An Engineered Human Fibroblast Growth Factor-1 Derivative, TTHX1114, Ameliorates Short-term Corneal Nitrogen Mustard Injury in Rabbit Organ Cultures

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PURPOSE. Organ cultures of rabbit corneas have been used to ascertain the effectiveness of a human fibroblast growth factor (FGF)-1 derivative (TTHX1114), lacking cysteine residues, to protect against and/or repair epithelial lesions following exposure to nitrogen mustard (NM).

METHODS. Rabbit corneas were exposed to NM and cultured for up to 14 days, with or without drug (TTHX1114). At specified times, tissue was examined by histopathology and graded by a novel composite scale. Proliferation was measured by 5-ethyl-2'-deoxyuridine (EdU) incorporation, and the expression of native FGF-1 and ADAM-17 after NM exposure was determined by immunofluorescence.

RESULTS. Rabbit corneas, exposed to a single dose of NM, showed a nearly complete loss of epithelial cells by day 6 but were significantly regenerated by day 14. When treated continuously with TTHX1114 following vesicant exposure, the losses remained at day 2 levels. The loss of keratocytes in the stroma was not affected by TTHX1114. EdU incorporation over the same time course showed a steady increase in tissue that had not been treated with TTHX1114, while corneas that were treated with the drug showed a higher percent incorporation initially, which then decreased, indicating the strong proliferative response to TTHX1114. ADAM-17 was not significantly altered by TTHX1114 treatment. Corneal epithelial FGF-1 disappeared after only 1 day following exposure to NM.

CONCLUSIONS. TTHX1114 is protective against NM-induced damage of the corneal epithelium, possibly by supplying an NM-resistant source of trophic support and by stimulating regeneration of new epithelial cells. These responses underscore the potential value of TTHX1114 as an anti-vesicant therapeutic.

Keywords: epithelial cell regeneration, fibroblast growth factor-1, cell proliferation, nitrogen mustard, FGF-1 expression, ADAM-17, keratocytes

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f the three main organ targets—skin, lung, and eye—of the vesicating agents used in biological warfare, such as the nitrogen and sulfur mustards (NM and SM), the eye is perhaps the most important because of the immediate (acute) ramifications of the insult and the potential for long-term (chronic) visual impairment.1 Exposure to these agents causes relatively rapid microvesication with deep epithelialization of the corneal surface2 that results in significant pain and degradation of vision. Increasing evidence indicates stromal and endothelial cell damage also occurs at the time of the original insult, and the extent of the injuries to these more posterior layers may be related to the onset of the chronic disease that is seen in more severely affected casualties.3 Steroids, such as dexamethasone, have had limited use in treating the acute phase damage to the epithelial layer after mustard exposure,4 which will generally heal itself in a few weeks with no intervention,5,6 and organ transplants remain the only effective treatment for the chronic phase of the disease, generally known as mustard gas keratopathy (MGK).7,8 SM is clearly more toxic than NM, and the knowledge base of vesicant effects is mainly derived from its study; however, NM, which is more amenable to laboratory manipulations, shows sufficiently similar responses by epithelial cells to be a useful paradigm for testing potential protective agents against vesicant damage.

Although the precise mechanisms that result in cell- or tissue-specific cytotoxocities following mustard gas exposure are unknown, it is appreciated that the mustards are effective alkylating agents and appear to act in large part by derivatizing biological macromolecules, particularly nucleic acids and proteins.9 These moieties differ in potency and effect; for example, SM is bifunctional and can cause chemical cross-linking as part of its reaction profile, while NM is only monovalent, and accordingly they do not necessarily share the same cellular targets. In proteins, cysteine has been shown to be the most reactive to NM,10 which is consistent with the fact that the thiol group of cysteine is known to be the most reactive nucleophile in this family of macromolecules. Because these residues are in polypeptides primarily found in intracellular locations, as are the nucleic acids, it is generally assumed that the greatest toxicity is associated with modifications by
vesicant acting intracellularly. Proteins that are normally exported through the endoplasmic reticulum/Golgi continuum to function in the extracellular space undergo a net oxidation in which their cysteine residues are converted to cystine to produce both intra- and interchain disulfide bonds,13 and these would not be expected to be significantly sensitive to alkylating agents. Exported proteins include most hormones and growth factors that interact with their target cells through cell surface receptors and regulate such processes as differentiation, proliferation, and maintenance of cell viability.

