Effects of Diclofenac or Ketorolac on the Inhibitory Activity of Cidofovir in the Ad5/NZW Rabbit Model

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PURPOSE. The goal of this study was to determine the effects of concurrent therapy with nonsteroidal anti-inflammatory drugs (NSAIDs) on the antiviral activity of cidofovir on adenovirus replication and the formation of subepithelial infiltrates in the Ad5/New Zealand White rabbit ocular model.

METHODS. According to two protocols, 20 rabbits were inoculated in both eyes with Ad5 topically to study adenovirus replication, and 20 rabbits were inoculated in both eyes topically and intrastromally to study the formation of subepithelial infiltrates. Animals were randomized to four masked treatment groups: group I, 0.5% cidofovir + artificial tears; group II, 0.5% cidofovir + 0.5% ketorolac tromethamine; group III, 0.5% cidofovir + 0.1% diclofenac sodium; and group IV, control + artificial tears. Cidofovir and control were administered to both eyes twice daily for 7 days, and artificial tears, ketorolac, and diclofenac four times daily for 14 days. Eyes were cultured on days 0, 1, 3, 4, 5, 7, 9, 11, and 14.

RESULTS. Compared with the control group, all cidofovir-treated groups demonstrated significant antiviral effects on adenovirus replication. There were no differences in adenovirus replication among the cidofovir-treated groups (I, II, and III), nor were there any differences among all groups (I–IV) in the formation of subepithelial infiltrates.

CONCLUSIONS. Concurrent treatment of ketorolac or diclofenac with cidofovir did not diminish its antiviral inhibitory activity on adenovirus replication, nor did it prevent the formation of subepithelial infiltrates in the rabbit model. (Invest Ophthalmol Vis Sci. 2001;42:158–162)

Adenoviruses (Ad) are the causative agents associated with the most frequent external ocular viral infection worldwide. At present, there is no US Food and Drug Administration (FDA)-approved antiviral therapy for the treatment of adenoviral ocular infections.

Cidofovir, (S-HPMPC), is a broad-spectrum antiviral agent with significant in vitro inhibitory activity against a number of DNA viruses (human cytomegalovirus [CMV], herpes simplex virus [HSV]-1 and -2, varicella-zoster virus, and adenoviruses). Previous prevention and treatment studies have shown that topical administration of cidofovir significantly reduces ocular viral titers, and the duration of viral shedding in the Ad5/New Zealand White rabbit ocular model10,11 and in the HSV-1/New Zealand White rabbit keratitis model.12 Cidofovir is currently FDA approved for the systemic treatment of CMV retinitis in patients with acquired immune deficiency syndrome (AIDS).

The role of topical anti-inflammatory agents in the treatment of adenoviral ocular infections remains controversial. The routine clinical use of topical corticosteroids is generally discouraged by most authorities. In experimental studies, topical 1% prednisolone acetate significantly enhanced Ad5 replication and prolonged Ad5 shedding,13 and the antiviral inhibitory activity of topical cidofovir was eliminated by local immunosuppression induced by 1% prednisolone acetate during concomitant therapy in the Ad5/New Zealand White rabbit ocular model.11

Nonsteroidal anti-inflammatory drugs (NSAIDs) are an important category of anti-inflammatory agents currently available for ophthalmic use. Topical NSAIDs have been shown to relieve symptoms of acute allergic conjunctivitis14 and to reduce inflammation associated with alkali burns of the cornea, herpetic uveitis, and ocular trauma. They have been shown to reduce postoperative inflammation after cataract15,16 and refractive surgery.16,17 Their value as local analgesics to reduce corneal sensitivity16,17 after traumatic abrasions18 and eximer laser refractive procedures has led to widespread use.19 Recently, topical NSAIDs have been implicated in a few cases with serious side effects (corneal melting and perforation) after cataract surgery,20 but no serious side effects have been reported after nonsurgical topical therapy.

