once thought to be a prerequisite for activation of the ADAM17 pro tease, but it is now known that it may not be necessary (Franzke et al., 2003; Srour et al., 2003). In vivo disruption of ADAM17 or disruption of the ADAM17 gene resulted in the death of mice due to developmental defects such as impaired epithelial maturation and failure to fuse eyelids (Peschon et al., 1998).
ADAM17 is one enzyme responsible for collagen XVII cleavage. ADAM9, ADAM10 and ADAM17 are all thought to be partially responsible for collagen XVII shedding (Franzke et al., 2009). The prediction that ADAM17 activation leads to shedding of collagen XVII was confirmed with keratinocytes derived from ADAM17-deficient mouse embryos. Compared with age-matched normal murine keratinocytes, shedding of collagen XVII was reduced by ~40% in ADAM17 deficient cells (Franzke et al., 2004; Franzke et al., 2002). Because both collagen XVII and ADAM17 are transmembranous proteins, their plasma membrane microenvironment can influence shedding. Cleavage of the ectodomain releases the cell from its binding partners and influences cell detachment, differentiation and motility (Areida et al., 2001; Brynskov et al., 2002; Doedens et al., 2003; Hirako & Owaribe, 1998; Hirako, Usukura, et al., 1998; Merchant et al., 2008; Sunnarborg et al., 2002; Y. Zhang et al., 2000; Zimina et al., 2005).

Previous studies of sulfur mustard injury have also examined collagen XVII. In corneal studies 24 hr post-exposure, an antibody against bullous pemphigoid antigen (BPA), which binds to several sites on collagen XVII, was absent at sites where the epithelium had cleaved from the basement membrane (Petrali et al., 2000; Petrali et al., 1997). The microbullae formed after a SM exposure is due to the separation of the epithelium from the stroma. The shedding of collagen XVII permits the separations observed after exposure, and is caused by activation of ADAMs. Therefore we tested whether an ADAM17 inhibitor would decrease the degree of separation manifested after vesicant exposure.
Hydroxamates are commonly used zinc binding group (ZBG) inhibitors previously found to be effective against MMP activation. Hydroxamates bind the catalytic zinc (II) ion in a bidentate fashion (chelating ligands that binds to metal in two places). This blocks substrate access to the active site and renders the metal incapable of peptide hydrolysis. Hydroxamate compounds have been shown by our work to be promising countermeasures when used for 24 hr, beginning 2 hr post-vesicant exposure. Hydroxamates inhibit ADAM17 activation as evidenced by a decrease in collagen XVII cleavage commonly seen as microbullae after vesicant exposure.

1.7 ADAM17 is activated by an ERK-induced phosphorylation

As mentioned earlier, ERK has been implicated as an upstream regulator of MMP-9. Inhibition of ERK by PD98059 demonstrated similar inhibitory effects as the MMP-9 inhibitor, doxycycline (Kim et al., 2005). Direct interactions of intracellular protein kinases (such as ERK) with cytoplasmic domains of ADAMs modulate shedding activity (Diaz-Rodriguez et al., 2002; Izumi et al., 1998; Poghosyan et al., 2002). Activated tyrosine kinase receptors rapidly induce ectodomain shedding through induction of the ERK signaling pathway without the need for new protein synthesis (Fan et al., 1999; Gechtman et al., 1999; Gutwein et al., 2000; Umata et al., 2001; K. P. Xu et al., 2001).

An activating modification of ADAM17 is phosphorylation. Phosphorylations of the cytoplasmic domain serine and threonine residues have been previously studied. Phorbol 12-myristate 13-acetate (PMA) and epidermal growth factor (EGF) treatments induced ADAM17 phosphorylation at threonine 735 by MAPK, increasing cleavage of
the transmembrane TrkA neurotrophin receptor (Diaz-Rodriguez et al., 2002). In contrast, PMA and EGF induced serine 819 phosphorylation of ADAM17 by ERK (Fan et al., 2003; Soond et al., 2005). The ability of MAP kinase signaling pathways to activate shedding without the need for new protein synthesis and the regulation of ectodomain shedding by ADAM17 suggest that the cytoplasmic domain may act as a signal transducer that regulates shedding by the protease domain (via a conformational change) in response to intracellular activities (Fan et al., 2003).

ADAM17 was found to be physically associated with active ERK. ADAM17 coprecipitates with activated ERK in mesangial cells & kidney cells, as seen with activated ERK immunoprecipitation followed by ADAM17 western blotting (Bell et al., 2010; Gooz et al., 2006). ERK1/2 is already known to mediate ADAM-dependent heparin binding epidermal growth factor (HB-EGF) shedding (Yin et al., 2009). Part of my hypothesis is that a wave of vesicant-induced ERK is responsible for phosphorylation/activation of ADAM17, and induces collagen XVII shedding. This phosphorylation after nitrogen mustard injury is observed within 10 minutes.

The activation of ERK may occur as follows: injury causes release of nucleotides. Nucleotides (UTP/ATP) become substrates for the G-protein coupled receptor, P2Y2 (Boucher et al., 2010; Kudirka et al., 2007; Yang et al., 2004). The purinergic P2Y2 receptor activates phospholipase C (PLC) and phosphatidylinositol hydrolysis, generating diacylglycerol and inositol 1,4,5-triphosphate, which stimulates protein kinase C (PKC) and cytosolic calcium (Ca^{2+}) mobilization, respectively (S. J. Chang et al., 2008). Calcium influx appears to initiate the ERK activation. The injury-induced ERK may proceed to activate ADAM17.
Rationale for Thesis Research

The effects of sulfur mustard are well documented, but therapies for exposed individuals is lacking. The ocular effects of sulfur mustard can be devastating for the person afflicted. Accidental exposures have occurred as leaks in canisters in storage facilities, and from post-war containers in coastal waters, and the possibility of these compounds being used in wars and terrorist attacks is very real. Exposure to SM can cause blindness and panic in those exposed as well as those around them. The ability to identify an effective countermeasure in the case of accidental or terrorist exposures would be an important contribution to U.S. defenses. The goal is to prepare therapies in a form that allows their storage in emergency response centers.

Using a model that allows visualization of cell layers by histology, and components of the basement membrane zone by immunofluorescence and western blot analysis, our research explored of a mechanism of SM injury. By using analogs of SM, we can safely mimic the effects from an SM exposure and prescreen countermeasures. After exposures we found observable separations between the epithelium and the stroma in nitrogen mustard exposed corneas. MMP-9 signaling increases over time preventing cell re-attachment, as opposed to decreasing over time as in a scrape wound. We hypothesized that the epithelial-stromal separations are caused in part by ADAM17 activation and cleavage of the basement membrane anchoring protein, collagen XVII. By identifying MMPs and transmembranous complexes as targets of vesicants, we proceeded to test drugs that inhibit MMPs and ADAM17. The countermeasures effectively improved the appearance of the epithelial-stromal junction as seen by preserved
epithelial-stromal attachments and decreasing ADAM17 activity assay. Our experiments identify candidates for future testing in *in vivo* sulfur mustard studies exposed rabbit eyes. We are just scratching the surface of vesicant therapeutics. The question remains whether the treatments we identify will only work on acute injury.
Statement of Hypothesis

Microblisters, or microbullae, caused by mustards are the result of ADAM17 cleaving collagen XVII, which, in turn, leads to separation of the epithelial and stromal layers. In addition, healing of this injury occurs slower than other corneal injuries because MMP-9 activity is prolonged, preventing wound repair of the basement membrane zone. I propose that inhibitors of ADAM17 and MMP will facilitate healing, and will test this in aims 1 and 2. In addition, I hypothesize that the activation of ADAM17 and MMP-9 by vesicants is quickly activated by ERK. ERK phosphorylates threonine 735 in the cytoplasmic tail of ADAM17, and induction of ERK signaling activates MMP-9 (possibly through induction of TGF-β). If true, the inhibition of ERK by PD98059 should reduce ADAM17 and MMP-9 activity levels.
SPECIFIC AIMS

Specific Aim 1: Determine whether the activation of ADAM17, the protease responsible for collagen XVII cleavage, is responsible for epithelial-stromal separation after mustard exposure, and determine whether ADAM17 inhibitors can attenuate cell layer separation.

Immunofluorescence assays have shown an upregulation of ADAM17 levels 24 hours after exposure. Changes in collagen XVII immunofluorescence have indicated cleavage in the basement membrane after exposure. Four ADAM17 inhibitors will be evaluated in the corneal organ culture model system. Effectiveness of the inhibitors will be evaluated by histological examination and fluorometric ADAM17 activity assay. Dose-response curves will be used to compare efficacy at increasing doses of inhibitor. Fluorometric ADAM17 activity assays will be used to measure precise amounts of active ADAM17, as well as used to determine IC50.

Specific Aim 2: Determine whether injury to the cornea is in part due to upregulation of matrix metalloproteinase-9 (MMP-9) remodeling of the extracellular matrix after wounding. If so, can the MMP-9 inhibitors, doxycycline and minocycline, contribute to timely healing of injuries?

An increase in MMP-9 activity is seen after SM exposure in skin (Shakarjian et al., 2006). MMP-9 initially helps remodel the tissue, but if activated too long, it is a source of tissue damage. Immunofluorescence analysis will be examined to visualize the area of upregulated protein and fluorometric activity analysis will be used to quantify
MMP-9 activity. The optimal dose of the MMP inhibitors doxycycline and minocycline will be determined, then the agents will be delivered as ophthalmic drops and in hydrogel form, beginning 2 hrs post exposure. Histological and immunofluorescence evaluations will determine the effectiveness of these compounds. Doxycycline results in organ cultures will be compared to in vivo studies of sulfur mustard exposure performed at Battelle Biomedical Research Center, Columbus, Ohio.

**Specific Aim 3:** Determine whether after vesicant exposure, ADAM17 and MMP-9 become activated by ERK following vesicant exposure. If so, determine whether the ERK inhibitor PD98059 reduces the activity of ADAM17 or MMP-9 after mustard exposure.

Previous studies have shown that ADAM17 is phosphorylated at threonine 735 (pT735) leading to its activation (Diaz-Rodriguez et al., 2002). Since injury-induced ERK is transient, we will use the MEK inhibitor, PD98059, immediately after exposure to determine when the cytoplasmic tail becomes phosphorylated, and inhibition is not possible. Analysis will include: (1) fluorometric assessment of ADAM17 activity; (2) western blots of pT735, the extracellular domain of ADAM17, and ERK; (3) ERK pull down assays to evaluate interactions between the cytoplasmic domain of ADAM17 and pERK. Signaling after injury can induce ERK and ultimately MMP-9, elucidating another application of the ERK inhibitor. Immunofluorescence and MMP-9 activity assay will be used to examine the role of MAPK in MMP-9 activation.
Figure 1.1 – The structure of the eye and the cornea. The cornea is located in the front the eye, which is not perfectly round in shape. The cornea contains three main layers, the epithelium, the stroma and the endothelium. While humans have an acellular Bowman’s layer just below the epithelium, rabbits do not appear to have this extra layer.

Figure 1.2 – Victims exposed to sulfur mustard often experience recurrent injuries known as “delayed mustard gas keratopathy”. The patient below is 12 years after mustard gas exposure. Massive calcification with opacification in both eyes, and painful corneal erosions (1.2A), right eye and (1.2B), left eye. Penetrating keratoplasty (cornea transplant) in the left eye three years after surgery (1.2C) which restored clarity of vision and reduced pain (Richter, et al, 2006).
Figure 1.3 – The structures of sulfur mustard and its derivatives. Since our laboratory is not approved to use sulfur mustard (only 4 in the US are), we used less potent derivatives: nitrogen mustard (NM) which induces a moderate injury, and 2-chloroethyl ethyl sulfide (CEES) which induces a mild injury. Sulfur mustard is a 2 arm (bifunctional) alkylator. CEES, or half mustard, lacks the second chloride atom so it is considered a 1 arm alkylator. And NM has a nitrogen atom in exchange for the sulfide atom. This is also a chemotherapeutic agent known as Mustargen. Modified from Shakarjian M P et al. Toxicol. Sci. 2009;114:5-19.
Figure 1.4 – The rabbit corneal organ culture model. The cornea is dissected from rabbit eyes with 2-3 mm of scleral rim still attached. The concavity of the endothelium is filled with DMEM and 0.75% agar to maintain shape. Medium is only added up to the scleral rim to keep cornea air lifted and cells differentiated. Medium, CEES, NM and counteragents were all added as 10 µl drops, drop-wise, over the central cornea.
Figure 1.5 – Eagle eye photos indicating cloudiness of corneas after nitrogen mustard (NM). The background “E” page is used to test clarity of vision through the cornea. This is more effective than a simple black or white piece of paper. Transparency of the cornea indicates all layers are functioning and there is no damage. After NM exposure, there is a definite change in cloudiness of the cornea, indicating stromal damage.
Figure 1.6-- H&E 10x micrographs show typical sections. The top section is an unexposed cornea simply cultured for 48 hrs. The second 24 hr post a 2 hr CEES exposed cornea, and we commonly see these areas of invagination where the epithelium dips into the stroma, and very few areas of epithelial separation. The next cornea is 24 hr post a 2 hr NM exposed cornea, and the most common phenotype here is the epithelium separating from the stroma in multiple spots if not the entire cornea. The last cornea is from Battelle, where they did a 4 hr SM exposure and 24 hrs later. The epithelium is completely separated from the stroma.
Figure 1.7 – The adhesion complex includes the hemidesmosome (yellow), the anchoring filaments (orange) and the anchoring fibrils (green). Note the bulbous head of collagen XVII extending from the hemidesmosome in the basal cell into the basement membrane before cleavage, and the cleavage just below the hemidesmosome (red box).

Modified from Kaisa Tasanen-Määttä, M.D., Ph.D., Collagen XVII in basement membrane biology and pathology (http://www.oulu.fi/iho/project2.html).
MATERIALS AND METHODS

Reagents

Rabbit eyes were purchased from Pel-Freez Biologicals (Rogers, AR). Agar, ciprofloxacin, HEPES buffer, RPMI 1640 vitamin solution, ascorbic acid, trizma base, EDTA, heptanal, 2-mercaptothiazoline, oleoyl chloride, hydroxyl-3-methoxyphenylacetic acid, CEES (also known as half mustard) liquid (cat. no. 242640), and NM powder (cat. no. 122564) were obtained from Sigma (St. Louis, MO). High glucose Dulbecco’s modified Eagle’s medium (DMEM), 100x MEM-NEAA, goat anti-mouse Alexa 488-tagged IgG, DAPI, and Prolong Gold were obtained from Invitrogen (Carlsbad, CA). Pen-Fix was from Richard Allen Scientific (Finland). Normal goat serum was obtained from Jackson ImmunoResearch (West Grove, PA). Optimal cutting temperature (OCT) embedding medium and cryomolds were from TissueTek, Sakura (Torrance, CA). Hematoxylin and Triton X100 were from Fluka. Sodium chloride was from JT Baker. Protease inhibitor cocktail tablets (EDTA free) (Cat#04 693 159 001) were from Roche (Nutley, NJ). LiquiTears™ (Major) were purchased from Medline. Innozyme TACE Activity Kit (Cat# CBA042) was purchased from Calbiochem (Germany). Furin inhibitors I and II (Cat#344930 & 344931), Fluorokine E Active MMP-9 kit, and antibodies against ADAM17 (extracellular, cat#MAB9304; cytosolic, cat# AF2129) were purchased from R+D Systems (Minneapolis, MN). Collagen XVII peptide Y0761 was designed by the Gordon laboratory, produced by Invitrogen, and used to generate antibodies in guinea pigs against full length rabbit collagen XVII. Mouse anti human
monoclonal antibody anti-MMP-9 from was Millipore (Bedford, MA). Fluorometric MMP-9 activity assay was from R+D Systems. Antibodies against pT735, pERK and ERK (Cat#s ab60996, ab50011 & ab54230, respectively), were purchased from Abcam (Cambridge, MA). Nitrocellulose membranes (0.2 µm) and secondary goat-anti-rabbit HRP, goat-anti-mouse HRP and donkey-anti-goat HRP were from Biorad (Hercules, CA). Chemiluminescent substrate and films were from Thermo Scientific. PD98095 (Cat# 9900) was from Cell Signaling Technology (Boston, MA). Doxycycline hyclate was from Professional Compounding Centers of America (PCCA) (Houston, TX).

