Ca\(^{2+}\) in alkali-burned corneas;\(^{10}\) if penicillamine inhibits both the synthesis and the catabolism of collagen, the net balance of its effect may be minimal.

It could be argued that, if the incisions are not deep enough, the wound healing process will be limited, and drugs will exert little effect on the results. However, it is clear from the postoperative K readings in this study that the cuts were of maximal depth, providing adequate scope for observing the effects of drug activity, if any.

The mechanism by which radial cuts modify corneal shape is not completely understood, and there is no evidence that wound healing is the main factor in determining the effect of radial keratotomy. Jester et al.\(^{11}\) showed that doubling the number of incisions from 8 to 16 did not affect either the corneal flattening or the amount of regression after radial keratotomy in non-human primate eyes. If wound healing were a major determinant in the outcome of this surgery, twice as many wounds should produce some difference in postoperative results.

However, it is still difficult to explain why our findings do not show the significant enhancement of surgical results obtained with drug treatment in previous studies. The conflicting results obtained with BAPN and the negative result obtained with D-penicillamine seem to suggest that the inhibition of collagen cross-linkage may not be a satisfactory approach to controlling the correction achieved by radial keratotomy. Further studies with larger numbers of animals and a standardized surgical technique are necessary to confirm the results reported here, and future investigations in primate eyes may be useful to demonstrate possible effects of such drugs in a model more similar to human eyes.

Key words: radial keratotomy, collagen cross-linkage, \(\beta\)-amino-propionitrile, D-penicillamine, rabbit, cornea

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References


Differential Protein Synthesis in Steroid-Treated Ocular Surface Epithelium

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Topical prednisolone and cortexolone, a known glucocorticoid receptor antagonist, differentially affected the synthesis of proteins in normal corneal epithelium and migrating conjunctival epithelium after complete corneal deepithelialization, as measured by 35S-methionine incorporation and SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel) electrophoresis. Application of either prednisolone or cortexolone to corneal epithelium resulted in similar protein synthesis patterns, each showing two new protein bands of about 15K and 32K. Cortexolone, but not prednisolone, initiated the appearance of several protein bands of different molecular weights in the migrating conjunctival epithelium, while treatment with prednisolone plus cortexolone resulted in a pattern of protein bands which resembled the saline-treated control. Crude extracts of prednisolone-treated migrating epithelium also enhanced the inhibition of phospholipase A\(_2\) activity, and this prednisolone-induced inhibition was reversed by cortexolone. Invest Ophthalmol Vis Sci 27:1005-1009, 1986

Several commercially available corticosteroid preparations, such as Pred Forte (1% prednisolone acetate;
Allergan Pharmaceutical Inc., Irvine, CA), when applied topically, inhibit reepithelialization of completely denuded rabbit cornea, which is normally accomplished by the migration of adjacent conjunctival epithelium.\(^1\) This inhibition is reversed by topical corticosterone (10%, 11-deoxy-cortisol), a specific glucocorticoid receptor antagonist which does not itself affect reepithelialization.\(^2\) Glucocorticoid action is receptor mediated and involves de novo synthesis of proteins, but studies on the mechanism of corticosterone action have been hampered by the fact that systemic corticosterone is inactivated by adrenal beta hydroxylation.\(^3\) In this study, the protein synthesis patterns of uninjured corneal epithelium and migrating conjunctival epithelium following complete corneal denudation in the rabbit were determined after topical corticosterone and prednisolone treatment.

**Materials and Methods.** New Zealand albino rabbits weighing 1.5–2 kg were used in this study. Complete corneal epithelial denudation was performed as described previously.\(^1\) A 50 \(\mu\)l dose of commercially available 1% prednisolone acetate (Allergan) or 10% corticosterone (Aldrich Chemical Co., Milwaukee, WI) was applied topically alone or in combination to both eyes 1 hr prior to complete corneal injury. Such treatment was continued up to 72 hr, three times daily. Both eyes of the control animals received 50 \(\mu\)l of topical 0.9% saline three times daily up to 72 hr following complete corneal injury. We treated rabbit eyes with drugs three times daily up to 72 hr because we had previously demonstrated that prednisolone treatment up to 72 hr initiated the inhibition of reepithelialization following complete corneal denudation.\(^1,2\)

In another set of experiments, 1% prednisolone, 10% corticosterone, or 0.9% saline treatment was administered as described above to both uninjured rabbit eyes.