Although no clinical studies have been performed to determine the effects of topical NSAIDs on the natural history of adenoviral ocular infections, experimental treatment with two topical NSAIDs (diclofenac sodium and ketorolac tromethamine) showed no stimulatory or inhibitory effect on adenoviral replication and no effect on the natural immune clearance of adenovirus from the eye.21 These NSAIDs also did not prevent the formation of subepithelial infiltrates in the Ad5/New Zealand White rabbit ocular model.22

The goal of the present study was to assess how topical NSAID (e.g., diclofenac sodium and ketorolac tromethamine) therapy would affect the established antiviral inhibitory activity of topical cidofovir and the formation of subepithelial infiltrates in the Ad5/New Zealand White rabbit ocular model. These results may support future clinical guidelines for the treatment of symptomatic adenoviral ocular infections with topical cidofovir and adjunct topical NSAIDs.

METHODS

Virus and Cells

A clinical adenoviral isolate was cultured from a patient with typical adenoviral keratoconjunctivitis who sought treatment at the Eye and Ear Institute of Pittsburgh. The isolate was serotyped by serum neutralization and found to be type 5. The isolate, designated Ad5 McEwen, was grown in A549 monolayers at 37°C in a 5% CO2-water vapor atmosphere, harvested, aliquoted, and frozen as stock virus at −70°C. Before use, the stock viruses were titered by using a standard plaque assay.

A549 cells, epithelial-like cells derived from human lung carcinoma (CCL-185; American Type Culture Collection, Rockville, MD), were grown and maintained in Eagle’s minimum essential medium (MEM) with Earle’s salts, supplemented with 6% fetal bovine serum, 2.5 μg/ml amphotericin B, 100 U penicillin G, and 0.1 mg streptomycin per milliliter (Sigma, St. Louis, MO).

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Experimental Drugs

Cidofovir (S-HPMPC, [(S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)-cytosine]), was supplied by Bausch & Lomb Pharmaceuticals (Tampa, FL) as a 0.5% solution. Control eye drops for cidofovir consisted of the vehicle alone. The NSAIDs, diclofenac sodium 0.1% (Voltaren Ophthalmic; CIBA Vision Ophthalmics, Atlanta, GA) and ketorolac tromethamine 0.5% (Acular; Allergan Pharmaceuticals, Irvine, CA) were purchased from the pharmacy at the University of Pittsburgh Medical Center. Artificial tears (0.5% carboxymethylcellulose [Sigma] in phosphate-buffered saline [PBS]) served as control drops for the NSAIDs.

Animals

Three to 4-b female New Zealand White rabbits were obtained from Myrtle’s Rabbitry, Thompson Station, TN. All animal studies conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) approval was obtained, and institutional guidelines regarding animal experimentation were followed.

Assessment of Ad5 Replication with Topical Inoculation Only

This study was performed in duplicate, with 20 rabbits used per experiment. After appropriate systemic anesthesia with 3/5 mg/kg ketamine and 10 mg/kg xylazine and topical anesthesia with proparacaine, the rabbits were inoculated with 50 μl (1.2 × 10⁶ plaque-forming units [pfu]/eye) of Ad5 McEwen in both eyes after 12 cross-hatched strokes of a number 25 sterile needle. Four—Twenty-four hours later, a total of 10 rabbits each from both experiments were randomly assigned to one of four topical coded treatment groups: group I, 0.5% cidofovir + artificial tears; group II, 0.5% cidofovir + 0.5% ketorolac tromethamine; group III, 0.5% cidofovir + 0.1% diclofenac sodium; group IV, control + artificial tears. Cidofovir and control were administered to both eyes twice daily for 7 days and artificial tears, ketorolac, and diclofenac four times daily for 14 days. Drops were administered at least 1 hour apart with cidofovir given first and fourth in the 6-drop regimen on days 1 through 7. Ocular swabbing to recover adenovirus from the tear film and corneal and conjunctival surfaces was performed on days 0, 1, 3, 4, 5, 7, 9, 11, and 14 after inoculation and frozen at −70°C pending plaque assay.