Organ culture of corneas

A rabbit cornea organ culture model system was used to evaluate healing after exposure to NM, followed by subsequent treatment with doxycycline or minocycline in the form of drops or hydrogel. Rabbit eyes were dissected by PelFreez (Rogers, AR) and stored in 4°C DMEM (with penicillin, streptomycin, amphotericin B and gentamicin) for transport to the laboratory. The entire cornea along with 2 mm scleral rim were dissected from the eye and placed with the epithelial side facing down into spot plates containing a small amount of DMEM to prevent drying of the epithelium. The corneal endothelial concavity was then filled with DMEM containing 0.75% agar at 50 °C. This mixture was allowed to set (usually within 4 min). Corneas were then inverted and transferred to 60 mm sterile pyrex tissue culture dishes and kept at 37 °C in a humidified 5% CO2 incubator in the presence of medium up to the scleral rim. Medium contained 500 mL high glucose DMEM, 5 mg ciprofloxacin, 5 mL of 100x MEM-NEAA, 5 mL RPMI 1640 vitamin solution, 50 mg L-ascorbic acid, and 50 mg L-ascorbic acid-2-P. To moisten the
epithelium, 500 µL of medium was added drop wise to the surface of the corneal epithelium every 6-8 h. All agents were added drop-wise to the central cornea.

**Exposure of cultured corneas to vesicants**

CEES was used to induce mild injury. NM was used to induce moderate-to-severe injury. CEES was purchased in liquid form. A 2M solution was made by adding 24 µl full strength CEES with 76 µl absolute ethanol. One microliter of the 2M CEES was added to 1999 µl medium (1 mM). Each cornea received 10 µl, twice (20 nmols). Nitrogen mustard powder was aliquoted into small vials and first dissolved in saline to 100 mM, and then medium to 10 mM (10 µl; 100 nmol).

Applications of 100 nmol NM (dissolved first in saline to 100 mM and then medium to 10 mM; 10 µl = 100 nmol) were dropped onto the central cornea. This was allowed to remain on the cornea for 2 hrs, while in the incubator. The 2 hr time period approximately simulates the time that would pass before an exposure is recognized (based on the delayed times for tearing and pain). Corneas were then washed by removing contaminated medium and replacing with fresh medium.

**Treating the corneas with counteragents**

After exposure corneas were incubated at 37°C for 2 hrs. The 2 hr time period approximately simulates the time that would pass before medical help is sought, since an exposure is not immediately recognized due to the delayed time before tearing and pain. Medium was then removed from the bottom of the culture dish.
Chapter 2: Either 20µl fresh LiquiTears™ containing 0.1% of the total volume of DMSO, or 20µl of a 15 µM, 50 µM, 150 µM, 500 µM ADAM17 inhibitor (0.3, 1, 3, 10 nmol per application, respectively) was added to corneal cultures. These compounds (NDH 4385, 4409, 4417, 4450) were first dissolved in 0.1% DMSO, then LiquiTears™ was added drop wise over the central cornea. Corneas were allowed to recover for 24 h at 37°C, receiving a total of 4 drop wise applications of LiquiTears™ or LiquiTears™ containing 0.3, 1, 3, 10 nmol of drug.

Chapter 3: The cornea was subsequently treated with either LiquiTears™ ophthalmic solution (pH 7.4), or LiquiTears™ containing doxycycline or minocycline. Ophthalmic drops used in these studies were prepared immediately before use. A dose-response was established with 25 nmoles, 75 nmoles or 225 nmoles per day (3 applications of 50 µl of 4.1 µg, 12.5 µg or 37.5 µg respectively; over the subsequent 24 hrs after exposure) of doxycycline or minocycline solution added drop wise to the central cornea.

Chapter 4: PD98059 was chosen because both MEK isoforms can activate the downstream ERK kinases. They activate ERK1&2 via two phosphorylations in a –Thr183-Glu-Tyr185- motif in the activation loop (Kolch, 2000). PD98095 powder was dissolved in 0.1% of the final volume DMSO and centrifuged at 10,000 rpm for 1 minute. LiquiTears™ was added while vortexing, and left in 50°C waterbath overnight to dissolve PD98059. After NM exposure and washing with fresh medium, either LiquiTears™ containing 0.1% DMSO or LiquiTears™ containing PD98059 originally dissolved in 0.1% DMSO (40 µl of 2.5 mM; 100 nmoles) was dropped on half of the control
(unexposed), or half of the exposed corneas for 10 minutes, or in one case 4 applications in 24 hours following the designated exposure time. Samples were then embedded in OCT and sectioned for histology, or frozen in liquid nitrogen for protein analysis.

**Hydrogel Preparation and Application**

*Chapter 2:  Hydrogels are a clear, synthetic, poly(ethylene glycol) (PEG)-derived drug delivery system applied as a liquid which solidified within minutes. Once solidified they resemble a contact lens. The hydrogels employed in this work were developed by Sree Anumolu, Manjeet Deshmukh, and Patrick Sinko to allow sustained release of drug over a 24 hr period. NDH4417 was chosen for hydrogel delivery to corneal organ culture injuries because of its beneficial results in ophthalmic drops. Hydrogels containing synthetic ADAM17 inhibitor, NDH4417, can be an advantageous vehicle for drug application. The hydrogel was prepared and applied as follows: the eight-arm PEG polymers containing the thiol groups (8-arm-PEG-SH, 20 KDa, 1 eq) were added to the eight-arm polymer composed of the N-hydroxysuccinimidyl ester groups (8-arm-PEG-NHS, 20 KDa, 2 eq) at room temperature in aqueous phosphate buffer. The two formed intermolecular crosslinks via thioester bonds. In a 1 ml vial, polymer 8 arm PEG-SH (20kDa, 5 mg) was dissolved in 100 µL PBS. In another vial 75 µmole drug (NDH4417) and 8-arm-PEG-NHS (10 mg) was dissolved in 100 µL PBS (pH 8.0). Both solution were mixed and applied on NM wounded rabbit corneal organ cultures, where the hydrogel was formed *in situ* within 80 seconds. A similar procedure was used for the preparation of hydrogel without drug. Control hydrogels formed in 45 seconds.*
Chapter 3: Minocycline and doxycycline loaded poly(ethylene glycol) hydrogels for healing vesicant-induced ocular wounds

Once an effective dose was established by histology, doxycycline and minocycline ophthalmic drops (12.5 µg in 50 µl, 3 times per day; 75 nmoles total) in LiquiTears™ were applied. Alternatively, doxycycline or minocycline-containing hydrogels (37.5 µg in 10 µl; 75 nmoles) were applied once per day.

Hydrogels were formed as stated above. In a third vial 75 mmole minocycline or doxycycline were mixed with 5 µL of each "thiol" and "NHS" polymer solution was added to the vial. Solutions were mixed and applied on NM-exposed rabbit corneas, where the hydrogel formed within 80 sec. A similar procedure was used for the preparation of hydrogel without drug. Those hydrogels solidified in 45 seconds.

Histology

After 24 hours, the corneas were put in cryomolds containing Optimal Cutting Temperature (O.C.T., Tissue-Tek) compound with the epithelial side facing down and placed on ice for 15 min before snap freezing them in liquid nitrogen to let bubbles raise away from the tissue. Corneas were stored at -80°C until sectioned for histology and immunofluorescence (IF) analysis. The 10 µm corneal sections (cut using a Microm HM505E cryostat) were stained using a modified hematoxylin & eosin (H & E) method. Briefly, the corneal sections were fixed in a Pen-Fix solution for 60 sec, dH₂O for 8 mins, Mayer’s hematoxylin for 8 mins, luke warm running tap water for 16 mins, eosin for 1 sec, dehydrated through graded alcohols (95% and 100%; 2 x 2 mins each), immersed in
xylene for 2 x 2 mins and coverslipped with Permount. Digital images were captured with a light microscope at 10x and 40x magnification. Corneas were viewed with a Leica Microscope DMLB Wetzlar GmbH with ProgRes software (Jenoptik). Measurement scale has been determined by stage micrometer.

**Assessment of the percent of preserved epithelial-stromal attachment preserved after nitrogen mustard exposure plus and minus countermeasures**

Micrographs taken at 10x were overlapped into a composite of the cornea across its entire diameter. The entire width of cornea was measured in mm, along with the width of each detached area. A sum of total detachment was measured using rulers in Photoshop and verified manually. The width of total detachments was divided by the measurement of entire width of the cornea. That number was multiplied by 100 to get the percent detachment. The percent attachment is 1 minus percent detachment. A dose-response graph was used to compare efficacy of the two inhibitors at increasing concentrations.

\[
1 - \frac{\text{(width of total detachments)}}{\text{(entire width of the cornea)}} \times 100 = \text{percent attachment}
\]

**Immunofluorescence**

Immuno reactivity was used to view the localization of specific proteins. Detection was accomplished by employing fluorescently labeled secondary antibodies. Slides were first fixed in 4°C methanol for 10 mins. Nonspecific binding was blocked for 1 hr with 5% normal goat serum (NGS).
Chapter 2: For ADAM17 detection, mouse anti-human ADAM17 monoclonal primary antibody (against the extracellular, activated domain) was applied to the slides at a 1:50 dilution in 1.5% NGS (10µg/mL). For collagen XVII detection, guinea pig monospecific polyclonal antibody against the ADAM17 cleavage site was applied to slides at a 1:500 dilution in 1.5% NGS. After 1 hr incubation, the slides were washed for 3 x 10 min in PBS with 0.05% Tween 20 (PBST). Negative control slides received PBST instead of primary antibody. The secondary antibody was goat anti-mouse IgG conjugated to AlexaFluor488 (for ADAM17) applied at 1:1000 in 1.5% NGS, or goat anti-guinea pig IgG conjugated to AlexaFluor488 (for collagen XVII) applied at 1:5000 in 1.5% NGS for a 1 hr incubation. After washing with PBST for 3 x 5 min, 0.4 mg/mL DAPI (4',6-Diamidino-2-Phenylindole) was applied for 5 min to counterstain nuclei. Prolong Gold was used to coverslip the slides.

Chapter 3: Sections were incubated with mouse-anti-human MMP-9 monoclonal antibody (5 µg/ml) dissolved in 1.5% NGS overnight at 4 °C. Sections were blotted and washed 4 x 10 mins with PBS/Tween and incubated for 1 hr at RT in the dark with Alexa-Fluor 488-conjugated goat anti-mouse IgG secondary antibodies (1:1000 in 1.5% NGS). The sections were washed with PBST, counterstained with DAPI for 5 min, mounted with Prolong gold and cover slipped. Negative controls replaced primary antibodies with PBS. Digital epifluorescent images were captured with a camera mounted to a fluorescence microscope. Corneas were viewed with a Leica Microscope DMLB.
Chapter 4: Sections were incubated with mouse anti-human monoclonal antibodies against ADAM17 ectodomain (8µg/mL), pERK (8µg/mL), and MMP-9 (5µg/mL) dissolved in 1.5% NGS. After 1 hr incubation, the slides were washed for 30 min in TBS with 0.05% Tween 20 (TBST). Negative control slides received TBST instead of primary antibody. The secondary antibody was goat anti-mouse IgG conjugated to AlexaFluor488 applied at 1:1000 in 1.5% NGS for 1 hr. After washing with TBST for 15 min, 0.4 mg/mL DAPI was applied for 5 min to counterstain nuclei. Prolong Gold was used to coverslip the slides. Digital fluorescent images were captured at 10x and 40x magnifications on a camera mounted to a Leica DMLB Wetzlar GmbH microscope at 494 nm excitation and 517 nm emission wavelengths.

**Protein Extractions**

*Chapters 2, 3, and 4:*

Frozen corneal samples were pulverized with a pulverizer gun, and by grinding in a mortar and pestle with liquid nitrogen. This was followed by homogenization with a Polytron generator (0.5 mm probe) at 20K rpm for 3 cycles of 30 seconds on wet ice. The sample was in low salt extraction buffer (25 mM Trizma Base, 200 mM NaCl, 10 mM EDTA; pH 7.4), 1% Triton X-100, a protease inhibitor cocktail, and furin inhibitors I and II (to prevent post-experimental ADAM17 activation). Samples were then centrifuged at 12K x g (rcf) for 30 min at 4°C. Supernates were collected on ice.
ADAM17 Activity Assay

*Chapters 2 and 4:*

Innozyme ADAM17 (TACE) Activity Kit was used to determine ADAM17 activity. The anti-human ADAM17 antibody-coated 96-well plate was washed twice with 400 µl wash buffer. Samples and standards (100 µl) were added to wells in triplicate. Unexposed corneas given LiquiTears™ alone were used as controls. Plate was covered with a plate sealer and incubated 1 hr with gentle shaking at room temperature. After 1 hr, plates were washed with 400 µl wash buffer 5 times. ADAM17 substrate (100 µl) was added to each well and incubated for 5 hours at 37°C. Fluorescence was measured at an excitation wavelength of 324 nm and an emission wavelength of 405 nm.

MMP-9 Activity Assay

*Chapters 3 and 4:*

Corneal samples were homogenized as described above for use in the MMP-9 activity assay. Protein extracts and positive standards (200 µl) were added to each well of a 96-well plate included in the kit and incubated for 2 hrs at room temperature on a plate shaker. After washing wells with wash buffer included in the kit, p-aminophenylmercuric acetate (APMA) was added to activate MMP-9 standards; samples were mixed with diluent buffer included with the kit. The plate was incubated again for 2 hrs at 37°C. After washing wells again, 200 µl of substrate supplied in the kit was added to each well and the plate was incubated in the dark overnight at 37°C. At the end of the incubation,
the plate was exposed to an excitation wavelength of 320 nm and read at an emission wavelength of 405 nm.

*In vivo* SM exposure of rabbit eyes and treatment with doxycycline as drops and hydrogels

*Chapter 3:*

SM exposures were performed at Battelle. Sixty-four New Zealand white rabbits were used in the experiment, and randomized into 7 groups (16 rabbits) per collection day. Those groups were: (1) no SM, no treatment (left eye); SM, no treatment (right eye), (2) no SM, dox drops (left eye); SM, dox drops (right eye); (3) dox drops (both eyes), (4) no SM, dox hydrogel (HG) (left eye); SM, dox HG (right eye), (5) dox HG (both eyes), (6) HG only (both eyes), and (7) naïve control (both eyes).