There are, however, a small number of regulatory proteins that are exported via a non-ER/Golgi pathway, and these moieties retain their cysteine residues in a reduced state12 after leaving the intracellular environment. As such they are potential targets of SM and NM modification and inactivation. Two important members of this select group are fibroblast growth factors-1 and -2 (FGF-1 and -2), which play a key role in potential targets of SM and NM modification and inactivation. leaving the intracellular environment. As such they are exported via a non-ER/Golgi pathway, and these cysteines by replacing two of the three native Cys residues with nonreactive residues and with the introduction of a new Cys residue (A66C) that forms an intrachain disulfide bond wherein A66C forms an intrachain disulfide bond with the remaining Cys at position 83. This entity retains its natural binding site for heparin, although it can function without it.23

The efficacy of both endogenously produced and exogenously added FGFs in the context of mustard gas injury may be limited by this sensitivity to cysteine modification. It is unclear to what extent the inactivation of native FGF-1 may contribute to epithelial cell loss in mustard-induced injury and whether residual vesicant would impair the ability of exogenously added drugs that were sensitive to cysteine modification. To assess the effect of native FGF-1 on vesicant injury and to determine whether an FGF-1 derivative lacking reactive thiol groups would be effective in mitigating mustard injury, a rabbit organ culture model, along with an objective histopathological grading scale, has been developed and the effectiveness of TTHX1114 in countering NM injury in the short term examined.

MATERIALS AND METHODS

Reagents

Human engineered FGF-1(TTHX1114) (C165/A66C/C117V whereby A66C forms an intrachain disulfide bond with C83) was prepared as a 140-amino acid protein commencing with N-terminal Phe by Trefoil Therapeutics, Inc. (San Diego, CA, USA). The expression construct used contained an amino terminal extension with a poly-His sequence for purification purposes that was removed by enterokinase; the resulting 140-residue product was further purified to essential homogeneity prior to use. Mouse monoclonal anti-human FGF-1 was obtained from Abcam (ab117640 [clone: 2E12]; Cambridge, UK), and donkey anti-mouse secondary antibody was from Jackson ImmunoResearch (DyLight 549; West Grove, PA, USA).

Corneal Organ Culture

Whole rabbit eyes were purchased (Pel-Freez, Rogers, AR, USA) and were rinsed in povidone iodide, washed in PBS (pH 7.4; Gibco, Grand Island, NY, USA) followed by corneal excision leaving a 2-mm scleral ring around the corneal periphery. Corneas were draped over 500 μL agar buttons that were precast in a gel drop mold (Scottcrew Enterprises, Spring Lake, MI, USA), composed of 1.5% agar in Dulbecco’s Modified Eagle’s medium (DMEM) with 4500 mg/L glucose, L-glutamine, and sodium pyruvate (Mediatech, Manassas, VA, USA) and placed in 20 × 100-mm Petri dishes. The buttons were secured to the dish using melted agar and placed in the dishes approximately 40 mm from the center or closer to the edge of the dish. Each dish was filled with 15 mL culture media composed of DMEM as described above, supplemented with Roswell RPMI 1640 vitamin solution 100 × 10-ML/L (R7256; MilliporeSigma, St. Louis, MO, USA), MEM amino acids 10 mL/L (no. 11140; Invitrogen, Carlsbad, CA, USA), antibiotic antimycotic 10 mL/L (no. 15240-062; Invitrogen), ascorbic acid 250 mM 1 mL/L (MilliporeSigma), ascorbic acid 2-P 225 mM 2 mL/L (MilliporeSigma), and 10 mg/L ciprofloxacin (Fluka/Sigma no. 17850; MilliporeSigma)24,25. The corneas in the Petri dishes were placed on a rocker platform with the corneas in each dish oriented such that when the rocker was at the maximum tilt, the corneas were exposed to air, and when halfway through one complete cycle, they were submerged in media (Fig. 1).