Assessment of Ad5-Induced Subepithelial Infiltrates with Topical and Intrastromal Inoculation

To study the effects of concurrent therapy with diclofenac sodium and ketorolac tromethamine and cidofovir on the formation of subepithelial corneal infiltrates, a second inoculation technique was needed (i.e., topical and intrastromal inoculation of adenovirus). This technique produces a reliable and reproducible number of subepithelial corneal infiltrates for grading. In contrast, the technique of topical inoculation alone, although excellent for titer studies, does not consistently produce a reliable number of subepithelial corneal infiltrates for grading.1,10,11,22,23 This study was performed in duplicate, with 20 rabbits used per experiment. After topical and systemic anesthesia, both eyes of the rabbits were inoculated with 50 μl (1.2 × 10⁵ pfu/eye) Ad5 McEwen intrastromally by using a 30-gauge short-beveled needle to form five focal blebs (dice pattern, 10 μl per bleb). The corneas were then scarified superficially (eight scratches) with a number 25 needle to form a square around the central intrastromal inoculations. Inoculation was completed by applying 50 μl (1.2 × 10⁶ pfu/eye) Ad5 McEwen. Twenty-four hours later, 10 rabbits each were randomly assigned to the same treatment groups and regimens, as previously described for the topical inoculation—only group. The extent of subepithelial immune infiltrate formation was determined by slit lamp examination of rabbit corneas on day 25 after injection (PI). The extent of subepithelial infiltrate formation was scored according to a scale of zero to four (zero, 0 infiltrates; one, 1 to 5 infiltrates; two, 6 to 10 infiltrates; three, 11 to 15 infiltrates; four, 16 + infiltrates).

Determination of Viral Titers by Plaque Assay

The ocular samples to be titered were thawed and diluted serially (1:10) for two dilutions. Each dilution (0.1 ml per well) was then inoculated onto A549 cells in duplicate wells of a 24-well plate. The virus was adsorbed for 5 hours at 37°C in a 5% CO₂ water vapor atmosphere. After adsorption, 1 ml of outgrowth media plus 0.5% methylcellulose was added to each well, and the plates were incubated at 37°C in a 5% CO₂ water vapor atmosphere for 7 days. The plates were stained with 0.5% gentian violet, and the number of plaques per well counted under a dissection microscope (×25). The viral titers were then calculated and expressed in plaque-forming units per milliliter. The viral titer data were presented as both the mean daily Ad5 titers and a global mean referred to as the mean combined Ad5 titers during the early phase (days 1–5) and late phase (days 7–14) of infection.

Evaluation of Serious Ocular Toxicity

All eyes were examined for signs of serious toxicity (corneal melting and perforation) before culture (during the acute phase of infection) and during slit lamp evaluation of subepithelial infiltrates (during the late phase of infection).

Statistical Analysis

After the completion of both experiments, the codes masking the treatment regimens were broken, and the data from each experiment were analyzed statistically. Data based on viral replication (Ad5 titers, duration of shedding, and Ad5-positive cultures/total) from experiments using the topical inoculation—only technique were combined and analyzed statistically. Similarly, data based on clinical scores of subepithelial infiltrates from experiments using the topical and intrastromal inoculation technique were combined and analyzed statistically. The statistical tests used included: analysis of variance (ANOVA), Kruskal–Wallis ANOVA, Duncan’s multiple comparisons for ANOVA, χ², and Monte Carlo randomization analyses. Significance was established at the P ≤ 0.05 confidence level. There was one outlying titer value (1.17 × 10³ pfu/ml) from a day 7 sample in the cidofovir + ketorolac group that was 3 logs greater than the mean ± SD (2.1 ± 8.0 × 10⁹ pfu/ml) of the 19 other day 7 samples, of which only two demonstrated a positive Ad5 culture. This data point exceeded our criterion for inclusion (greater than three SDs from the mean) and was eliminated from all titer, Ad5-positive eyes, and duration of shedding analyses.

RESULTS

Assessment of Ad5 Replication

Ad5 Ocular Titers. Compared with the control + artificial tears group, treatment with cidofovir + artificial tears, cidofovir + ketorolac, and cidofovir + diclofenac reduced the mean daily adenoviral titers on days 7 and 9 (P = 0.003; P = 0.02, respectively: ANOVA and Duncan’s multiple comparisons; Fig. 1). There were no differences among the cidofovir treatment groups on any day. There were no differences among the treatment groups in the mean combined Ad5 titer during the early phase of infection (days 1–5; Table 1). In contrast, during the late phase of infection (days 7–14), the mean combined Ad5 titers demonstrated an antiviral effect between the three cidofovir treatment groups and the control (Table 1). The cidofovir + artificial tears (2.8 ± 11.9 × 10⁹ pfu/ml), cidofovir + ketorolac (0.6 ± 16.9 × 10⁶ pfu/ml), and the cidofovir + diclofenac (3.1 ± 17.5 × 10⁶ pfu/ml) groups all demonstrated lower mean combined Ad5 titers than the control + artificial tears group.
Ad5-Positive Cultures per Total. A significant antiviral effect was seen overall from days 1 through 14 in eyes treated with cidofovir + artificial tears (79/160; 49%), cidofovir + ketorolac (71/159; 45%), and cidofovir + diclofenac (81/160; 51%), which demonstrated fewer Ad-positive cultures compared with the control + artificial tears group (100/160; 63%; \( P \leq 0.042; \chi^2 \) analysis). There were no differences among the cidofovir treatment groups.