Collection days were 1, 3, 7, 28 days post-exposure. To the right eye of 3 anesthetized rabbits per collection day (group 1), 0.4 ml neat SM (5 mg; 30 nmol) was directly applied onto the central cornea. Eyelids of the exposed eye were held open for 5 min using an ocular speculum and then manually closed 3 times to spread any residual SM over the cornea. A second set of 3 animals per collection day (group 2) were exposed to SM as described, but 4 hrs after exposure were treated with one 50 µl drop of doxycycline (12.5 µg) applied 3 times per day (75 nmols) to the central cornea until time of sacrifice. A third set of 3 animals per collection day (group 4) were exposed to SM as above, and then at 4 hrs post-exposure, they received 25 µl of hydrogel plus doxycycline solution (37.5 µg, 75 nmols), pipetted into the lower eyelid pocket under the center of the eye instead of over the cornea as in our *ex vivo* studies. A new hydrogel was applied
daily. For this treatment group, 3 rabbits were sacrificed at each collection day. Controls rabbits included unexposed animals receiving doxycycline drops 3 times per day (group 2, left eyes, and group 3), unexposed animals receiving daily hydrogels without drug (group 6), and unexposed animals receiving daily hydrogels containing doxycycline (group 4, left eyes, and group 5) until the day of sacrifice. Unexposed and untreated rabbits were also used as naïve controls (Gordon, et al., 2010).

Keratectomy to inflict corneal injury

Chapter 3:

Whole rabbit eyes obtained from PelFreez were fixed to a 60mm culture plate containing solid wax. A 3.5 mm biopsy punch was given a quarter twist to penetrate the epithelium and basement membrane, into the upper stroma. The edge of the punch was pulled up with sharp forceps. The tissue was evenly split between lamellar layers, giving a flat look to the wound. Eyes were placed in DMEM with HEPES for 10 mins before corneas were dissected and collected (0 hr), or filled with agar and place into culture dishes for organ culture (24 hr and 48 hr).

Western Blotting

Chapter 4:

SDS-PAGE gels were run with 10 µg of reduced protein sample diluted in dH2O with loading buffer in each well of a 7.5% gel for 1.25 hrs at 80 volts. Gels were transferred onto nitrocellulose transblot membrane for 1 hr at 100 volts. Nonspecific binding was blocked overnight in 5% bovine serum albumin (BSA) at 4°C. Blots were
washed with TBST for 30 mins. Primary antibodies against pT735 (the binding site of ERK on the cytoplasmic tail of ADAM17, 1:5000), the ectodomain of (active) ADAM17 (1:1000), and ERK (1:1000) were prepared in 1% BSA, submerged for 1 hr, and washed again in TBST for 30 mins. Secondary goat-anti-rabbit IgG conjugated to HRP (pT735; 1:100,000) or goat-anti-mouse IgG conjugated to HRP (ERK, ADAM17, 1:20,000) were used for 1 hr and again washed with TBST for 30 mins. Chemiluminescent substrate was added for 1 min before exposing to film.

**Immunoprecipitations**

*Chapter 4:*

Anti-mouse IgG agarose beads were washed with PBS and centrifuged three times at 3000 x g for 5 mins to remove excess liquid a total of three times, with 1 hour of rocking in PBS at room temperature in between spins. Five microliters of anti-ERK antibody was added to the beads and rocked for 1 hr. The amount of protein added to beads depended on the amount of protein available and the binding capacity of the beads. Protein extracts were added to the beads and rocked at 4°C overnight. SDS-PAGE gels with 40 µl of beads (approximately 172 µg of protein, according to binding capacity) were loaded into each well of a 7.5% gel. Gels were run at 80 V for 1.25 hrs, transferred to membranes at 100 V for 1 hr. Membranes were blocked as previously described above and probed with antibodies against the cytoplasmic domain of ADAM17 (the domain bound to phosphorylated ERK) (1:200), pERK (1:5000) and ERK (1:500) in 1% BSA and washed with TBST for 30 mins. Secondary donkey-anti-goat IgG conjugated to HRP (ADAM17; 1:50,000) and goat-anti-mouse IgG conjugated to HRP (pERK, ERK;
1:20,000) were used for 1 hr before 30 mins of washing in TBST, chemiluminescent substrate for 1 min, and exposure to film.
CHAPTER II:

ADAM17 INHIBITORS COUNTERACT EPITHELIAL DE-ADHESION RESULTING FROM COLLAGEN CLEAVAGE AFTER CEES AND NITROGEN MUSTARD OCULAR EXPOSURE
2.1-- Abstract:

Sulfur mustard (SM) is a potential bioterrorist agent that can cause vision loss in those exposed. It has been observed that SM, the related nitrogen mustard (NM), and 2-chloroethyl ethyl sulfide (CEES, half mustard) induce epithelial-stromal separations in the cornea. CEES, NM and SM exposure increases ADAM17 (aka TNFα converting enzyme, TACE) activity, which contributes to cleavage of transmembranous collagen XVII, a component of the hemidesmosome and anchoring complex. The goal is to determine whether rabbit corneal organ cultures exposed to CEES or NM would inhibit detachment if ADAM17 inhibitors were applied. Rabbit corneal organ cultures were exposed onto central corneas 100 nmoles of NM or 20 nmoles of CEES. After 2 hrs, contaminated medium was replaced with fresh medium alone, or medium plus one of four synthetic ADAM17 inhibitors. Corneas were analyzed by light and immunofluorescence microscopy, and by ADAM17 activity assay. We conclude that vesicant exposure leads to activation of ADAM17 and cleavage of collagen XVII at the epithelial-stromal junction. The 2 hr post-exposure application of ADAM17 inhibitors preserves the integrity of the BMZ and attenuates the loss of the epithelium from the stroma.

**Key Words:** ADAM17, TACE, nitrogen mustard, CEES, corneal injury, hydroxamate
**Abbreviations:** ADAM17, a disintegrin and a metalloprotease 17; TACE, tumor necrosis factor-α converting enzyme; BMZ, basement membrane zone; NM, nitrogen mustard; CEES, 2-chloroethyl ethyl sulfide

**2.2-- Introduction:**

Collagen XVII (BP180, BPAG2) is a transmembranous protein located within the hemidesmosome. Hemidesmosomes are a component of the adhesion complex responsible for strongly linking basal epithelia to the stroma. Other components of the adhesion complex are the anchoring filaments and the anchoring fibrils. During cell migration after a wound, hemidesmosomes must detach from the basement membrane which means that collagen XVII must be cleaved. Each collagen XVII molecule is a trimer of three 180 kDa α chains (Franzke et al., 2002). The intracellular domain is 466 amino acids, and is followed by a 23 amino acid transmembrane domain, and an extracellular domain of 1,008 amino acids. The C-terminal ectodomain consists of 15 collagenous subdomains (COL 1-15) and 16 short stretches of non-collagenous sequences (NC 1-16) (Giudice et al., 1992). Purified bovine collagen XVII has revealed that the molecule has a globular cytoplasmic head (Hirako et al., 1996). Both the cytoplasmic and the extracellular part of the also transmembranous α6β4 integrin binds to collagen XVII (Borradori et al., 1999; Hirako, Usukura, et al., 1998; Jones et al., 1994; McMillan et al., 2003; Nievers et al., 1999). The collagen’s intracellular domain interacts with the β4-integrin subunit and is essential for the incorporation of the collagen into the hemidesmosome. The extracellular domain binds to the α6-integrin subunit (Borradori et al., 1999). The extracellular domain NC16A between the plasma membrane and the
collagenous Col15 domain is functionally important because it plays a role in both ectodomain shedding and in the proper folding of the triple helical structure of collagen XVII (Franzke et al., 2009).

The ectodomain of collagen XVII is proteolytically shed from the cell surface to release a shorter form of the collagen into the extracellular matrix. Human collagen XVII molecules are cleaved from their original 180 kDa size to a 120 kDa extracellular fragment at the noncollagenous (NC16A) domain (Borradori et al., 1996; Burgeson et al., 1997; Hirako & Owaribe, 1998; Hirako, Usukura, et al., 1998; Jones et al., 1994). The cleavage releases the ectodomain, and is catalyzed by a group of enzymes collectively referred to as sheddases. (Black et al., 1998; Franzke et al., 2003). ADAM9, ADAM10 and ADAM17 are some of the enzymes, or sheddases, targeting collagen XVII as a substrate (Franzke et al., 2009).

The ADAM (a disintegrin and a metalloprotease) family is a transmembranous family of enzymes that effect cell adhesion, migration, proteolysis, signaling and can be involved in ectodomain shedding (Edwards et al., 2008). Matrix metalloproteinases (MMPs) differ from ADAMs because the are associated with degradation of the extracellular matrix, while ADAMs are generally cell associated or transmembranous and have more variable substrates (Hooper, 1994; Newton et al., 2001).

ADAM17 (aka TNFα converting enzyme, TACE) is a member of the ADAM family of metalloproteinases. ADAM17-deficient mouse embryos were used to confirm their collagen XVII cleaving properties. Compared with age-matched normal murine keratinocytes, shedding of collagen XVII was ~40% reduced in ADAM17-deficient cells (Franzke et al., 2004; Franzke et al., 2002). Figure 2.1 is a schematic of the
conformational change seen with ADAM17 activation and the region of collagen XVII cleavage.

Our work shows that mustards cause cleavage of collagen XVII via activation of ADAM17. This agrees with previous sulfur mustard injury studies that show at 24 hr post-exposure, bullous pemphigoid antigen (BPA), which binds to several sites on collagen XVII, was absent at sites where the epithelium had been cleaved from the basement membrane (Petrali et al., 2000; Petrali et al., 1997). Our studies were performed with the related mustards, CEES and NM, which also cause ADAM17 activation 24 hr post-exposure. Therefore, we tested whether ADAM17 inhibitors were able to attenuate injury when applied at 2 hr post-exposure. Figure 2.2 shows corneas in culture, and how they are exposed to the mustards and the inhibitors.

Three of the hydroxamate compounds utilized in this study were new materials [and were prepared by Dr. Ned Heindel, C. Jeffrey Lacey, Abhilash Pillai, Sherri C. Young, and Robert A. Flowers II (Lehigh University)]. The structures are seen in figure 2.3. All were shown to bind cationic iron and zinc and to inhibit matrix metalloproteinases (MMPs). These structures were originally selected for synthesis and screening because their de-hydroxylated counterparts, which are neither MMP inhibitors nor metal ion binding ligands, had previously been shown to be excellent topical anti-inflammatory pharmaceuticals (Casillas et al., 2000; Janusz et al., 1993). The conversion of the nor-hydroxyl to the N-hydroxyl counterparts (hydroxamates) was considered likely to add MMP inhibitory effects to the already potent components and hence increase the potential anti-inflammatory effects. Hydroxamate MMP inhibitors are well known to possess anti-inflammatory activity (Hu et al., 2007; Muri et al., 2002). Abbott
Pharmaceutical’s clinically-effective hydroxamate and MMP inhibitor, ABT-518 (aka NDH4450 in our studies), was prepared by a modification of the published Abbott pathway and was included in the cornea study.

The three new hydroxamates described below [designated NDH4409 (N-hydroxyolvanil), NDH4385 (retro-OH-7), and NDH4417 (retro-OH-8)] were tested for anti-inflammatory activity in a mouse ear inflammatory model using 0.75 µmol 12-O-tetradecanoylphorbol-13-acetate (TPA) per ear as the inflammatory stimulant. In the assay, results are expressed as percentage reductions of edema and inflammation relative to TPA alone, and has been previously described (Young et al., 2011). All three hydroxamates were potent inhibitors of the TPA-induced inflammatory response in the mouse ear: NDH4409 (N-hydroxyolvanil) = 64%; NDH4385 (retro-OH-7) = 50%; and NDH4417 (retro-OH-8) = 78% reduction of inflammation (Heindel, unpublished data). NDH4450 (ABT-518) was not evaluated in this model. Compounds were dissolved in DMSO, 0.1% of final volume, and centrifuged at 10,000 rpm for 1 minute. LiquiTears™ was added between vortexings, and the solution was incubated in a 50°C waterbath overnight to completely dissolve the hydroxamates.

Briefly, Dr. Heindel’s group was able to synthesize NDH4409 [(9Z)-N-Hydroxy-N-[(4-hydroxy-3-methoxyphenyl)methyl]-9-octadecenamide also known as N-Hydroxyolvanil] from oleoyl chloride, 2-mercaptothiazoline, dichloromethane, and triethyl amine carefully monitored with thin layer chromatography. Synthesis of NDH4385 [N-heptyl-N-hydroxy-2-(4-hydroxy-3-methoxyphenyl)acetamide (also known as N-hydroxy-heptylhomovanillamide or Retro-OH-7)] and NDH4417 [N-octyl-N-hydroxy-2-(4-hydroxy-3-methoxyphenyl)acetamide (also known as N-hydroxy-
octylhomovanillamide or Retro-OH-8)] were made from similar four-step reactions. These hydroxamates, were the N-hydroxy counterparts of a promising fatty acid amide topical anti-inflammatory (Janusz et al., 1993). The four steps included synthesis of (1) N-(benzyloxy)heptan-1-imine, (2) N-(benzyloxy)heptylamine, (3) N-benzyloxy-N-heptyl-2-(4-hydroxy-3 methoxyphenyl)acetamide, and (4) N-heptyl-N-hydroxy-2-(4-hydroxy-3-methoxyphenyl)acetamide. Synthesis of 4450 [N-[(1S)-1-((4S)-2,2-Dimethyl-[1,3]dioxolan-4-yl)-2-(4-(4-(trifluoromethoxyphenoxy)phenylsulfonyl)ethyl]-N-hydroxyformamide also known as Abbott ABT-518] was modeled after the previously published method (Wada et al., 2002).

2.3-- Results:

Histology

Injury was gauged mainly on the epithelial-stromal separations (the microbullae which form) after an injury, and the ability of the countermeasure to inhibit the cell layer separations. We used the monospecific collagen XVII antibody because it was designed to show a fluorescent signal only when the full length form is intact. If it has been cleaved, there will not be any collagen XVII signal. The ectodomain specific ADAM17 antibody was used because there is a conformational change which occurs with ADAM17 activation. The ectodomain conforms to a position to facilitate shedding of substrates. The ectodomain specific ADAM17 antibody will identify the conformational change (i.e. activation).

The submaximal dose (3 nmol) of each ADAM17 inhibitor was chosen for examination. The submaximal dose is the dose lower than the highest dose tested, which
is commonly indicated for clinical use. Corneal organ cultures were exposed to CEES or NM applied directly to the central cornea, and then allowed to incubate at 37°C for 2 hrs before washing. After washing, cultures were treated with 1 of the 4 ADAM17 inhibitors, applied 4 times over a 24 hr period, and then the tissue was prepared for histological examination. Cultures were frozen in OCT, sectioned, then either stained with H&E, reacted with an extracellular domain ADAM17 antibody or reacted with a collagen XVII monospecific antibody for the full length form of the molecule.