NM Exposure

Corneas were processed for organ culture as above and maintained in culture for 2 days to allow recovery from the enucleation/excision process. Two days post dissection (designated as day 0 of treatment), the media was drained from the dishes and 10 μL of freshly prepared 10 mM NM (mechlorethamine) (MilliporeSigma) in culture media was applied to the center of the cornea. After 2 hours at room temperature, the corneas were rinsed three times with culture media and then placed in culture media alone or in culture...
media containing TTHX1114, 100 pg/mL, and returned to the incubator. Control or naive corneas were not exposed to NM but were subjected to the same room temperature rinse and media change protocol.24,25

Fixation and Staining of Corneas
At specified time points post NM exposure (days 2, 4, 6, 9, and 14), the corneas were harvested and fixed with 2% paraformaldehyde in PBS for 2 hours at room temperature and processed for paraffin embedment. The corneas were then cross-sectioned at a thickness of 4 μm and stained with hematoxylin and eosin (H&E) for histopathological evaluation.26

Corneal Histologic Grading Score
In vitro corneal epithelium health at the lesion site for each cornea was evaluated by three criteria: (1) epithelial differentiation, (2) basal layer orientation, and (3) epithelial intracellular adhesion (EIA) (Table 1; Fig. 2). Scores were assigned to each group based on a scale from 1 (no or minimal change) to 5 (loss of epithelial structures) for each criterion. The score for the three criteria was summed to give an overall rating that ranged from 3 (best) to 15 (complete loss of the epithelial layer). Thus, the lower the score, the healthier the tissue. The mean and standard deviation was calculated for each group and time point (n = 8).

Stromal Histologic Grading Score
In vitro corneal stromal health at the lesion site for each cornea was evaluated by two criteria: (1) stromal structure and (2) keratocytes (Table 2; see also Fig. 7). Scores were assigned using the scales described in Table 2 and the two criteria scores summed to give an overall rating that ranged from 2 (best) to 9 (worst). Mean and standard deviation were calculated for each group and time point (n = 8).

<table>
<thead>
<tr>
<th>Grading Score</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
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<tbody>
<tr>
<td>Epithelial Epithelium</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Basal Layer</td>
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<td></td>
<td></td>
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<tr>
<td>EIA (intracellular adhesion)</td>
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</table>

Each corneal cross-section at the lesion site was evaluated for total epithelial differentiation, BL, and EIA. Scores for the three criteria were then summed, and mean and standard deviations generated.

TABLE 1. Histopathological Grading Scale for Vesicant Damage

<table>
<thead>
<tr>
<th>Total Epithelial Differentiation</th>
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<tbody>
<tr>
<td>1. Normal three layer, good differentiation</td>
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<tr>
<td>2. Disruption in layers, differentiation</td>
</tr>
<tr>
<td>3. Single layer only</td>
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<tr>
<td>4. Single layer, partial coverage</td>
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<tr>
<td>5. No epithelium present</td>
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<table>
<thead>
<tr>
<th>Basal Layer (BL)</th>
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</thead>
<tbody>
<tr>
<td>1. Columnar formation proper orientation</td>
</tr>
<tr>
<td>2. Columnar and rounded with minor orientation changes</td>
</tr>
<tr>
<td>3. Rounded, erratic orientation</td>
</tr>
<tr>
<td>4. Rounded, erratic orientation, partial coverage</td>
</tr>
<tr>
<td>5. No BL present</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>EIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No breaks in EIA</td>
</tr>
<tr>
<td>2. Mild breaks, vesicles appear in EIA</td>
</tr>
<tr>
<td>3. Moderate breaks, vesication in EIA</td>
</tr>
<tr>
<td>4. No EIA observed</td>
</tr>
<tr>
<td>5. No epithelium present</td>
</tr>
</tbody>
</table>

Using Vectashield hard set antifade mounting medium with PBS with 0.05% Tween-20, sections were coverslipped with PBS with 1.5% NGS. After washing three times with PBS with 0.05% Tween-20, and incubated for 1 hour with goat anti-mouse temperature, washed three times with PBS with 0.05% Tween-20, and incubated for 1 hour with goat anti-mouse secondary antibody diluted 1:400 in 1.5% normal donkey serum with PBS/Tween-20, applied to designated sections, and incubated at 4°C, overnight. Sections were washed again 3 × 10 minutes. Donkey anti-mouse secondary antibody diluted 1:400 in 1.5% normal donkey serum with PBS/Tween-20 was applied accordingly and incubated 1 hour at room temperature in the dark. Sections were washed 3 × 10 minutes in PBS/Tween-20. (Negative controls were sections with no primary but with secondary antibody and no primary-no secondary antibody.) Slides were washed 3 × 10 minutes and mounted with mountant and DAPI (Prolong Gold; Invitrogen-Life Technologies, Eugene, OR, USA). Digital images were captured using an inverted fluorescent microscope using a camera and imaging software (Zeiss and ProgResCaturePro; Jenoptik Optical Systems, Jena, Germany).