During the early phase of infection (days 1–5), there were no differences among any of the groups based on Ad5-positive cultures (Table 1). However, during the late phase (days 7–14), an antiviral effect was demonstrated in which the cidofovir + artificial tears (6/80; 8%), cidofovir + ketorolac (5/79; 4%), and cidofovir + diclofenac (9/80; 11%) groups all demonstrated fewer Ad-positive cultures compared with the control + artificial tears group (24/80; 30%; \( P < 0.007; \chi^2 \) analysis). There were no differences among the cidofovir + artificial tears, cidofovir + ketorolac, and cidofovir + diclofenac groups.

The percentage of daily Ad5-positive cultures are displayed graphically in Figure 2. Compared with the control + artificial tears group, significantly fewer Ad-positive cultures were demonstrated for the cidofovir + artificial tears group on day 7 (\( P = 0.0099 \)) and day 9 (\( P = 0.0099 \)), for the cidofovir + ketorolac group on day 7 (\( P < 0.02 \)) and day 9 (\( P = 0.0099 \)), and for the cidofovir + diclofenac group on day 9 (\( P < 0.02; \) Monte Carlo randomization test). There were no differences among the cidofovir treatment groups on any day.

Assessment of Adenoviral Subepithelial Infiltrate Formation

Treatment with cidofovir + artificial tears (score, 3.0 ± 0.9), cidofovir + ketorolac (2.6 ± 0.9), and cidofovir + diclofenac (2.3 ± 1.0) demonstrated no differences in subepithelial infiltrate scores compared with the control + artificial tears-treated eyes (2.8 ± 1.0; Kruskal–Wallis ANOVA; Table 1).

Evaluation of Serious Ocular Toxicity

There were no cases of corneal melting or perforation in any of the eyes treated with topical NSAIDs, cidofovir, or artificial tears in any of the experimental studies.