CEES and NM exposure altered corneal epithelial thickness and the basement membrane zone (BMZ) as previously noted (Anumolu, et al., 2010; Gordon, et al., 2010). Vesicant injury was variable and occurred focally. For example, 24 hr after the 2 hr CEES exposure (20 nmols) there was an increase in the number and depth of epithelial cells invaginating into the stroma (Figure 2.4F) than unexposed cultured corneas (Figure 2.4A). At low magnification, the NM-exposed (100 nmol) samples also presented an increase in the number and depth of areas with downward epithelial hyperplasia penetrating into the stroma (not shown), and usually more than 50% of the epithelium was detached from the stroma 24 hr after exposure (Figure 2.4K). Unexposed corneas showed normal width and depth of epithelium and minimal invaginations into the stroma with and without treatment applications (Fig 2.4A-E).

Four applications of the inhibitors were added over the 22 following the termination of mustard exposure. All four inhibitors appeared to inhibit the injury appearing after CEES exposure (Figs. 2.4G-2.4J). The same was not true for nitrogen mustard exposures. H&E examination showed NDH4417 (Retro-OH-8) was the most effective at inhibiting epithelial-stromal detachment post-NM exposure (Fig. 2.4M).
NDH4385, NDH4450 and NDH4409 showed phenotypes that likely precede epithelial loss (Figs. 2.4L, 2.4N, 2.4O, respectively). The histology of unexposed corneas treated with the inhibitors was the same as the unexposed/untreated controls, indicating the inhibitor drugs themselves did not cause epithelial-stromal separation (Figs. 2.4B - 2.4E).

**Immunofluorescence of collagen XVII is preserved when active ADAM17 levels are decreased**

An antibody reactive against the extracellular domain of ADAM17 (i.e. active form) shows that the enzyme is present 24 hr after CEES and NM exposures (Fig. 2.5F, 2.5K, respectively). The signal is easily visualized because of the conformational change with ADAM17 activation (Le Gall et al., 2010). Most unexposed samples show no signal, with the exception of NDH4450 which appears to induce some ADAM17 with this assay (Fig. 2.5D). Inhibitor treatments on CEES exposed samples reduced ADAM17 signal (Fig. 2.5G-I). NDH4409 is the only exception with some visible signal (Fig. 2.5J). In NM treated samples, ADAM17 immunofluorescence staining was strongest in the corneas receiving no inhibitor. Antibody reactivity was spotty in inhibitor-treated samples, indicating at least partial enzyme inhibition (Figs. 2.5L-O). The corneas treated with NDH4417 showed the greatest inhibition by the scarcity of ADAM17 staining (Fig. 2.5N). This result agrees with the H&E histology which showed NDH4417 to be best at attenuating epithelial-stromal separation (Fig. 2.4M).

The collagen XVII peptide was designed by our laboratory and used to generate antibodies at Invitrogen. The unique antibody against this monospecific collagen XVII
peptide (Figure 2.6) will only react if the protein remains intact and is not cleaved. Cleavage disrupts the epitope. The unexposed samples retain integrity as seen by a green line of full length collagen XVII signal in the BMZ. The exposed CEES and NM-exposed samples given no treatment (Figs. 2.6F, 2.6K) do not show full length collagen XVII. Collagen XVII immunoreactivity analysis of samples receiving inhibitor treatments indicates that compound NDH4417 was most effective in maintaining the integrity of the BMZ after vesicant exposure (Figs. 2.6H, 2.6M). In addition, NDH4450 (Figs. 2.6I) after CEES exposure, and NDH4409 (Figs. 2.6J, 2.6O) after both CEES and NM exposure maintained some BMZ integrity with this analysis. Many of the corneas presented some fluorescence in the apical epithelium after both antibodies. This result is more common in corneas exposed to NM, or treated with inhibitors. Corneas are known to express autofluorescence signals as seen here in the apical cells (Joyce et al., 1997). Distressed or dying cells display higher amounts of autofluorescence (Godbey et al., 2000).

**Hydrogel Application of effective drug, NDH4417, as drug delivery system**

The histology of corneas treated with NDH4417 as an ophthalmic drop, as opposed to a hydrogel with sustained release, indicates a beneficial advantage to the alternative drug delivery system after NM exposure. The results are shown in figure 2.7. The hydrogel appears as a blue porous body in the H&E photos. The hydrogel with or without drug had no adverse effects on unexposed corneas.

Corneas exposed to NM show separation of the epithelium from the stroma as well as cell-cell separations. The cells do not appear to be necrotic, but would have fallen off had the hydrogel not been there, as seen by the upward migration of nuclei which usually
preceeds detachment. The hydrogel containing NDH 4417 increased epithelial-stromal junction preservation after exposure, and has a phenotype resembling controls. The NDH4417 hydrogel appears to be a beneficial route of application for the compound screened as most effective against injury. This compound in the hydrogel should be tested in vivo as one of the next steps in finding an approved drug applicable to microbullae prevention after SM exposure.

**Dose-Response Assessment**

A dose-response assessment (Figure 2.8a) was done to compare the efficacy of each inhibitor drug in preserving epithelial-stromal attachment after NM exposure, using the equation \[100 - \left(\frac{\text{width of detached cornea}}{\text{total width of cornea}}\right) \times 100\]. This was only done after NM and not with CEES because epithelial-stromal detachment was not commonly seen after CEES exposure. With the exception of the 0.3 nmol application, NDH4417 (solid line) was the most effective of all doses up to 10 nmol, followed by NDH4409, NDH4385, and NDH4450. There is a significant decrease in epithelial-stromal attachment when measuring the unexposed controls compared to the NM-exposed corneas without treatment (Figure 2.8b).

ADAM17 activity was fluorometrically assessed from protein extracts of each sample. ADAM17 activity inversely correlates with epithelial-stromal attachment (Figure 2.8c), indicating that ADAM17 is at least in part responsible for epithelial-stromal detachment. The IC50 of ADAM17 was measured by finding the dose corresponding to half ADAM17 activity without treatment. NDH4417 had the lowest IC50, followed by NDH4385, NDH4409 and NDH4450 (Table 2.1). There is a significant increase in
ADAM17 activity after mustard exposure compared to unexposed corneas with no treatment (Figure 2.8d).

2.4-- Discussion:

Mechanisms responsible for the pathology of mustard injuries are not clearly understood. Here we show activation of ADAM17 may be one mechanism causing pathology by demonstrating that vesicant exposure and activation of the enzyme leads to cleavage of collagen XVII at the epithelial-stromal junction. This favors development of separations between the cell layers (i.e., formation of microbullae). ADAM17 activity levels from protein extracts of corneas exposed to NM, followed by treatment with inhibitors, is associated with the extent of epithelial-stromal detachment in the presence or absence of ADAM17 inhibitors. This suggests that ADAM17 is at least in part responsible for microbullae formation after nitrogen mustard exposure.

Sulfur mustard induces loss of integrity of the basement membrane zone in the cornea. Collagen XVII immunofluorescence can be used to assess the integrity of the basement membrane zone post-exposure to sulfur mustard derivatives. NM exposure activates ADAM17 and cleaves collagen XVII, leading to cell layer separation. Half mustard (CEES), NM and sulfur mustard are all vesicants that cause damage to the basement membrane zone of the skin and cornea. Our data demonstrates that ADAM17 and collagen XVII can be used as markers to assess vesicant injury.

The corneal organ cultures exposed to CEES and NM can be used to identify counteragents that may be effective against SM exposure. Once organ culture experiments are screened, promising counteragents can proceed to testing in animal
corneas exposed to SM. Two of the hydroxamate compounds (NDH4417 and NDH4409) examined in these experiments have shown promise as countermeasures when used for 24 hr, beginning 2 hr post exposure. NDH4417 appears to be the most efficacious ADAM17 inhibitor and is a candidate for future \textit{in vivo} studies.

There is currently no ideal treatment, short of corneal transplant, effective against acute or chronic sulfur mustard injury. Hydroxamates may be good candidates for therapy after mustard exposure and should be pursued. They are commonly used zinc binding group inhibitors previously found to be effective against MMP activation. NDH4417 has augmented the already superior vanilloid-like activity of octyl homovanillamide with a metal ion binding potential to inhibit ADAM17 activity. Hydroxamates bind the catalytic zinc (II) ion in a bidentate (chelating ligands that binds to metal in two places) fashion, blocking substrate access to the active site and rendering it incapable of hydrolysis (Puerta et al., 2004). We have shown that hydroxamates can be used to inhibit ADAM17 activation and the subsequent collagen XVII cleavage commonly seen as microbullae after vesicant exposure.
Figure 2.1 -- Schematic of ADAM17 conformational change after activation, and cleavage of collagen XVII at the NC16A domain. Modified from Franzke et al. 2002.
Figure 2.2 -- A schematic of the rabbit corneal organ culture system. Medium is only added up to the scleral rim (approximately 2 ml per 60 mm pyrex petri dish containing 3-4 corneas).
Figure 2.3 -- Four ADAM17 inhibitors were provided by Dr. Ned Heindel of Lehigh University. The drugs were administered after the 2 hour exposure period, and the contaminated medium was removed. Fresh medium was added with ADAM17 inhibitor in LiquiTears™ ophthalmic solution with 0.1% DMSO of the total volume. Each dose was either 0.3, 1, 3, or 10 nmol doses and they were given 4 times over the following 24 hrs.

![Chemical structures of ADAM17 inhibitors](image)

- NDH 4385 (Retro OH-7)
- NDH 4417 (Retro OH-8)
- NDH 4450 (ABT-518) (phenoxyphenyl sulfone retrohydroxamate)
- NDH 4409 (Olvanil-hydroxamate)
Figure 2.4 -- H&E photos with and without inhibitors. This first column shows unexposed sections. None of the compounds had a negative effect on the cornea. After NM exposure there was separation of the epithelium from the stroma, and both apoptotic and necrotic bodies. Most of the ADAM17 inhibitors, with the exception of NDH 4417, an upward migration away from the basement membrane, indicating future epithelial detachment. Scale bar = 100µm
Figure 2.5 -- Immunofluorescence staining with anti-extracellular ADAM17 Ab (green, Alexa488) Counterstain is DAPI (blue). The ectodomain-specific ADAM17 antibody should only give a signal if it is active because of the conformational change. It is clearly seen after NM exposure, but not in the control corneas. Each inhibitor retained some of ADAM17 activity with the exception of NDH 4417. Scale bar = 100µm
Figure 2.6— Immunofluorescence staining with collagen XVII Ab (green, Alexa488). Counterstain is DAPI (blue). Collagen XVII Ab is a monospecific antibody for only the full length molecule. The control cornea expresses collagen XVII as an uninterrupted green line which is preserved with NDH 4417 treatment. Scale bar = 100µm
Figure 2.7 -- The poly(ethylene glycol) hydrogel contained NDH 4417 directly in it and this was applied on the cornea (once) similar to a contact lens. In the second column the hydrogel (which appears as blue and porous) alone (E) and the hydrogel containing NDH 4417 (G) did not have a negative effect on the cornea. The hydrogel without the drug was not beneficial to the cornea after NM exposure (F). However the NM exposed cornea given the hydrogel with NDH 4417 (H) had results just as good if not better than 4417 given as an ophthalmic drop (D).
Figure 2.8 -- The graph shows quantification of the epithelial-stromal separations as measured by 1 minus the (width of detached cornea divided by the width of total cornea x 100). This was measured from H&E sections and showed no significant difference (2.8A). Some corneas were frozen and homogenized for protein extraction and these were tested with an ADAM17 activity kit. ADAM17 activity decreases as epi-stromal separation increases and that NDH4417 was the most effective at reducing ADAM17 levels at all doses (2.8C). Below is a comparison of exposed vs. unexposed levels. Epithelial-stromal attachment observed before and after NM exposure (2.8B); and ADAM17 activity increase after exposure similar correlation with detachment (2.8D). The results were expressed as means +/- SD, and analyzed by using 2-sided students t-tests. A value of p < 0.05 was considered statistically significant.
Table 2.1 -- The hydrophilicity of each compound is indicated by the cLogP value (increased value indicates more lipophilicity). The IC50 is the concentration of inhibitor necessary to decrease ADAM17 activity to 50% of its initial value without inhibitor. The NDH 4417 had the lowest, followed by NDH 4385, then NDH 4409 and NDH 4450. There was no correlation between hydrophilicity and IC50.

<table>
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<tr>
<th>Compound</th>
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CHAPTER III:

UPREGULATION OF MMP-9 CAUSED BY NITROGEN MUSTARD EXPOSURE IS INHIBITED BY DOXYCYLINE AND MINOCYCLINE
3.1 – Abstract

Sulfur mustard is a terrorist agent that leads to degradation to the basement membrane zone in the cornea. The injuries can range from mild to severe, and can recur over time. One of the contributors to the extent of damage is the upregulation of matrix metalloproteinase-9 (MMP-9). MMP-9 is responsible for reorganization of the extracellular matrix after an injury has occurred. While this response is beneficial for other types of injuries, mustard injuries prolong upregulation of MMP-9, reaching maximal levels later, preventing re-epithelialization of the cornea. In these studies an ex vivo culture model system was used to induce injury with a 2 hour NM exposure. This model is efficient for screening the efficacy of doxycycline and minocycline applied as an eye drop, or within a sustained release hydrogel. The use of doxycycline and minocycline reduced levels of MMP-9 and improved the histological phenotype of organ-cultured corneas exposed to mustard compounds. Several tetracycline derivatives were tested for their ability to inhibit the activation of MMPs by binding to zinc or calcium in the catalytic site preventing further substrate activation. The culture model results led to doxycycline being advanced for in vivo testing on rabbits exposed to sulfur mustard as an eye drop and within a hydrogel.

**Key Words:** nitrogen mustard, MMP-9, doxycycline, minocycline, hydrogel

**Abbreviations:** NM, nitrogen mustard; MMP-9, matrix metalloproteinase-9; HG, hydrogel
3.2 – *Introduction*

Corneal injuries from vesicants range from mild to severe, depending on the agent, its concentration, and the duration of exposure. The effects of exposure are not felt immediately, but take approximately 2 to 4 hrs to manifest (Papirmeister et al., 1985). Sulfur mustard (2,2-dichlorodiethyl sulfide, SM) was used in the past by terrorists, and is considered a likely agent to be used again in the future. SM is extremely dangerous because even mild ocular exposures cause visual disturbances, panic, and a fear of blindness. Unlike the skin, the cornea does not blister after exposure to vesicants. Instead, microbullae are formed at the basement membrane zone (BMZ). These microbullae are focal separations between the epithelial and stromal cell layers, with disruption of the BMZ. There is yet to be an FDA–approved countermeasure against vesicant exposures to the cornea.

Controlled processing of extracellular matrix molecules is essential for the detachment and migration of epithelial cells after an injury. Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes important in degrading the extracellular matrix after tissue injury. The activity and inhibition of MMPs is strictly regulated (Nagase et al., 1999). MMPs are responsible for degrading the extracellular matrix after wounding to allow for provisional matrix deposition that allows cells to migrate and repair the region (C. Chang et al., 2001).

In skin, sulfur mustard causes progressive upregulation of MMP-9 activity increasingly past the 7th day post SM exposure (Shakarjian et al., 2006). Similar effects have been noted in animal studies of the cornea (Kadar et al., 2009). Thus agents that inhibit MMPs such as doxycycline and minocycline may represent potential therapeutics.
Doxycycline and minocycline are tetracycline derivatives and remain widely used as antibiotics. Current studies have demonstrated that they are beneficial drugs affecting many mammalian cell functions, including cell proliferation, migration and matrix remodeling in vitro (Nelson, 1998; Yao et al., 2007), functioning completely separate from their anti-microbial action. Doxycycline inhibits gelatinase, collagenase and stromelysin (Gilbertson-Beadling et al., 1995; Golub et al., 1991; Ryan et al., 1996), and has been used to reduce tissue degradation in aortic aneurysms and arthritis and to inhibit tumor cell invasion and metastasis (Fife et al., 1995; Seftor et al., 1998; Tamargo et al., 1991).