**RESULTS**

**Corneal Histologic Grading Score**

As a means of making a more quantitative assessment of the NM-induced damage to the corneal epithelium, a composite grading score that evaluates three distinct parameters was devised: (1) epithelial differentiation, (2) basal layer orientation, and (3) EIA. In each case, five levels of increasing severity were defined (Table 1), ranging from a normal, unlesioned epithelium (level 1) to a complete loss of the epithelial cell layer (level 5). A number (level) was assigned for each category for any given image and then summed to give the final score. An image corresponding to each level in the three different categories is shown in Figure 2. In category 1 (top row), the corneal epithelium was graded for overall differentiation and integrity. As damage progressed, there was a loss of epithelial organization and layering, progressing to partial epithelial coverage and loss of epithelial layer completely. Noteworthy is the reduction in the top and middle layers in 2, with elimination of the middle layer in 3 and no discernible layering in 4, with no cells at all in 5. In category 2 (middle row), there was a decrease in density of the basal layer and rounding of basal layer cells at level 2, a lack of columnar structure of the basal layer at level 3, rounded and erratic cells at level 4, and no cells at all at level 5. In category 3 (bottom row), EIA analysis showed there were gaps between basal layer cells at level 2, gaps in all cell layers at level 3, extensive cell separation at level 4, and no cells at all at level 5.

**Effect of TTHX1114 on Corneal Epithelial Cells in Organ Culture**

A 2-week time course of corneal epithelium response to NM exposure at day 0 is shown in Figure 3a (top row). By comparison to the scale illustrated in Figure 2, all components of the composite score were at or near 5, yielding final values approaching 15 by day 6. These findings are consistent with previous reports of vesicant-induced epithelial damage. By day 14, natural regenerative processes had largely restored the epithelium to level 2 to 3 in all categories. The bottom row of this figure shows the same experimental time course carried out over a 14-day period, with a return to normal morphology.

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**Table 2. Histopathological Grading Scale for Stromal Vesicant Damage**

<table>
<thead>
<tr>
<th>Stromal Structure</th>
<th>Corneal Histologic Grading Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No disruption noted</td>
<td>1. Present, in normal orientation to epithelial layer</td>
</tr>
<tr>
<td>2. Mild separation of keratocytes from stroma</td>
<td>2. Abnormal shape and orientation to epithelial layer mild</td>
</tr>
<tr>
<td>3. Severe separation of keratocytes from stroma</td>
<td>3. Abnormal shape and orientation to epithelial layer moderate</td>
</tr>
<tr>
<td>4. Abnormal shape and orientation to epithelial layer severe</td>
<td>5. No keratocytes present below epithelial layer</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Keratocytes</th>
<th>Stromal Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Present, in normal orientation to epithelial layer</td>
<td>5. No keratocytes present below epithelial layer</td>
</tr>
<tr>
<td>2. Abnormal shape and orientation to epithelial layer mild</td>
<td>4. Abnormal shape and orientation to epithelial layer severe</td>
</tr>
<tr>
<td>3. Abnormal shape and orientation to epithelial layer moderate</td>
<td>3. Abnormal shape and orientation to epithelial layer moderate</td>
</tr>
<tr>
<td>4. Abnormal shape and orientation to epithelial layer severe</td>
<td>2. Abnormal shape and orientation to epithelial layer mild</td>
</tr>
<tr>
<td>5. No keratocytes present below epithelial layer</td>
<td>1. Present, in normal orientation to epithelial layer</td>
</tr>
</tbody>
</table>

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**ADAM17 Imaging**

Rabbit corneas from the organ culture system were embedded in ornithine carbamoyltransferase (OCT) (TissueTek; Sakura Finetek USA, Torrance, CA, USA), frozen using a dry ice/isopentane bath, and stored at −70°C until use. Cryosections (6 μm) on slides were fixed in −20°C methanol for 10 minutes and nonspecific binding blocked by incubation with 5% normal goat serum (NGS) (MilliporeSigma) in PBS with 0.05% Tween-20 (MilliporeSigma). Sections were incubated for 1 hour in 5 μg/ml antibody to human ADAM-17 (MAB 9304; R&D Systems, Minneapolis, MN, USA) in PBS with 1.5% NGS at room temperature, washed three times with PBS with 0.05% Tween-20, and incubated for 1 hour with goat anti-mouse immunoglobulin G (IgG)-Alexa Fluor 488 conjugate (Invitrogen) 1:1000 in PBS with 1.5% NGS. After washing three times with PBS with 0.05% Tween-20, sections were cover-slipped using Vectashield hard set antifade mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA).17,18

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**FGF-1 Expression**

For FGF-1 immunostaining, corneas were placed in OCT and set on an ice bath for 30 minutes, then frozen on the surface of liquid nitrogen.