**Table 1.** Effects of NSAIDs on the Antiviral Activity of Cidofovir

<table>
<thead>
<tr>
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<th>Group I (Cidofovir + Artificial Tears)</th>
<th>Group II (Cidofovir + Ketorolac)</th>
<th>Group III (Cidofovir + Diclofenac)</th>
<th>Group IV (Control + Artificial Tears)</th>
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</thead>
<tbody>
<tr>
<td>Combined Ad5 titer (pfu/ml)</td>
<td>1.2 ± 2.6 \times 10^5 (n = 80)</td>
<td>4.8 ± 12.0 \times 10^4 (n = 80)</td>
<td>7.1 ± 12.5 \times 10^4 (n = 80)</td>
<td>1.1 ± 2.0 \times 10^5 (n = 80)</td>
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<td>Early phase (n = 80) (days 1–5)</td>
<td>2.8 ± 1.9 \times 10^5 (n = 20)</td>
<td>4.9 ± 1.2 \times 10^4 (n = 20)</td>
<td>3.1 ± 17.5 \times 10^4 (n = 20)</td>
<td>3.8 ± 11.5 \times 10^4 (n = 20)</td>
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<tr>
<td>Duration of shedding (days) (n = 20)</td>
<td>5.6 ± 1.9 (n = 20)</td>
<td>4.9 ± 1.2 (n = 20)</td>
<td>5.8 ± 1.8 (n = 20)</td>
<td>7.5 ± 2.3 (n = 20)</td>
</tr>
<tr>
<td>Ad5-positive cultures/total (%)</td>
<td>79/160 (49%) (n = 14)</td>
<td>71/159 (45%) (n = 14)</td>
<td>81/160 (50.6%) (n = 14)</td>
<td>100/160 (64%) (n = 14)</td>
</tr>
<tr>
<td>Early phase (days 1–5)</td>
<td>73/80 (95%) (n = 80)</td>
<td>68/80 (85%) (n = 80)</td>
<td>72/80 (90%) (n = 80)</td>
<td>76/80 (95%) (n = 80)</td>
</tr>
<tr>
<td>Late phase (days 7–14)</td>
<td>6/80 (8%) (n = 80)</td>
<td>3/79 (3.8%) (n = 79)</td>
<td>9/80 (11%) (n = 80)</td>
<td>24/80 (30%) (n = 80)</td>
</tr>
<tr>
<td>Subepithelial infiltrate scores (n = 20)</td>
<td>3.0 ± 0.9 (n = 20)</td>
<td>2.6 ± 0.9 (n = 20)</td>
<td>2.3 ± 1.0 (n = 20)</td>
<td>2.8 ± 1.0 (n = 20)</td>
</tr>
</tbody>
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* \( P \) = not significant among all groups.
\( \dagger \) \( P \leq 0.05 \) when compared with the control + artificial tears group.
\( \ddagger \) \( P \) = not significant when compared with the cidofovir + artificial tears group.
\( \hat{\ddagger} \) One outlying point on day 7 in group II was removed from the analyses, because it did not meet the inclusion criteria.
\( n \) = 79.
At present, there is no specific antiviral treatment for adenoviral ocular infections. The ongoing development of cidofovir as a topical antiviral agent has the potential to revolutionize treatment. Although an effective antiviral agent would be expected to kill adenovirus, it would not necessarily be expected to reduce immediately the secondary inflammation and attendant discomfort associated with an active infection. Optimally, a rational therapeutic approach would combine a topical antiviral with a topical anti-inflammatory agent to provide symptomatic relief for the patient without delaying the normal immune clearance of virus. Furthermore, the choice of a topical anti-inflammatory agent must not antagonize the inhibitory activity of the antiviral, cidofovir.

Corticosteroids are known to inhibit both the cyclooxygenase and lipoxygenase inflammatory pathways, other cellular enzymes, and direct expression of cytokines and lymphokines that activate the immune system. Previously, we reported that the antiviral efficacy of topical cidofovir was eliminated by local immunosuppression induced by 1% prednisolone acetate treatment in the Ad5/New Zealand White rabbit ocular model.11

NSAIDs (e.g., diclofenac sodium and ketorolac tromethamine) represent a class of anti-inflammatory agents currently available for ophthalmic use. NSAIDs block only the cyclooxygenase pathway and appear to have no direct effect on im-

FIGURE 2. Percentage of Ad5-positive cultures for each culture day for the cidofovir + artificial tears (C), cidofovir + ketorolac (C), cidofovir + diclofenac (C), and control + artificial tears (C) groups.11 Day on which all treatment groups demonstrated significantly fewer Ad5-positive cultures compared with the control + artificial tears group (P < 0.02, Monte Carlo randomization test). The therapeutic shift to the left is again demonstrated by comparing the cidofovir treatment groups to the control + artificial tears group.

Based on those results, we proposed that topical diclofenac or ketorolac may be desirable alternatives to topical steroids as adjunct therapy with cidofovir.

The antiviral efficacy of topical 0.5% cidofovir was not affected when used in combination with either topical 0.5% ketorolac tromethamine or 0.1% diclofenac sodium. Furthermore, the effect of the combined use of topical cidofovir with topical ketorolac or diclofenac was not different from the use of cidofovir alone with regard to formation of subepithelial infiltrates. Although topical corticosteroids alone and in combination with cidofovir inhibited formation of subepithelial infiltrates, the formation of these infiltrates is believed to be immune based and these data support the observed differential effect of topical steroids rather than their anti-inflammatory activity.

Based on the Ad5/New Zealand White rabbit ocular model, our projected clinical guideline suggests that treatment with topical NSAIDs diclofenac sodium and ketorolac tromethamine may not adversely effect the antiviral inhibitory activity of 0.5% cidofovir during the treatment of acute adenoviral ocular infections. The clinically proven anti-inflammatory and topical analgesic effects may provide patient comfort by reducing ocular inflammation and local pain and irritation. However, a controlled clinical trial remains the best way to establish the value of concomitant NSAIDs and cidofovir therapy.

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