The corneal organ culture model is used as a screening tool to test the efficacy of doxycycline and minocycline applied as an eye drop, or within a hydrogel with sustained release. Doxycycline is a long-acting tetracycline, well recognized for its therapeutic efficacy in treating MMP-mediated ocular surface diseases such as epithelial erosions and sterile corneal ulcerations. Treatment with doxycycline attenuates acute and delayed ocular injuries caused by SM exposure (Amir et al., 2000; Kadar et al., 2009). The drug is a relatively inexpensive, FDA approved antibiotic that likely promotes wound healing by reducing inflammation and protease activity. It is, however, not FDA approved for ocular use. Minocycline is less well studied in the eye, and has been shown to generally induce more adverse effects.
3.3 – Results

Dose-response of doxycyline and minocycline as a countermeasure against nitrogen mustard *ex vivo*

Ophthalmic drops used in these studies were prepared immediately before use (50 µl containing 4.1 µg, 12.5 µg or 37.5 µg doxycycline or minocycline; 3 applications per day) in LiquiTears™. Unexposed control corneas were given only LiquiTears™. Four corneas were evaluated for wound healing efficacy in each treatment group. Half of the unexposed corneas and half of the exposed corneas were given doxycycline or minocycline in solution, which was applied drop wise three times over a 24 hr time period for totals of 25, 75 or 225 nmoles per day (calculated from above).

Histological evaluations with H&E staining are shown in figure 3.1A. Unexposed control corneas exhibited a normal phenotype of multilayered epithelium, attached to stroma with minimal hyperplasia into the stroma and an intact basement membrane zone (BMZ). Severe damage to the epithelium 24 hr after NM causes epithelial cell sloughing, epithelial cell dissociation and invagination into the stroma. Where the epithelium is detached from the stroma, the epithelial cells are separated, losing their cell to cell junctions. Where the epithelium is still attached, the basal cell nuclei appear to be more distant from the stroma than in controls.

Neither drug improved the histologic appearance after a NM exposure at a 25 nmol dose. At 75 nmols, the doxycycline treatment appears to produce corneas with a thinner, but healthier and attached-looking epithelium. The minocycline treated cornea had a phenotype likely to precede detachment at this dose because of upward cell migration away from the basement membrane. Improved cellular appearance occured
with 225 nmol of minocycline, as seen by epithelial cell nuclei appear closer to the basement membrane.

The measured amount of epithelial-stromal attachment was used to gauge effectiveness of the compounds relative to controls. The percent epithelial-stromal attachment preserved was found by dividing the (width of detachments) by the (entire width of cornea) x 100. This number is the percent detachment, which was subtracted from 100 to find the percent attachment. The dose-response curves after 1 day of treatment (Figure 3.1B) reveals that the 75 nmole dose was most effective for doxycycline, while the 225 dose was most effective for minocycline. Having a lower effective dose is advantageous because the amount of active ingredient needed is lower, and the product is less expensive to perform further testing and develop products. However, minocycline is also less stable than doxycycline after mixing with solutions (Barry et al., 1978).

**Effectiveness of doxycycline and minocycline hydrogels compared to ophthalmic drops**

Unexposed and untreated corneas exhibited a normal epithelium with and without a hydrogel application (Figure 3.2). Unexposed corneas given doxycycline or minocycline drops appeared normal with no further damage to the cornea visualized except for increased typical cell growth. Nitrogen mustard exposed corneas that were given 75 nmoles (3 x 50 µl of 12.5 µg) of doxycycline drops in 24 hrs show histological improvement compared to nitrogen mustard exposed corneas without any treatment. There is less sloughing of cells and fewer apoptotic bodies, but not comparable to the
multilayered, intact epithelium of the control phenotype after 1 day (Figure 3.2A). The same dose used in minocycline drops was not as effective. The epithelium still contains an amorphous basal cell layer, apoptotic bodies, and unhealthy epithelial cells.

Half of the exposed and unexposed corneas received 10 µl of hydrogel containing 37.5 µg of doxycycline or minocycline once per day. The hydrogel was formed on top of the cornea and solidified there to be similar to a soft contact lens. The hydrogels covering the corneas were remoistened with medium three times per day. The hydrogels were applied by Manjeet Deshmukh (Dr. Patrick Sinko’s laboratory, Rutgers University).

The hydrogels were applied as a solution that gelled a few seconds after instillation onto the cornea. The hydrogel formed a thin, transparent film and likely because of its high water content, it remained in place for the entire duration of the study. Histology of control corneas demonstrated an epithelium with normal thickness and an intact stroma. The unexposed corneas treated with a hydrogel without drug, or a doxycycline or minocycline-containing hydrogel were very similar in phenotype to the unexposed controls, demonstrating that the hydrogel did not injure the cornea.

In NM exposed samples administered a hydrogel without drug, the epithelium remained detached from the basement membrane in most areas. Histologically, the hydrogels without drug did not prevent NM injury including epithelial cell separation from the stroma, and loss of cell-cell junctions. In contrast, the PEG-doxycycline hydrogel showed improvement over the NM-exposed corneas, but was not as effective as the drug in solution. Although the cells appear healthier with the doxycycline-loaded hydrogel, the basal cells would slough off if given more time as seen by small epithelial-stromal detachments. The doxycycline-loaded hydrogel may be slower to provoke wound
healing. The advantages of the hydrogel are less neovascularization seen with *in vivo* studies (Gordon et al., 2010), and it’s an easier vehicle to store and easily prepared at emergency response centers. A disadvantage is that it takes longer to catch up with the effectiveness of the ophthalmic drop.

NM exposed samples treated with minocycline drops continued to show epithelial cells that were detached from the stroma and separated from adjacent cells after 24 hrs. There were apoptotic bodies present and sloughing of cells in some areas. PEG-minocycline hydrogels did not improve the phenotype of nitrogen mustard exposed corneas when used at a daily dose of 75 nmol. The epithelium appeared to be more multilayered, but there were many areas of epithelial-stromal separations and increased apoptotic bodies.

**Immunofluorescence (IF) detection of MMP-9 in vesicant-exposed corneas treated with doxycycline or minocycline**

MMP-9 is a corneal epithelial product upregulated by wounding and is specifically localized first at the basal surface of the basal epithelial cells. In a normal wound MMP-9 spreads distally throughout the wound site, in a matter of a few hours correlating with remodeling at the basement membrane zone. Very low amounts are seen in unwounded corneas. In the controls there is little, if any, MMP-9 staining (green, figure 3.3A) under the basal epithelial cells in the basement membrane zone. The staining at the apical epithelial cells is typical of the corneal epithelium’s autofluorescence. It is commonly observed in the cornea and is non-specific. Nuclei are stained blue by DAPI.
In the NM-exposed corneas, an increase in MMP-9 staining was observed at the basement membrane zone, reflecting the greater wounding by NM. The corneas exposed to NM, then treated with doxycycline in solution showed a less intense level of fluorescence, and improved epithelium. Most areas showed the epithelium to be in contact with the stroma. The corneas exposed to NM then treated with minocycline drops showed an even more intense fluorescence because the basal cells present are not healthy and the matrix is degraded. In this treatment group, there remained many areas where the epithelial cells were totally detached from the stroma. For NM-exposed samples treated with doxycycline and minocycline hydrogels, there is strong fluorescence because the cells are still present (unlike the NM samples), but they are likely to be in the process of dying from their appearance of cell migration away from the basement membrane.

Quantification of epithelial-stromal attachment was assessed to compare NM exposed samples to those treated with doxycycline or minocycline in ophthalmic drops to those in sustained released hydrogel (Figure 3.3B). The LiquiTears™ solution or the hydrogel without drug did not preserve any attachment, as both were similar measurements. Minocycline treatment after NM exposure in drops or hydrogel was also maintained around equal measurements, and not much more attachment was noted compared to no treatment at all. It is concluded that that minocycline treatment was not effective at this dose, regardless of the vehicle used. The only substantial difference was doxycycline drops compared to a doxycycline-loaded hydrogel. The doxycycline drops were most effective at this dose compared to any other treatment group. At the 24 hr post exposure time, the doxycycline-loaded hydrogel did not increase epithelial-stromal attachment beyond levels of corneas receiving no treatment.
MMP-9 Fluorometric Quantitation Assay

Figure 3.1C shows the effectiveness of doxycycline vs. minocycline in ophthalmic drops at increasing doses against MMP-9 activity induced by NM. As the histology suggested, doxycycline was more effective at reducing the activity of MMP-9 (Fig 3.1C). However, the difference from the minocycline inhibition was not as much as expected. Neither drug substantially reduced MMP-9 levels, as the greatest inhibition was only 17% of control.

Figure 3.3C shows the effectiveness of ophthalmic drops vs. hydrogels (which were only tested at 75 nmol, a similar dosing by ophthalmologists for off-label use). These results showed there actually was a reduction in MMP-9 activity with minocycline treatment after NM exposure that was not noticeable with histology. The doxycycline drops and hydrogel significantly reduced MMP-9 activity, as did minocycline, but there was no significant difference between doxycycline and minocycline. This suggests that these compounds may be effective in ways other than MMP reduction.

Doxycycline drops and hydrogels show effectiveness in vivo with SM exposed rabbit eyes

The previous data shows that corneal organ cultures can be used for the preliminary evaluations of drugs. Doxycycline proved most effective against injury in the cultures, therefore it was used in an animal study at Battelle. Rabbit eyes were exposed in vivo to SM followed by delivery of doxycycline as ophthalmic drops 3 times per day. Rabbit eyes were analyzed at 1 day, but because of the greater extent of injury with SM,
healing was followed for 28 days and analyses were also collected at 3, 7, and 28 days after exposure.

At 1 day post-exposure, the ulcerated corneal tissue had not yet begun to heal from the severe SM exposure. Gross examination of the rabbit eyes clearly demonstrated that corneal edema clouded some eyes by 3 days. Doxycycline was not apparently effective at these two time points (Fig. 3.4). However, with drop wise delivery of doxycycline for 7 days, a significant improvement was observed compared with eyes receiving no treatment. The doxycycline hydrogels produced the same improvement in corneal clarity as the drops. By 7 days post exposure, edema adversely affected transparency by increasing the corneal thickness. Treatment with doxycycline as drops or hydrogels decreased corneal thickness toward more normal values. Between the 2 doxycycline groups, the drop wise application resulted in less edema than the hydrogel application. By 28 days, the corneal epithelium appears to be thinner even without any treatment as in unexposed eyes samples. Treatment for 28 days with doxycycline drops or hydrogel applications did not negatively impact the eyes, but presented a normal phenotype similar to untreated samples (Figure 3.4). The doxycycline hydrogel did, however, decrease the amount of neovascularization caused by SM more than eyes receiving doxycycline drops and eyes not receiving any treatment by day 28.

**Keratectomy injuries show initial MMP-9 activation followed by a decrease after 48 hrs, while nitrogen mustard injury increases MMP-9 expression**

*Ex vivo* corneas were given keratectomized. Those exposed to NM were collected and sectioned for immunofluorescence analysis of MMP-9. Some were collected for
protein extractions to quantify active MMP levels. It took approximately 10 mins from the time the keratectomy was complete for corneas to be frozen for analysis (depicted as 0 hr), or to be put in culture for the 24 and 48 hr post keratectomy analyses (Fig 3.5A). The NM samples were cultured and either exposed for 10 min, washed with medium and collected as 0 hr samples, or exposed for 2 hrs and left in the 5% CO₂, 37°C incubator for 24 hrs or 48 hrs post-initial exposures.

Immunofluorescence sections stained with MMP-9 (green) and DAPI (blue) were photographed on the edge of the wound bed for keratectomies, and at the center of the cornea for NM-exposed samples (Fig 3.5B). The keratectomy caused an immediate upregulation of MMP-9 in the 0 hr sample. The 24 hr keratectomy sample showed less expression, but was still present at the edge of the wound bed. The 48 hr keratectomy sample was completely re-epithelialized and no MMP-9 expression was present once the injury was healed.

The 0 hr NM sample had low MMP-9 levels, as compared to the 0 hr keratectomy sample. The 24 hr NM sample showed an intense increase in MMP-9 levels and an even more dramatic increase after 48 hrs. This is opposite of what is observed with keratectomy injuries. MMP-9 activity, assessed from isolated protein, showed a similar pattern (Fig 3.5C). Therefore, MMP-9 levels are not similarly upregulated in all corneal injuries that destroy the basement membrane zone. With vesicants, the MMP-9 levels remain upregulated for longer, and increase with time, whereas after keratectomy, the levels decrease.
3.4—Discussion

The extracellular matrix is a complex network of protein constituents including collagens, elastin, glycoproteins (such as laminin, fibronectin, and nidogen), as well as various proteolycans and glycosaminoglycans. The dynamic equilibrium of the extracellular matrix under physiological conditions is a consequence of the balance between the regulation of synthesis and degradation of extracellular matrix components. MMPs represent a group of enzymes involved in the degradation of most of the components of the extracellular matrix and therefore participate in tissue remodeling associated with pathological situations and wound healing (Corbel et al., 2000).

Gelatinases such as MMP-9 are known to degrade almost all basement membrane constituents, including type IV collagen, nidogen, laminin, and gelatin (denatured fibrillar collagen). In skin during acute inflammation, polymorphonuclear neutrophils (PMNs), eosinophils, monocytes, and lymphocytes are recruited from the circulation at specific sites within tissue. These recruited cells ingest and kill microorganisms, remove foreign and cellular debris, and assist in tissue repair by modulating the activity of surrounding mesenchymal and endothelial cells. Inflammatory cells perform some of their functions by releasing MMPs into the pericellular space. MMPs are secreted to digest proteins, but when present in excess they can also over-process the extracellular matrix environment, disrupt resident cells and stimulate further inflammation (Corbel et al., 2000; Tetley, 1993).

MMPs commonly contain three domains. The first is the N-terminal hydrophobic (pre)domain, the propeptide domain, and the catalytic zinc-binding domain. The catalytic domain of MMPs has a cleft containing the catalytic Zn++ in which the substrate is
bound and then cleaved. One potential explanation for the effectiveness of doxycycline and minocycline is that tetracyclines bind to Zn$^{++}$ or Ca$^{++}$ in the active site of with MMPs, blocking the active site inducing changes that render the proenzyme susceptible to fragmentation during activation preventing any new substrates (Giasson et al., 1994b; Golub et al., 1998; Ollivier et al., 2007; Smith et al., 1996; Yao et al., 2007). Direct coordination with the active site (containing the zinc atom) of the catalytic domain blocks the access of the catalytic site to the substrate (Corbel et al., 2000; Van Wart et al., 1990).