OCT-embedded sections were allowed to air dry before outlining sections with a PAP pen. Sections were rehydrated with 3 × 10 minute washes of PBS/Tween-20. Sections were blocked with 5% normal donkey serum in PBS/Tween-20 for 1 hour at room temperature. Mouse monoclonal anti-human FGF-1 was diluted to 30 μg/mL in 1.5% normal donkey serum/PBS/Tween-20, applied to designated sections, and incubated at 4°C, overnight. Sections were washed again 3 × 10 minutes. Donkey anti-mouse secondary antibody diluted 1:400 in 1.5% normal donkey serum with PBS/Tween-20 was applied accordingly and incubated 1 hour at room temperature. Sections were washed 3 × 10 minutes in PBS/Tween-20. (Negative controls were sections with no primary but with secondary antibody and no primary-no secondary antibody.) Slides were washed 3 × 10 minutes and mounted with mountant and DAPI (Prolong Gold; Invitrogen-Life Technologies, Eugene, OR, USA). Digital images were captured using an inverted fluorescent microscope using a camera and imaging software (Zeiss and ProgResCaturePro; Jenoptik Optical Systems, Jena, Germany).
out with 100 pg/mL of TTHX1114 in the media. In contrast to the severe losses seen at day 4 and particularly at day 6 in the NM-lesioned corneas without TTHX1114, the drug-treated corneas show significantly less damage with only level 2 to 3 lesions throughout the 2-week period. Grading of images from groups of corneas ($n = 8$ per time point per condition) using the scale shown in Table 1 shows that the NM-induced damage peaks at 6 days and that treatment with TTHX1114 post NM exposure protects the corneas from damage, with the TTHX1114-treated corneas maintaining a lower (better) score at days 4, 6, and 9 (Fig. 4) with $P$ values for the individual comparisons at these three times of 0.0014, 0.0005, and 0.0314, respectively. By comparison, the lesioned corneas not treated with drug showed this score only on days 2 and 14, being significantly higher (indicating greater damage) for the other three time points (days 4, 6, and 9).

The effect of TTHX1114 on corneas maintained under the same conditions (but not exposed to NM) is shown in Figure 3.
incorporation and the NM-only corneas having greater EdU
14, with the TTHX1114-treated corneas having lower EdU
0.138, 0.069, and 0.083 at days 2, 4, 6, 8, and 14, respectively.

Thus, there is a trend toward a greater EdU incorporation in
the cells that have already been exposed to TTHX1114 at short
time periods after NM exposure, and this pattern inverts at day
3b. There was no significant difference between those exposed
to TTHX1114 and those that were not. All images were graded
with a score of 3 (data not shown). Interestingly, there was no
evidence from these images of any hyperproliferative activity.

EdU Incorporation

To determine the extent to which TTHX1114 affords its
protective effect against NM-induced cell damage/loss as
manifested in the day 4 through day 9 period (Figs. 3a, 4) by
stimulating the formation of new epithelial cells via proliferative
stimuli, corneas exposed to NM and then treated with and
without TTHX1114, as described in Figure 3, were treated with
EdU (at 24 hours prior to the indicated time) and the labeled
tissue analyzed by fluorescence microscopy (Fig. 5a, top row).
The images were collected from the regions around the central
lesion that were still populated with viable cells capable of
proliferative responses. Clearly, corneas not exposed to
TTHX1114 showed an increasing number of EdU-labeled cells
from day 2 to 14 (Fig. 5b), with the percentage of cells
incorporating EdU increasing from 19% to 68% (P = 0.016,
Student's t-test), presumably reflecting the proliferation of
epithelial cells that accompanies the normal regenerative
process. The presence of TTHX1114 provides a rather different
picture (Fig. 5a, bottom row). There is a much greater
percentage of EdU-labeled cells at day 2, and this number
diminishes over the next 12 days (Fig. 5b), reaching about the
same level of incorporation as the untreated cells at day 2.
Pairwise comparisons of NM versus NM/TTHX groups using
the Student’s t-test produce 2-tailed P values of 0.086, 0.40,
0.138, 0.069, and 0.083 at days 2, 4, 6, 8, and 14, respectively.
Thus, there is a trend toward a greater EdU incorporation in
the cells that have already been exposed to TTHX1114 at short
time periods after NM exposure, and this pattern inverts at day
14, with the TTHX1114-treated corneas having lower EdU
incorporation and the NM-only corneas having greater EdU
incorporation.