Doxycycline has been shown to effectively treat MMP mediated ocular surface diseases and inhibit inflammatory cytokines, tumor necrosis factor (TNF)-α and interleukin-1 (IL-1) (Amir et al., 2000; De Paiva et al., 2006; Solomon et al., 2000). Doxycycline is not FDA approved for SM exposure, but is frequently prescribed off-label by the ophthalmologists because of its observed positive effect on wound healing. Doxycycline and minocycline also show anti-angiogenic activities in vivo (Lee et al., 2004), desirable in corneal wound healing. Both drugs inhibit human umbilical vascular endothelial cell (HUVEC) proliferation and tube formation, tumor cell proliferation and migration in vitro (Bettany et al., 1998; Fife et al., 1997; Fife et al., 2000).

In the current study, the results suggest that doxycycline and minocycline, delivered by the ophthalmic drop, will be as or more effective than doxycycline or minocycline applied in hydrogel form at the 24 hr post-exposure time point for treatment of mustard injuries. The hydrogels prepared and evaluated in the current study are normally biodegradable (but not in our model system), and for the most part optically transparent. They show resistance to external forces and provide sustained drug release for up to for 24 hours. These formulations can be administered as a solution which rapidly forms a
hydrogel capable of withstanding shear forces in the eye. Permeability studies show that the barrier property of the cornea is compromised when exposed to vesicants, further allowing drug access to the cornea (Anumolu et al., 2010; Dursun et al., 2001).

Other tetracycline derivatives were tested against NM exposure in the same manner as reported here (Gordon et al., 2010). These derivatives were sancycline, t-butyl sancycline and dedimethyl amino tetracycline. Immunofluorescence data revealed a reduction in MMP-9 levels compared to NM-exposed samples without treatment, but no remarkable difference in MMP-9 levels between drugs. Each showed good MMP-9 inhibition, but the other tetracycline derivatives did not facilitate healing as well as doxycycline, which showed healthy cells and attached cell layers when visualized with histology compared to the three other compounds. This leads us to conclude that other unidentified properties, besides MMP inhibition, are responsible for the beneficial effects seen with doxycycline and should be investigated further.
Figure 3.1 – Histology of the dose-response experiment using doxycycline vs. minocycline inhibition of epithelial-stromal separations (A) as a response. Doxycycline at 25 nmoles was not an improvement compared to no treatment at all. At 75 nmoles, doxycycline was enough to improve the appearance so that the epithelium stayed attached to the stroma, but the minocycline was not much improved until 225 nmoles. The measurement of epithelial-stromal attachment shows more clearly that 225 nmoles is a toxic dose with doxycycline (B). Protein extractions were used in an MMP-9 activity kit which shows doxycycline decreases more activity compared to minocycline, but neither reduced it by as much as we thought it would. MMP-9 activity in corneal protein extractions at various doses when used after NM is shown in (C). Scale bar = 100 µm.
B.

Percent Epithelial-Stromal Attachment Preserved with Doxycycline and Minocycline after NM exposure (+/- SD)

C.

MMP-9 activity at increasing doses of Mino vs. Dox (+/- SD)
Figure 3.2 – Use of minocycline and doxycycline ophthalmic drops vs. hydrogels (HG) with and without NM exposure. A dose of 75 nmoles of doxycycline was most effective in maintaining epithelia integrity, and is often prescribed by ophthalmologists for off-label use at this dose; we also used that dose to load the hydrogels. The right column is the results of the hydrogel experiment. The hydrogel itself for the most part did not have any effect on the cornea, and the results are worse than with the ophthalmic drops. Histology shows normal control corneal and separated epithelium in nitrogen mustard exposed corneas. NM-induced separations were inhibited most effectively by doxycycline drops (next page).
Figure 3.3 – MMP-9 levels shown with immunofluorescence of anti-MMP-9 antibody (green), (DAPI=blue) Scale bar = 100 μm (A). There is some autofluorescence in the apical epithelium here which may occur right before cell death. Each treatment, with the exception of doxycycline drops, still shows some MMP-9 expression. Again epithelial-stromal attachment was quantified and only the doxycycline drops show improvement in epi-stromal separations by student’s t-test; p<0.05 (B). MMP-9 activity was measured on untreated, doxycycline treated, and minocycline treated samples as drops vs hydrogel and doxycycline was significantly decreased as seen by 2-way ANOVA; p<0.05 (C).
B. Percent Epithelial-Stromal Attachment Preserved after NM exposure with Doxycycline or Minocycline drops or hydrogel after 1 day (+/- SD)

C. MMP-9 activity in Nitrogen Mustard exposed samples with Dox or Mino treatment as drops vs. hydrogel (+/- SD)
Figure 3.4 – Results of *in vivo* sulfur mustard-exposed rabbit studies. The *in vivo* studies were performed by Battelle and given either doxycycline drops or a doxycycline hydrogel, both at 75 nmoles. The first row shows an actual picture of the rabbit eye which appears cloudier after 3 days. At 1 day, doxycycline treatment does not improve histology because epithelia are still separated from the stroma, but with 3 days of treatment the histology is improved and continues to improve from 7 to 28 days. The third rows are unexposed corneas that either received no treatment throughout the study, or received dox drops or received dox hydrogel treatment.
Figure 3.5 – A keratectomy was used to determine whether MMP-9 is upregulated for a longer period of time than a keratectomy injury. A biopsy punch was inserted into the stroma and the overlying epithelium was removed (A). Corneas were collected at 0, 24 and 48 hrs. MMP-9 expression is highest in the keratectomy immediately after injury and is completely healed after 48 hrs. With NM, the injury continues to get worse at 48 hrs. That is verified both with immunofluorescence and activity assay. Immunofluorescence reveals an increase in MMP-9 (green) after nitrogen mustard exposure with time, the opposite of MMP-9 levels seen after keratectomy (B). The chart indicates MMP-9 activity assay supports the immunofluorescence data (C). The difference in MMP-9 activity 48 hrs after the wound was significantly significant **p<0.01 (next page).
A.

B. Keratectomy

Nitrogen Mustard

0 hr 24 hr 48 hr

C.

MMP-9 Activity post Keratectomy vs. NM-exposure (±SD)

MMP-9 Activity (ng/ml)

0 HOUR 24 HOUR 48 HOUR
CHAPTER IV

ERK ACTIVATION PHOSPHORYLATES ADAM17 IN NITROGEN MUSTARD-EXPOSED CORNEAS
4.1 -- *Abstract:*

Sulfur mustard (SM) and nitrogen mustard (NM) exposure causes separation of the corneal epithelial-stromal junction, resulting in microbullae (or microblisters). Separation of the cell layers is, in part, due to activation of ADAM17, which cleaves the hemidesmosomal transmembranous anchoring component, collagen XVII. ERK (extracellular signal-related kinase) may be responsible for the phosphorylation of threonine (T735) in the cytoplasmic tail of ADAM17, known to cause its activation; it may also be indirectly responsible for inducing matrix degradation by MMP-9. These experiments were performed to determine whether ERK and ADAM17 have a direct interaction, and whether the interaction is attenuated by the addition of the MEK (MAPK/ERK kinase) inhibitor, PD98059. MEK is upstream of ERK, and therefore the inhibitor also affects ERK. Corneal organ cultures were exposed to 100 nmoles of NM applied onto the central corneas for 0, 5 or 10 mins, followed by washing with medium or medium plus 100 nmoles PD98059. The medium or medium plus inhibitor was left in the culture at 37°C for either 10 min or for 24 hrs. Corneas were then frozen in OCT for analysis by light and immunofluorescence microscopy, or frozen in liquid nitrogen for protein extraction for westerns or activity assays. Immunofluorescence indicated the presence of pERK, the extracellular (activated) domain of ADAM17, and MMP-9. Pull down assays were performed to demonstrate an association between ERK and ADAM17. Immunoprecipitated ERK was analyzed on western blots probed with antibodies against the cytosolic domain of ADAM17, ERK, and pERK. It was found that ADAM17 quickly becomes associated with ERK after a 10 minute NM exposures, and it is phosphorylated. This activation leads to cleavage of collagen XVII and separation of the epithelial and
stromal layers. The ERK inhibitor PD98059 inhibited activation of ADAM17 when applied immediately after the brief NM exposures.

**Key Words:** ADAM17, NM, ERK, PD98059, collagen XVII, MMP-9

**Abbreviations:** ADAM17, a disintegrin and a metalloprotease 17; NM, nitrogen mustard; ERK, extracellular signal related kinase; MMP-9, matrix metalloproteinase-9; MEK, MAPK/ERK kinase

**4.2 -- Introduction:**

Vesicants are blister-causing agents in the skin, and microbullae-inducers in the cornea. These microblisters are painful and may lead to vision loss. Previous studies showed that nitrogen mustard targets the basement membrane zone in the cornea, and induces epithelial detachment from the underlying stroma (Cho et al., 2000). This is partly due to activation of ADAM17, the sheddase for the transmembranous hemidesmosomal protein, collagen XVII (Franzke et al., 2002). Cleavage of collagen XVII allows the basal epithelial cells to detach from the basement membrane and the underlying stroma. While epithelial shedding is part of normal wound healing, if it is prolonged or too extreme, severe negative effects are observed such as infection. With this in mind, we sought the mechanistic cause of ADAM17 activation. Direct interactions of intracellular protein kinases with cytoplasmic domains of ADAMs have been postulated as modulators of shedding activity (Diaz-Rodriguez et al., 2002; Izumi et al., 1998; Poghosyan et al., 2002).
Activation of ADAM17 may be dependent upon MAPK activation. MAPK (mitogen activated protein kinases) are activated by diverse stimuli ranging from cytokines, growth factors and cellular stress. The basis for activation of these groups of cell signaling molecules is the transfer of phosphate moieties. The basic arrangement of all MAP kinase signaling systems consists of a three-kinase model: a MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK kinase (MAPKK), which phosphorylates and activates a MAPK (Kolch, 2000). ERK (extracellular signal-regulated kinase) is a MAPK in its signaling cascade, and the pathway already has been shown to be involved in corneal epithelial wound healing (Glading et al., 2000; Klemke et al., 1997). ERK is phosphorylated as a result of the Ras (G-protein, with signaling beginning at the cell surface) / Raf (the MAPKKK) / MEK (the MAPKK) / ERK (the MAPK) cascade (Kolch, 2000).

MEK (also known as MAPK/ERK kinase) needs phosphorylation of 2 serine amino acid residues to become activated, and when this occurs, ERK will be activated. ERK also requires 2 phosphorylation sites to become activated (Y. Zhang et al., 2006). The 2 amino acid phosphorylation sites (Tyrosine-185 and Threonine-183) lay one residue apart in the ERK phosphorylation site. The -T-X-Y- motif (particularly the Tyr-185 side chain) blocks access of substrates to the active site when not phosphorylated (inactive). A conformational change occurs upon phosphorylation, displacing Tyr-185, and creating a lip structure which allows substrate binding (Cobb et al., 1995). The phosphorylation lip is where ERK transfers phosphate molecules to substrates such as ADAM17.
We hypothesize that an important post-translational modification of ADAM17 is phosphorylation of the cytoplasmic domain. Serine-proline or threonine-proline is the minimum phosphorylation sequence on any substrate to be phosphorylated by ERK (Davis, 1993; Jacobs et al., 1999; Schaeffer et al., 1999; Widmann et al., 1999). The cytoplasmic domain of ADAM17, particularly phosphorylation of its threonine residues, has been studied previously. Since the peptide sequence -Pro-Gln-Thr735-Pro- contains the only Thr-Pro dimer in the cytoplasmic domain of ADAM17, it is the likely phosphorylation site (P. Xu et al., 2010).

Docking sites (or binding sites for ERK on a substrate) may determine which sites are phosphorylated (Tanoue et al., 2000). A common binding motif on a substrate of ERK is –P-x-x-P- as found in PI-3 kinase and src tyrosine kinase for docking with ADAMs 12 and 15, respectively (Kang et al., 2000; Poghosyan et al., 2002). The -Pro-Gln-Thr735-Pro- motif located in the intracellular domain of ADAM17 indicates that the docking site also contains the phosphorylation site. This suggests that ERK and ADAM17 may physically associate, allowing the ADAM17 intracellular domain to mediate “inside-out” regulation of metalloprotease activity (Soond et al., 2005).

Support for the threonine 735 phosphorylation site discovery comes from previous studies (Diaz-Rodriguez et al., 2002; Fan et al., 2003; Soond et al., 2005). Phorbol 12-myristate 13-acetate (PMA) and epithelial growth factor (EGF) induced phosphorylation of ADAM17’s Thr-735 via ERK-induced cleavage of the transmembrane TrkA neurotrophin receptor (Diaz-Rodriguez et al., 2002). PMA and EGF also induce Ser-819 phosphorylation of ADAM17 via ERK (Fan et al., 2003; Soond et al., 2005). The regulation of ectodomain shedding of transmembranous
molecules by ADAM17, and the ability of most MAP kinase signaling pathways to activate shedding without the need for new protein synthesis suggests that the cytoplasmic domain may act as a signal transducer that regulates shedding (Fan et al., 1999; Gechtman et al., 1999; Gutwein et al., 2000; Umata et al., 2001; K. P. Xu et al., 2001) via a conformational change, in response to intracellular activities (Fan et al., 2003).

ERK may also be involved in MMP-9 activation. NM induces an increase of MMP-9 expression and activity that can be attenuated by application of the MMP inhibitor, doxycycline (Cho et al., 2000; Genersch et al., 2000; Kim et al., 2005). MMP-9 degrades the basement membrane extracellular matrix after injury so that a provisional matrix can be laid down for cells to migrate on to close the wound. When MMP-9 levels persist for several days, the degradation of matrix inhibits wound closure. This is the case with NM exposure. Transforming growth factor (TGF)-β1 indirectly stimulates MMP-expression. Doxycycline was found to inhibit TGF-β1-induced MMP-9 activity through the ERK signaling pathway in human corneal epithelial cells (Kim et al., 2005). Ten ng/ml of TGF-β1 activated ERK in as little as 15 minutes. The inhibitory action of the MMP inhibitor, doxycycline, against ERK phosphorylation was comparable to that of the MEK inhibitor, PD98059 (Kim et al., 2005). This suggests that ERK affects the levels of both MMP-9 and ADAM17 in NM-exposed corneas. We evaluated the effect of ERK on MMP-9 activity also using the MEK inhibitor.
4.3 – Results:

**A MEK/ERK inhibitor facilitates recovery from short NM exposures**

NM affects the extracellular matrix between the corneal epithelial and stromal cell layers, also known as the basement membrane zone. First, the gross appearance of control corneas and corneas exposed to NM plus or minus ERK inhibitor was examined. Figure 4.1 shows H&E stained corneas at 40x. H&E staining was used to evaluate the interaction between the epithelium and the stroma. Figure 4.1A is an unexposed control cornea, which was not exposed to NM or the ERK inhibitor PD98059. The epithelial layer is about 5 cell layers thick and has a fairly uniform appearance throughout. The basement membrane is intact as it is in 4.1F, which shows an unexposed cornea treated with PD98059. In panel F, one can observe mild undulations in the epithelial-stromal border which are normal for organ cultured corneas. Except for this, the epithelial-stromal junction looks very similar to that of figure 4.1A. There is no visible separation between the stroma and epithelium.