Effect of TTHX1114 on ADAM17 Expression

Following NM Treatment

As NM attacks the epithelial cell layer, it induces microbullae
at the epithelial-stromal junction, which is partially caused by
cleavage of collagen XVII by ADAM17, and inhibition of
this metallopeptase has been shown to be effective in
preventing epithelial-stromal separation.24 To assess whether
TTHX1114 exerted any of its protective/proliferative re-
response on NM-treated corneas, ADAM17 was imaged by
immunofluorescence over 6 days following the NM lesion at
both the site of the lesion (central) and in the periphery (Fig.
6). In naive cornea, ADAM-17 expression was seen localized
to the area of the epithelial cells facing the basement
membrane. As shown in the top row, at days 1, 4, and 6
following NM exposure, there was only a modest expression
of ADAM17 in the periphery, and it was unchanged in this
time frame. Little or no ADAM17 was detected in the lesion
area during this period, consistent with a lack of epithelial
cells in the center at these time points. When TTHX1114
was added (bottom row), there was a pronounced increase
(compared to NM exposed) of ADAM17 at day 1 in both the
lesion and peripheral areas. However, at the later time
points, the effect of TTHX1114 on ADAM17 expression in
the lesion site in treated corneas was basically identical to
the untreated tissues, with little ADAM-17 expressed in the
epithelial cells in the lesion area. ADAM-17 levels in the
peripheral cells remained similar to the naive cornea. Thus,
TTHX1114, in contrast to expectations, did not lower
ADAM17 and in fact increased its expression in cells
removed from the lesion site. In all cases, ADAM-17
expression was localized in the epithelial cells to (1) the
basement membrane facing area of the basal layer and (2)
perinuclear cytoplasmic vesicles.

Effect of TTHX1114 on Stromal Keratocytes

As is well appreciated,27 NM penetrates the stroma (and
beyond), resulting in the loss of the keratocytes that populate
this part of the cornea (see Fig. 5a). Stromal tissue from in vitro
rabbit corneas, fixed and stained as described above, was
evaluated for morphologic changes (Table 2). Changes in
keratocyte morphology and loss were noted associated with
the lesion site beginning with day 2. However, over all time
points (days 2–14), no further changes in stromal organization,
edema, or integrity was noted, either below or adjacent to the
NM lesion sites (Fig. 7). TTHX1114 had no effect on these cells
over this time frame. Keratocytes are known to be responsive
to FGF-1, so the lack of response observed here may indicate
that TTHX1114 does not readily penetrate the stroma to reach
any reservoir of keratocytes that could serve as a source for
new cells.

Expression of Native FGF-1 Following NM

Treatment

To confirm that NM exposure impacts the levels of native FGF-
1 in corneal epithelium, exposed corneas were analyzed by
immunofluorescence at 1 and 3 days following NM treatment
(Fig. 8). As compared to the controls, the NM-treated tissue
showed no detectable FGF-1 in the epithelium after 1 day,
although there were clearly detectable cells present. However,
by day 3, a significant amount of FGF-1 was observed,
indicating that FGF-1 expression is part of the normal
regenerative process and that this process had already begun
by this time point (in agreement with the EdU data shown in
Fig. 5).
FIGURE 5. (a) Effect of TTHX1114 on corneal epithelial cells following NM damage as measured by EdU incorporation. Corneas in organ culture were treated with NM as described followed by incubation in media with (bottom row) or without (top row) TTHX1114 (100 pg/mL). Twenty-four hours prior to the times indicated, EdU-containing (10 pg/mL) media was added. Corneas were harvested at the indicated times post NM lesioning, fixed and stained for EdU with counterstain for Hoechst as described, and analyzed for EdU incorporation using fluorescence microscopy. Images were taken and analyzed from the peripheral cornea between the limbus and the edge of the NM lesion. (b) Percent of EdU incorporation in corneal epithelial tissue with (dashed) and without (solid) TTHX1114 treatment as a function of time. In vitro rabbit corneal epithelia peripheral to the NM lesion was counted for total number of EdU-labeled cells (numerator) divided by total number of basal cells (denominator).
DISCUSSION

Cell viability is maintained in vivo by a complex network of factors and cell-cell interactions, which provide a combination of hypertrophic and hyperplastic stimuli that support it. A significant portion of these activities are provided by humoral substances such as tissue growth factors and other endocrine-like modulators that arise locally (autocrine and/or paracrine) or systemically. As a rule, these agents, such as epidermal growth factor, platelet-derived growth factor, and the transforming growth factor β family, are disulfide bond–containing entities. However, FGF-1 and -2, important members of this family, are disulfide bond–containing growth factors. They are involved in various cellular processes, including proliferation, differentiation, and migration. They play a crucial role in the healing of the corneal epithelium after injury.

**FIGURE 6.** Induction of ADAM-17 by TTHX1114 treatment following NM exposure in rabbit corneal organ cultures. Corneas were cultured in vitro as described and treated with NM, rinsed, and cultured in media only (Control) or media supplemented with TTHX1114 (100 pg/mL). The naive section is a central corneal image from a cornea maintained in culture for 2 days but not treated with NM or TTHX1114. At the indicated times, corneas were embedded in OCT, frozen using a dry ice/isopentane solution, sectioned and stained with anti-ADAM17 antibody (green), and counterstained with DAPI (blue). Images were taken from the central cornea and from the peripheral cornea away from the area of NM damage. Sections were stained with anti-ADAM-17 (green) and DAPI (blue).

**FIGURE 7.** Effect of TTHX1114 on stromal keratocytes as a function of time following NM lesioning. Recovery from NM damage with (dashed) and without (solid) TTHX1114 was evaluated by a histopathological grading scale (see Table 2). Values from the two categories were summated for each cornea to give the final grade. The dosing and administration of both NM and TTHX1114 were as described in Figure 3a.

**FIGURE 8.** NM exposure reduces native FGF-1 levels in corneas cultured in vitro. Corneas were cultured and exposed to NM as described. Naive corneas or corneas 1 or 3 days after NM exposure were embedded in OCT, frozen, and 8- to 10-μm cryosections made and mounted on slides. Naive corneas were cultured in vitro for times matching the NM-exposed corneas. Slides were stained with (FGF-1) or without (negative control) anti-FGF-1 (Abcam 2E12) followed by donkey anti-mouse IgG (DyLight 549) as secondary antibody (red) and counterstained with DAPI (blue).
type of regulator, are exceptions in that they do not contain disulfide bonds and the three cysteines that are present are sensitive to modification that leads to inactivation. As a result, native FGF-1 (or -2) would be expected to be readily modified by NM and the vesicant would eliminate this source of trophic support for responses to damage. In the experiments described herein, topical application of vesicant to the cornea would be expected to most severely affect the cells closest to the ocular surface but be less damaging, that is, produce fewer modifications, in cells that are closer to the posterior side as the concentration of the vesicant decreased by diffusion. These more modestly impaired cells are much more likely to be resuscuable through trophic support. In the absence of this support (because their source of trophic stimulation, i.e., native FGFs, has been inactivated by the vesicant), these same cells (or a significant percentage of them) would not survive, as observed in the control samples of Figure 4. Indeed, in the absence of any external trophic support, the epithelial cell losses usually extend across the entire lesion site, and the formation of new epithelial cells must occur in the areas surrounding the lesion or in the periphery (limbus). While from these data it is not possible to determine the relative contributions of new epithelial cell formation, stimulated by TTHX1114 and a protective effect afforded by providing trophic support to replace that normally provided by native FGF-1, it seems likely that both mechanisms underlie the overall amelioration observed.

NM is an alkylating agent that has been shown to modify Lys and His side chains as well as those of Cys. Addition likely occurs via the initial formation of a reactive aziridinium, intermediately followed by covalent reaction. All nucleophiles in proteins are pH sensitive (except the thioether of methionine), and lysine is poorly reactive at neutrality, but the other two would likely be modified under the conditions of these experiments. Cys is about an order of magnitude more reactive than His. Thus, any proteins containing Cys, where their modification impacts activity, will be particularly susceptible to mustard gas. It may be imagined that the cytoplasm of mammalian cells is rich in proteins that generally meet this description, and thus it may be further concluded that once NM has penetrated the plasma membrane, cellular damage that is largely irreversible will occur. It would be of substantial interest to understand how TTHX1114 can offset such damage via trophic stimulation.