After 0, 5 or 10 min NM exposures (4.1B-D), there is downward hyperplasia of the epithelium, which appears to invade into the stroma. In the 10 min NM-exposed cornea (4.1D), damage to the epithelial-stromal border is visible, as seen by an indistinct basement membrane. Evidence of cell death is visible by the small, pyknotic nuclei in the basal layer of the epithelium. The epithelial cells are less regular in appearance; the epithelium appears thicker than the control, and the cells are less organized. Figures 4.1G-I show the corneas exposed to NM and then treated for 10 min with PD98059. The integrity of the basement membrane zone appears improved compared to the NM-exposed cornea, but are not as intact as the control. In the cornea exposed to NM for 10
minutes and allowed to incubate at 37°C for 24 hr (4.1E), the basal cells and the epithelial-stromal interface appear to have healed with time although the nuclei appear swollen. The 24 hr treatments with PD98059 after a 10 min NM exposure (4.1J) shows smaller epithelial nuclei, and an intact BMZ, more like the unexposed control (4.1A).

**pERK and activated ADAM17 are reduced by PD98059 treatment**

Figure 4.2 shows the immunofluorescence of activated ERK, denoted as pERK, in corneas exposed to the same conditions as figure 4.1 (pERK=Alexa488, green). Figure 4.2A, the control cornea, has very low levels of green stain. The PD98059 control section (4.2F) also has little to no pERK expression. In the cornea exposed to NM which is immediately washed off (i.e., exposed for 0 min) (4.2B), ERK expression is much more intense, and is even more so with NM exposure left on the cornea for 5 min (4.2C). After a 10 min NM exposure (4.2D), pERK remains upregulated, but not as intensely as seen at 5 min. The 10 min NM-exposed cornea which was washed, then incubated at 37°C for 24 hrs had a greatly reduced level of pERK expression than the sample washed and collected immediately after the exposure. Figure 4.2H-J shows the corneas exposed to NM, then treated with ERK inhibitor. Activated ERK is undetectable in these corneas.

Immunofluorescence expression of the extracellular domain of ADAM17 is shown in figure 4.3. This antibody reacts with ADAM17 that has undergone a conformational change to the activated enzyme form. The unexposed samples show little activated ADAM17 (4.3A, 4.3F). Cells appear normal and there is no sign of basement membrane injury. In the corneas exposed to NM (Fig 4.3B-4.3E), activated ADAM17 expression is evident in the BMZ. In corneas that received treatment with the ERK
inhibitor after NM exposure (figure 4.3G-4.3J), the level of activated ADAM17 is attenuated, but not completely eliminated.

Although pERK and activated ADAM17 are present in corneas exposed to nitrogen mustard, they are present in very low levels in unexposed corneas and in corneas exposed to PD98059 alone. Furthermore, pERK and ADAM17 are attenuated in NM-exposed corneas that received inhibitor treatment. PD98059 binds to inactive MEK molecules, preventing downstream phosphorylation to occur. PD98059 itself was not toxic to the corneal cells.

**ADAM17 activity is reduced only when PD98059 is applied at 0 min and 5 min**

An ADAM17 activity assay (Figure 4.4) was performed to quantify the amount of active enzyme after NM exposure, with and without subsequent PD98059 treatment. The corneas that received a 10 min treatment of PD98059 after 0 and 5 min NM exposure had significantly reduced activity of ADAM17 compared to corneas that were given no treatment for 10 minutes after NM exposure. This may be because the inhibitor was able to interfere with the ability of pERK to phosphorylate ADAM17 at these early time points. After 10 min of exposure to NM, however, it appears that pERK has already phosphorylated ADAM17, causing its activation and this could not be reduced with a 10 min PD98059 treatment. When the corneas were exposed to NM for 10 min, then followed by 24 hr treatment with the inhibitor, the ADAM17 levels were not much different than without inhibitor. This indicates that events involving ERK were likely complete. For statistical analysis each experiment was performed in triplicate. The results
were expressed as means +/- SD, and analyzed by using 2-sided students t-tests. A value of p < 0.05 was considered statistically significant.

Western blots were used to verify these results (Figure 4.5A). Membranes probed with an antibody against phosphorylated threonine 735 in ADAM17 (pT735) showed a similar expression pattern to the ADAM17 activity assay. The level of pT735 (i.e. the activated form) was decreased with PD98059 application, and was very low 24-hr post exposure with or without inhibitor. Detection of the extracellular domain of ADAM17 remained upregulated at all time points, but was slightly inhibited with PD98059 treatment after 0 min and 5 min NM exposures, similar to our immunofluorescence results. This suggests that at the 24 hr time point, when activity of ADAM17 is still observed, that it may be activated by phosphorylation at a site other than T735, or activated by furin and other proprotein convertases which activate ADAM17 by cleaving the prodomain.

Co-immunoprecipitation assays (Figure 4.5B) were performed to examine the association between ERK and ADAM17 since pERK binds to the cytoplasmic tail of ADAM17. Protein extractions were immunoprecipitated with anti-mouse IgG agarose beads bound to an anti-ERK antibody (all forms), and run on SDS-PAGE gels that were transferred to western blots probed with either the cytosolic domain of ADAM17, pERK or ERK. The cytosolic domain of ADAM17 co-precipitated with ERK, and decreased with time after NM exposure, as did pERK, indicating that the interaction between ERK and ADAM17 early on is strong if not inhibited.
MMP-9 levels are reduced with PD98059 when used for 24 hrs

Immunofluorescence and fluorometric activity analysis were used to test whether MMP-9 was induced through the ERK pathway (Fig 4.6). Immunofluorescence analysis showed little to no MMP-9 levels until after a 10 min exposure (Fig 4.6A). The most observable MMP-9 expression was seen 24 hrs after the 10 min NM exposure. In this case, four PD98059 treatment applications over the 24 hrs post injury reduced the MMP-9 signal greatly. These results indicate that MMP-9 is expressed at somewhere between 10 mins to 24 hrs after NM exposure in this system, and that PD98059 is able to attenuate the MMP-9 levels.

MMP-9 activity was quantified showing similar results (Figure 4.7). MMP-9 activity levels did not significantly rise until 24 hrs post a 10 min exposure. Although MMP-9 was not immunodetectible after a 10 min NM exposure followed by 4 treatments of PD98059 over 24 hr (Fig 4.6J), activity of the enzyme was detectible, and was significantly elevated over the 0, 5, and 10 min NM exposures treated for only 10 min with the ERK inhibitor. These results support the previous observation that MMP-9 expression must take between 10 mins to 24 hrs to become significantly upregulated, and that the ERK inhibitor, PD98059 can reduce levels of MMP-9 when applied 4 times over a 24 hr post exposure period. The discrepancy between the results of the 24 hr applications of PD98059 after a 10 min NM exposure, (with immunofluorescence and with MMP-9 activity assay) could mean that the activity assay is more sensitive, or that homogenized extracts are more accurate and less subjective than single sections.
4.4 -- Discussion:

We tested whether interfering with an initial signaling pathway induced by injury would identify how ADAM17 is activated after mustard exposure and whether blocking the signaling pathway might be a potential therapy for vesicant injury. These results suggest that PD98059 treatment is not an ideal countermeasure for civilian exposures since in these studies it must be used within 10 mins to be effective against basement membrane zone (BMZ) degradation and ultimately microbullae formation. People exposed to sulfur mustard do not feel the exposure for hours. Other signaling pathway inhibitors may be more effective countermeasures. These experiments do, however, elucidate one mechanism for ADAM17 activation and a potential activation mechanism for MMP-9 after nitrogen mustard exposure.

NM exposure results in activation of ADAM17 and thereby contributes to collagen XVII cleavage, allowing cell layer separation. To verify that NM activated ADAM17 via the ERK family, we utilized PD98059, an inhibitor of phosphorylation/activation of MEK (ERK kinase) (Alessi et al., 1995). When MEK is phosphorylated, it phosphorylates ERK to pERK which is released in the cytosol to phosphorylate available substrates (Adachi et al., 1999; Kolch, 2000). These experiments revealed by immunoflourescence microscopy with a pERK antibody that the signaling molecule was upregulated by NM exposure. Phosphorylation of ERK led to phosphorylation of ADAM17, activating the enzyme. Thus, ERK activation is a link between NM exposure and BMZ damage. Evidence that pERK phosphorylated ADAM17 was demonstrated by immunoprecipitation assays with ERK antibody. ERK co-
precipitated with phosphorylated ADAM17. Immunofluorescence coupled with activity assays confirmed its enzymatic activity.

In normal mesangial cells and kidney cells, the physical association between active ERK and ADAM17 has also been demonstrated by ADAM17 co-precipitation with activated ERK (Bell et al., 2010; Gooz et al., 2006). Our results support the claim that injury-induced ERK activation is responsible for phosphorylation of threonine 735, activation of ADAM17, and downstream collagen XVII shedding after nitrogen mustard exposure as a consequence of MAPK “inside-out” signaling. T735 in the ADAM17 cytoplasmic domain participates in the docking of ERK to the enzyme (Diaz-Rodriguez et al., 2002), and mutation of T735 decreases the ADAM17 phosphorylation by MAPK (P. Xu et al., 2010).

One of the initial steps of ERK activation in corneal wounding is the release of nucleotides, (UTP/ATP), which become substrates for the G-protein coupled receptor, P2Y2 (Boucher et al., 2010; Yang et al., 2004). The purinergic P2Y2 receptor activates phospholipase C (PLC) and phosphatidylinositol hydrolysis, generating diacylglycerol and inositol 1,4,5-triphosphate, which stimulates protein kinase C (PKC) and cytosolic calcium (Ca^{2+}) mobilization, respectively (S. J. Chang et al., 2008). The calcium influx is thought to be responsible for the injury-induced ERK activity that results in ADAM17 phosphorylation (Jacob et al., 2002).

In addition to its collagen XVII cleaving properties, ADAM17 contributes to other injury responses. For example, a second function of ADAM17 is the liberation of EGFR (epidermal growth factor receptor) ligands by sheddase activity (Fig. 4.8). Thus, physical injury to the cornea initiates wound healing by ERK activation, which leads to
phosphorylation and activation of ADAM17 that can result in EGFR activation (Soond et al., 2005; Yin et al., 2009). ADAM17 cleaves the pro-domain of EGFR ligands such as heparin binding (HB)-EGF, resulting in a soluble ligand which can activate the EGF receptor (Kenny, 2007). A downstream result of EGFR activity is Raf-MEK-ERK intracellular signaling, necessary for proliferation and cell survival. The timing for this to occur can be anywhere between 10 mins and several hours, initiated in response to a variety of stimuli, including wounding and G-protein coupled receptor (GPCR) ligands (Fini et al., 1996; Yin et al., 2009). The healing seen in our experiments at 24 hrs after injury (i.e. the 1 day post exposure ADAM17 and MMP-9 activity) may be due to a second wave of ERK signaling, induced by EGFR signaling stimulated by ADAM17 from the first wave of ERK signaling (see schematic, figure 4.8). EGFR activation contributes to the progression of carcinomas by promoting cell proliferation and cell survival (Yarden, 2001), and interference with EGFR activation is the basis for some cancer therapies (W. Zhang et al., 2007).

ERK may also have an indirect role in the induction of MMP-9 which results in matrix disruption by mustards. This may occur via TGF-β1, since this has been shown to stimulate MMP-9 expression through the ERK pathway (Kim et al., 2005; D. Q. Li et al., 2001). However, this was not our focus, so we did not test this in the cornea. It was apparent that it would take a 24 hr incubation period after a 10 min NM exposure before the highest amount of MMP-9 levels were seen. This is very different from ADAM17 expression levels, which were highest immediately after a 10 min incubation period.

The MMP-9 gene promoter contains AP-1 (activator promoter-1) binding sites in its gene promoter region (Fini et al., 1998; Fini et al., 2005; Vu et al., 1998). AP-1
transcriptional activity is typically regulated by a MAPK such as ERK. Therefore, upstream triggers of MMP-9 are likely to involve ERK. Our results indicate that, even though MMP-9 was only turned on at 24 hr post exposure, the ERK inhibitor PD98059 inhibited downstream MMP-9 activation. Whether this was a direct or indirect interaction was not examined.

This information is useful in elucidating new targets for countermeasures against mustards. Our data is a beginning toward understanding the mustard-induced disruption of the adhesion complexes at the corneal epithelial-stromal border, and the subsequent disorganization of the extracellular matrix. We suggest the mechanism for microbullae formation may be ERK signaling the activation of ADAM17 and MMP-9. More experiments need to be done to test the effectiveness of PD98059 in vivo, as well as additional ERK inhibitors and optimal times of application.
Figure 4.1 -- H&E micrographs. The left column was not given the ERK inhibitor (PD98059). These were given either 0, 5 or 10 minute exposures, and then a 10 minute incubation period. The last row got a 24 hr incubation period. There were no microbullae formed, but instead an increase in epithelial invaginations which were prevented with a 10 min PD98059 treatment after exposure (right column). The hyperplasia seen with NM exposure at short time points is attenuated after 24 hrs even with no treatment. Unexposed samples and samples treated with PD98059 show normal epithelium. Scale Bar = 100 µm.
Figure 4.2—Activated ERK immunofluorescence signal (Alexa 488; green) as visualized in the nuclei of basal epithelial cells and in the extracellular space due to ADAM17 binding and phosphorylation. Scale Bar = 100 µm.
Figure 4.3 -- ADAM17 ectodomain fluorescence staining (Alexa488, green) as seen in unexposed controls, and samples exposed to nitrogen mustard with (right column) and without PD98059 treatment (left column). ADAM17 was seen almost immediately, and was attenuated with PD98059. Blue = DAPI. Scale Bar = 100 µm.
Figure 4.4 -- ADAM17 activity assay indicated that ADAM17 activity can be significantly inhibited by PD98059 if applied within 10 min of NM exposure. N=4 *p<0.05
Figure 4.5 -- Western blots correlate with ADAM 17 activity data and immunofluorescence data. Western blots (4.5A) demonstrate that phosphorylation of threonine 735, which activates ADAM17, occurs immediately after NM exposure, and is inhibited by PD98059, as indicated by the activity assays. This inhibition of ADAM 17 activity was observed only when the 10 minute PD98059 treatment was applied after the 0 of 5 min NM exposures. It did not appreciably inhibit ADAM17 if the NM exposure was 10 min or longer. Pull down assays were performed after NM exposure, and anti-ERK probed beads were able to immediately bind to the cytosolic domain of ADAM17, where T735 is located (4.5B), indicating a direct interaction. A schematic of the ADAM17 structure (4.5C) shows where antibody epitopes reside. Pro = prodomain, Cat = catalytic domain, CRD = cysteine rich domain, TM = transmembrane domain, CT = cytoplasmic domain. The location of threonine 735 is indicated. Modified from (X. Li et al., 2007).
Figure 4.6 -- MMP-9 was not seen prior to 10 minutes post-NM exposure and was not fully visually expressed until after 24 hrs by immunofluorescence (E). After 24 hrs the MMP-9 signal was present and it appears reduced with PD98059.
**Figure 4.7** -- MMP-9 activity analysis also did not show any significant increase in activity until after 24 hrs. The spike was decreased somewhat by PD98059. N=4 *p<0.05
Figure 4.8 -- The hypothesized schematic of ADAM17 activation by ERK phosphorylation at the T735 position in the cytoplasmic tail. (1.) pMEK phosphorylates ERK (2.) pERK phosphorylates ADAM17 at the T735 position (3.) ADAM17 is activated and cleaves collagen XVII from the hemidesmosome causing epithelial-stromal separation (4.) ADAM17 also cleaves the pro-domain of EGFR ligands, creating soluble ligands able to bind to EGFR. This allows further downstream ERK signaling, which is necessary for epithelial proliferation and wound healing.
CHAPTER V

CONCLUSION

The objective of the experiments in this dissertation was to elucidate the mechanisms of mustard toxicity to the basement membrane zone of the cornea, as well as to use the identified mechanisms to develop countermeasures. The first two specific aims determined that the enzymes ADAM17 and MMP-9 were upregulated after exposure. These two enzymes may be the cause of one of the most damaging injuries to the cornea, namely the separation of the epithelium from the stroma. Using inhibitors of these two enzymes, we found improvement in healing in an organ culture system and in \textit{in vivo} exposed eyes. An ideal countermeasure for civilian mustard exposures would need to be effective when applied about 4 hr after the initial exposure, as opposed to co-incident with the exposure or pre-exposure. This is because civilians in terrorist situations would not feel the effects for hours, as has been demonstrated by accidental exposures from canisters buried in the ocean after World War I. Because of the restriction on use of sulfur mustard, and because of the range of injuries produced by mustard exposures, corneal organ cultures were exposed to CEES (half mustard) and NM (nitrogen mustard) to identify counteragents that might be effective against SM exposure. Once organ culture experiments were completed, promising counteragents proceed to testing in animal eyes exposed to SM.
The first goal of these experiments was to optimize the culture system, and to evaluate whether the injury caused by CEES and NM was the same as with SM. The culture system worked ideally. Corneal dissections were performed the day before the exposures, the corneas were equilibrated overnight, then the organ cultures were exposed the next morning immediately after the CEES or NM was mixed into medium. Mustards take time to induce injury. Blistering seen in skin exposures is often not seen until 24 hrs later. Once the histology was performed on the organ culture sections, it became clear that a two-hour CEES or NM exposure was ideal to mimic the corneal SM injury at 24 hr post exposure, as demonstrated by comparison with rabbit eyes exposed \textit{in vivo} to sulfur mustard at Battelle.

The experiments in specific aim 1 show that activation of ADAM17 is an important cause of pathology after mustard injuries. The activation of this enzyme leads to cleavage of collagen XVII, an important transmembranous component of the hemidesmosome at the epithelial-stromal junction. This cleavage leads to separation of the cell layers (i.e., formation of microbullae). Microbullae are painful and ineffective repair of them can cause scarring, opacity of the cornea and loss of vision. In some mustard-exposed individuals, separations between cell layers reoccur many years after the exposure, thus becoming a life-long problem for victims, and to date, the only successful treatment is corneal transplant. If neovascularization of the cornea has occurred, the risk of transplant failure is high.

Protein extracts from corneas exposed to NM, then treated with synthetic ADAM17 inhibitors, were evaluated for their ADAM17 activity levels. The increase in activity of ADAM17 correlated with the increased extent of epithelial-stromal
detachment. This suggested that ADAM17 at least in part was responsible for microbullae formation. ADAM17, along with ADAM9 and ADAM10, cleave collagen XVII (Franzke et al, 2009). Immunofluorescence with antibodies against collagen XVII and the ectodomain of ADAM17 support the role of ADAM17 in microbullae formation by visualizing the loss of collagen XVII in the BMZ when ADAM17 levels are increased. The inhibitors decreased the separations of the epithelium and stroma. ADAM17 inhibitors, i.e. hydroxamates, may be candidates for therapy of mustard injuries. The hydroxamate compounds tested in the experiments in specific aim 1 have shown to be promising countermeasures when used for 24 hr, beginning 2 hr post exposure. NDH4417 appears to be the most efficacious ADAM17 inhibitor and is a candidate for future in vivo studies. NDH4417 reduced ADAM17 levels to the point where the collagen XVII signal by immunofluorescence was equivalent to controls.

Matrix metalloproteinases are matrix molecules known to play a role in mustard injuries. MMPs are secreted to perform routine matrix maintenance and to remodel extracellular matrix after injury. When present in excess, they may become overactive and destroy the extracellular matrix environment, disrupt resident cells and stimulate inflammation (Corbel et al., 2000; Tetley, 1993). MMP-9 degrades almost all basement membrane constituents, including type IV collagen, nidogen and laminin, and gelatins. Thus it seemed likely that MMP-9 might be involved in the matrix remodeling steps that result in epithelial-stromal separation. Our work (Gordon et al., 2010), and the work of others (Kadar et al., 2009) showed this to be true. Since the tetracycline family is composed of agents with MMP-inhibitory action, we tested some of these. Doxycycline was chosen because it is routinely used off label as a topical application for eye
infections. Doxycycline also inhibits outward vessel remodeling in response to increased blood flow (Ramamarthy et al., 1993; Tronc et al., 2000). This is an important effect because neovascularization is an effect of sulfur mustard exposure. In vivo studies in SM-exposed rabbit eyes demonstrated that doxycycline retarded neovascularization over the course of a month after exposure (Gordon et al., 2010).

In specific aim 2, the organ culture results show that doxycycline and minocycline, delivered by an ophthalmic drop, have a better 24 hr post-exposure histology than doxycycline or minocycline applied in hydrogel form. In previously reported work (Gordon et al., 2010) we tested the hydrogels containing doxycycline against SM injury for up to 28 days. By 7 days, the doxycycline hydrogel was just as effective at reducing thickness of the cornea as the doxycycline drop. The hydrogel did a better job of preventing neovascularization (~48% reduced) in SM-exposed eyes compared to those receiving doxycycline as a drop (~30% reduced), which was much improved compared to SM-exposed eyes receiving no treatment.

In an effort to understand the mechanism by which ADAM17 and MMP-9 are activated, a literature search revealed that ERK signaling was potentially involved (Diaz-Rodriguez et al., 2002, Kim et al., 2005). To verify whether this mechanism is employed after NM exposure, we monitored the ERK activity after exposure plus and minus PD98059, an inhibitor of upstream MEK phosphorylation. The experiments in specific aim 3 revealed that NM exposure induced the ERK pathway activation of ADAM17, a contributor to collagen XVII cleavage. ERK signaling may also contribute to the upregulation of MMP-9. Increase in TGF-β1 is a common response to injury and has been shown to stimulate MMP-9 expression (Kim et al., 2005). Cellular responses by
TGF-β receptors are transduced through intracellular signaling pathways during inflammation and wound healing. The experiments in specific aim 3 suggest that the MAPK/ERK kinase (MEK) inhibitor, PD98059, can reduce levels of ADAM17 when used within 10 minutes of exposure and can also reduce MMP-9 levels when treatment is continued for 24 hours. ADAM17 phosphorylation is the initial effect causing the shedding of collagen XVII and epithelial-stromal separation, while MMP-9 is expressed later and targets nidogen and laminin hindering the re-establishment of a healthy cell layer. ERK signaling appears to be activated within minutes after exposure, and is still exerted in different ways 24 hrs later. The use of PD98059 as a countermeasure against mustard exposure would only be practical for inhibiting the 24 hr injury phenotype, since the ocular effects are felt hours later. More experiments with PD98059 will have to be performed on rabbits exposed to sulfur mustard to determine whether PD98059 is useful at extended times post exposure. The experiments in this study do, however, elucidate a possible mechanism for ADAM17 and MMP-9 activation seen after nitrogen mustard exposure.

The hope is that the information in this dissertation is useful for elucidating new targets for countermeasures against disruption of the basal epithelial cell adhesion complexes and disorganization of the extracellular matrix after mustard exposures. More experiments need to be done to test the effectiveness of NDH4417, minocycline, and PD98059 in vivo, and their time line of efficacy. If hydrogel delivery is more efficacious, it would make storage of these drugs in emergency response centers easier. Therefore, this must be evaluated as well.
CHAPTER VI

FUTURE WORK

Additional in vivo studies should be performed to test efficacy of countermeasures in rabbit eyes

Although this work gives some insight on mechanisms and therapies after vesicant-induced injury, there is much more work to be done. Most of the countermeasures tested in these studies, with the exception of doxycycline, have not yet been tested in the in vivo rabbit eye. The benefits of the in vivo model will incorporate systemic responses more indicative of how humans will respond to the treatment after an exposure. The removal of drug by tearing and blinking will be able to be evaluated in animal eyes. The most promising candidates in these studies, NDH4417, minocycline, and PD98059, should be tested next. Because only a few facilities are permitted by the government to use sulfur mustard, such experiments are very costly.

The first compound that should be tested in vivo is the ADAM17 inhibitor, NDH4417. This compound targets the enzyme responsible for epithelial-stromal separation and has proved effective in decreasing epithelial-stromal separations. Also, combination treatments with NDH4417 as an initial treatment used up to approximately 3 days, followed by doxycycline for up to 28 days to avoid the long-term MMP-9 upregulation effects may also be an effective strategy. There will most likely be a combination therapy needed. At which time to switch therapies or whether a co-administration is necessary will have to be determined.
It would also be interesting to see what happens in a co-administration study with sulfur mustard added along with the ERK inhibitor, PD98059. Our *ex vivo* studies indicate that PD98059 would be effective against ADAM17 activation if used within 10 minutes and against MMP-9 if used at 4 doses over 24 hr. The timeframe in an *in vivo* model would have to be determined to elucidate at what point therapy would ameliorate BMZ damage.

Topical minocycline appears to be more effective when used at higher doses than doxycycline. The 225 nmol dose of minocycline was just as effective as doxycycline at the 75 nmol dose, if not more effective. Additional studies to determine whether the higher dose of minocycline is more effective than doxycycline when the therapy is applied for 28 days should be done.

*Tear film/MMPs*

While exploring the competence of *in vivo* studies, the existence of the tear film should be considered. There are natural proteinase inhibitors present in preocular tear film (PTF) and cornea that prevent excessive degradation of normal healthy tissue. In damaged corneas, total proteolytic activity in the tear fluid was found to be significantly increased compared to uninjured eyes (Ollivier et al., 2007). These results indicate that enzymes secreted from the preocular tear film can contribute to healing after injury. Whether they become overactive after sulfur mustard exposure is unknown. This cannot be evaluated in our model system.
Prolonged healing – will it be effective in recurrent injuries up to 20 yrs post exposure?

So far these studies have been designed to treat the acute exposure effects to reduce pain and potential vision loss. Our evaluation of effectiveness includes how quickly the cornea heals after exposure, with the least amount of proteinases being activated for the least amount of time. Studies have not been able to document the effectiveness of drugs for suppressing recurrent injuries caused by sulfur mustard. It has been well documented that sulfur mustard injuries can arise again after 10 or 20 years (Amir et al., 2000; Balali-Mood et al., 2005, 2006; Ghasemi et al., 2009; Javadi et al., 2005; Kadar et al., 2001). No in vitro corneal system or an in vivo rabbit model is able to simulate the delayed sulfur mustard keratopathy seen in war veterans. Unfortunately the work presented here will not give insight on whether or not these compounds can suppress future occurrences, or whether they will still be effective against long-term recurrent injuries. Still, even identifying a good therapy for acute mustard injury is a step forward, since few have yet been identified. Doxycycline in the 2 in vivo rabbit studies holds the most promise.

Other effects of sulfur mustard not tested in these experiments

Neovascularization

While performing in vivo sulfur mustard with doxycycline treatment studies, neovascularization was noted after 3 days (Gordon et al., 2010; Kadar et al., 2009). This led us to develop a model to induce neovascularization for the purpose of screening anti-
angiogenic drugs for combination therapy. Neovascularization in the cornea can obstruct vision and indicates a poor prognosis for long term sight. Fungal infections have been shown to increase neovascularization caused by vascular endothelial growth factor (VEGF) induction (Yuan et al., 2009). Adding VEGF directly to corneal organ cultures, possibly in a hydrogel, should induce neovascularization in a model suitable for prescreening anti-angiogenic compounds that would go on to in vivo testing.

Reactive Oxygen Species

ROS induce injury to the cells and is associated with aging, cancer and various degenerative diseases. ROS generated by phagocytic cells during the immune response are essential for the elimination of harmful pathogens, and may possibly play a role in corneal cell proliferation, adhesion and wound healing (Chanock et al., 1994; Huo et al., 2009a; Huo et al., 2009b; Nomura et al., 1994). The impact of oxidative stress on an organism exposed to sulfur mustard has been well documented, but the exact shifts of reductants/oxidants is not fully recognized (Pohanka et al., 2009). Antioxidant protective mechanisms are diminished after sulfur mustard exposure, but it is still not known whether the resulting ROS is a direct effect, or a secondary effect due to inflammation (Paromov et al., 2007).
PARP

Sulfur mustard is a bifunctional alkylating agent at the molecular level. It reacts with proteins and nucleic acids. Poly(ADP-ribosyl)ation is a post-translational modification leading to DNA adducts in the cell (Debiak et al., 2009). The reaction is catalyzed by poly(ADP-ribose) polymerases (PARPs), encoded in humans by more than a dozen different genes (Debiak et al., 2009). The majority of the DNA alkylation consists of adducts at the N-7 position of guanine (61%) and N-3 position of adenine (16%) (Ludlum et al., 1994). Di-adducts occur within the same or complementary DNA strands, yielding intra- and interstrand cross-links (Ludlum et al., 1986).

The link between PARP and sulfur mustard was first reported by Papirmeister, et al., 1985. It was concluded that massive induction of DNA strand breaks led to activation of PARP-1 and consequent depletion of cellular NAD+ resulting in a collapse of ATP production. This was followed by protease release and blister formation. PARP links DNA damaging events to late pathology of blistering seen in skin. Studying PARP in order to learn more about the events leading to skin blisters could provide additional mechanisms of pathology. This has not yet been addressed in the cornea.

Other proteolytic enzymes with integrins as substrates

Epithelial-stromal separation is the result of enzymes which target transmembranous basement membrane components. Although ADAM17 is the main sheddase for collagen XVII, ADAM9 and ADAM10 also target collagen XVII. Whether
ADAM17 inhibitors reduce the activity of these enzymes is unknown. The indication of specific inhibitors for them would be advantageous. These drugs, while not in existence as of yet, could be developed for the purpose of inhibiting corneal erosions.

New studies are now suggesting that MMP-9 can also cleave α6β4 integrin (MaryAnn Stepp, JBC, in press), also located in the hemidesmosome. This discovery continues to stress the importance of identifying beneficial MMP-9 inhibitors for mustard injuries. Not only is MMP-9 responsible for prolonged degrading of the matrix and preventing cells from reattaching, it also targets ECM components such as gelatin, nidogen and laminin. We are barely scratching the surface of what can be done to preserve the extracellular matrix at the BMZ after mustard exposure. The future holds many exciting prospects.
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PUBLICATIONS

A DeSantis Rodrigues, RA Hahn, CJ Lacey, N Heindel, DR Gerecke, M Babin, KKH Svoboda and MK Gordon. ADAM17 Inhibitors Counteract Collagen XVII Disruption of the Basement Membrane Zone from CEES and Nitrogen Mustard-induced Ocular Injury (in preparation for dissertation)


A DeSantis Rodrigues, RA Hahn, JA Beloni, DR Gerecke, M Babin and MK Gordon, KKH Svoboda. Amino-Plex Counteracts Ocular Injury from the Vesicants CEES and Nitrogen Mustard in Whole Corneal Organ Cultures (in preparation)

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