The application of NM to the organ cultures is necessarily difficult to quantify. In the experiments reported herein, NM was added topically as a drop of reagent solution in a fixed volume. Over the course of several experiments, some variation was observed in the severity of the lesion introduced (e.g., Fig. 3b, day 6), but the time of response, with and without TTHX1114 treatment, was not altered. When NM was delivered by soaking a 6-mm circle of filter paper with reagent and placing it on the anterior surface of the cornea, it was determined that the NM lesion extended to the limbus, although it did not appear to penetrate the stroma to the same extent. Thus, this method of application tended to diffuse the vesicant laterally over a larger surface area with less penetration (data not shown). This method was not used in the studies reported herein. These differences related to drug application also underscore the difficulty in comparing responses to damage introduced by different vesicants, for example, NM and SM.

The observations in this report support the view that TTHX1114 exerts a positive effect on NM-induced lesions of the corneal epithelium. In other experiments, TTHX1114 has also been shown to induce a similar response in vivo to lesions introduced by SM in rabbits (unpublished observations). Although the onset of the cell losses was faster than in the organ culture experiments, the overall impact of TTHX1114 on

Healing Vesicant Damage of the Corneal Epithelium

modified by NM, 10 and the vesicant would eliminate this

been observed in other paradigms involving cell damage and
delayed until new FGF-1 can be synthesized (Fig. 6).

When the corneal epithelium is first exposed to vesicant,
tissue over a period of 2 hours such that it mimicked the natural lubrication of eyelid blinking and kept the corneas moist (Fig. 1). This arrangement also allowed the tissues to be constantly bathed in media containing the test drug sample.

When the corneal epithelium is first exposed to vesicant, there is a delay of several hours before symptoms are detected by the victim of the attack. During that time, the agent is clearly causing damage to the cells of the corneal surface, and to the extent that it penetrates the stroma, on the keratocytes located there as well. Increasing evidence indicates that it can also reach the endothelial layer on the posterior surface and inflict damage on these cells. Indeed, the development of the chronic problems of mustard gas injury, or MGK, seems to be at least in part dependent on the extent of damage incurred by the endothelial cell layer during the acute phase of the insult. There is certainly epithelial cell loss that occurs during this period (up to 2 days) that results from the modification of proteins/nucleic acids essential for viability. These data do not specifically address the mechanisms by which TTHX1114 reduces NM damage and then aids in restoring epithelial function, but they suggest that two activities are likely involved. The reduction (improvement) in histopathological score and the observation that the epithelial layer is never completely eliminated suggests a reduction in the overall cytotoxicity of the NM and a potential anticytotoxic effect. On the other hand, the healing of the lesion requires the production of new epithelial cells, undoubtedly preceded by the migration of uninjured cells, from the periphery. The EdU data support this increased proliferation of the epithelial cells, and the lack of ADAM-17 in the epithelial cells that are protected or regenerating in the center of the lesion also indicates that cell adhesion to the stroma may be enhanced, favoring a role for cellular migration as well.

The protective/anticytotoxic actions of trophic factors have been observed in other paradigms involving cell damage and death. For example, FGF is known to protect against radiation damage that can otherwise lead to cell death. These effects are generally attributed to distinct changes in metabolism and other cell functions, and determining which of these may apply to protection against vesicant damage would be informative as a follow-up study. In the experiments described herein, topical application of vesicant to the cornea would be expected to most severely affect the cells closest to the ocular surface but be less damaging, that is, produce fewer modifications, in cells that are closer to the posterior side as the concentration of the vesicant decreased by diffusion. These more modestly impaired cells are much more likely to be resuscuable through trophic support. In the absence of this support (because their source of trophic stimulation, i.e., native FGFs, has been inactivated by the vesicant), these same cells (or a significant percentage of them) would not survive, as observed in the control samples of Figure 4. Indeed, in the absence of any external trophic support, the epithelial cell losses usually extend across the entire lesion site, and the formation of new epithelial cells must occur in the areas surrounding the lesion or in the periphery (limbus). While from these data it is not possible to determine the relative contributions of new epithelial cell formation, stimulated by TTHX1114 and a protective effect afforded by providing trophic support to replace that normally provided by native FGF-1, it seems likely that both mechanisms underlie the overall amelioration observed.

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the healing process as measured by pachymetry and corneal fluorescein staining was similar to those reported here. These experiments were conducted with a twice daily topical dosing regimen of TTHX1114. Taken together with the organ culture results described in this report, it suggests that TTHX1114 is a potentially useful drug for addressing vesicant damage to the corneal epithelium that could be delivered in an eyedrop fashion.

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References