**Protein synthesis:** Samples of rabbit corneal epithelium or migrating conjunctival epithelium at 72 hr following complete corneal epithelial denudation were obtained by scraping the corneal surface with a sharp blade. The tissues were incubated in oxygenated Krebs-Henseleit solution for 1 hr at 37°C with 35S-methionine (50 uCi, spec. activity 800 Ci/mM; Amersham, Inc., Arlington Heights, IL). Tissues were then homogenized. The homogenates were placed in 0.01 M Tris-HCl containing 0.001 M EDTA and 0.001 M iodoacetic acid (pH 7.6) were analyzed by electrophoresis through 12.5% sodium dodecyl sulfate (SDS) containing polyacrylamide gels (PAGE).\(^4\) The gels were dried on Whatman paper. The synthesis of different proteins was detected by autoradiography using XAR-5 (Eastman Kodak Co., Rochester, NY) film.\(^4\)

**Phospholipase A\(_2\) assay:** Phospholipase A\(_2\) activity was measured by a modification of the method by Blackwell et al\(^5\) using radiolabelled phosphotidylcholine-palmitoyl-arachidonyl as a substrate. Briefly, reaction sets for the phospholipase assay comprised the following: 1 ml Tris–HCl buffer (pH 8.0) containing 50 mM CaCl\(_2\) (optimal concentration) and 200 ng porcine pancreatic phospholipase A\(_2\) enzyme (Sigma Chemical Co., St. Louis, MO); crude extract of migrating conjunctival epithelium (treated three times daily up to 72 hr with either 1% prednisolone, 10% corticosterone, 1% prednisolone plus 10% corticosterone, or 0.9% saline), or without extracts, and 200 nCi of phosphotidylcholine, L-alpha-1-palmitoyl-2-arachidonyl (arachidonyl-1-\(^{14}\)C) (spec. activity 40–60 mCi/mM, New England Nuclear Corp., Boston, MA). The samples were incubated for 1 hr at 4°C before addition of the substrate. The reaction is too rapid at 37°C to allow accurate rate measurements, but a 2-min incubation at room temperature permitted measurements over a linear range. At the end of the 2-min incubation period, the reaction was quenched with 1 ml ice-cold methanol. The samples were extracted in chloroform. The organic phase was separated and evaporated completely with N\(_2\). Labelled \(^{14}\)C-arachidonic acid was separated from unhydrolyzed phospholipid by TLC on silica gel, using chloroform:methanol:acetic acid (90:10:1) solvent system. The radioactivity at the fatty acid and phospholipid zones was estimated by conventional liquid scintillation counting technique. The percent inhibition of the phospholipase A\(_2\) activity by the crude extracts was calculated using the amount of radiolabelled arachidonic acid released (100%) from phospholipid by phospholipase A\(_2\) enzyme.

The investigation utilizing animals, as described in this manuscript, conforms to the ARVO Resolution on the Use of Animals in Research.

**Results.** Effects of prednisolone and corticosterone on protein synthesis: Corticosterone and prednisolone had similar effects on protein synthesis in the corneal epithelium. Treatment of uninjured corneal epithelium with topical corticosterone or prednisolone (but not with saline) resulted in the synthesis of two new protein bands, in the region of 15K and 44K, as detected by the incorporation of 35S-methionine (Fig. 1A, B).

However, prednisolone and corticosterone had different effects on protein synthesis in migrating conjunctival epithelium. A new 13K protein band was detected in prednisolone- and prednisolone plus corticosterone-treated cells, when compared to saline-treated migrating conjunctival epithelium. With corticosterone treatment, five new protein bands (17K, 18K, 23K, 35K, 44K) were detected, but the 13K band seen in the prednisolone- or prednisolone plus corticosterone-treated migrating conjunctival epithelium was absent (Fig. 2A, B). Treatment with prednisolone plus corticosterone re-
sulted in a pattern of protein bands which resembled saline-treated controls, except for the presence of the 13K band (Fig. 2A, B).

Inhibition of phospholipase activity in vitro: In four different experiments (n = 4), we assayed phospholipase activity. There was some residual anti-phospholipase A₂ activity (12 ± 8%) in the crude extracts of saline-treated migrating conjunctival epithelium. When the crude extract of 1% prednisolone-treated (applied topically 3 times daily up to 72 hr) migrating conjunctival epithelium was tested for its activity, the anti-phospholipase A₂ activity was enhanced to 54 ± 8%. Furthermore, 1% prednisolone plus 10% cortexolone treatment (topically applied three times daily up to 72 hr) antagonized (lowered to 25 ± 13%) the prednisolone-induced anti-phospholipase A₂ activity, while similar treatment with 10% cortexolone alone did not markedly inhibit the phospholipase activity (21.6 ± 12, n = 3).

Discussion. Our earlier work demonstrated that corticosteroids such as prednisolone, fluorometholone, and dexamethasone inhibit PMN release into rabbit tear fluid following corneal injury.² The anti-inflammatory effect of corticosteroids is believed to be due to induction of proteins such as macrocortin (M.W. 15,000), lipomodulin (M.W. 40,000), and renocortin (M.W. 16,000 and 40,000) in a variety of cells. These proteins inhibit phospholipase A₂ activity, which is re-
Fig. 2. A, Electrophoretic patterns of the 35S-labelled proteins of migrating conjunctival epithelium at 72 hr following de-epithelialization. A 50 μl dose of commercially available 1% prednisolone (P) acetate solution or 10% cortexolone (C) or both drugs simultaneously (P + C) was applied topically 1.5 hr prior to complete corneal epithelial denudation and three times daily (9 AM, 1 PM, and 5 PM) thereafter. Control animals received 50 μl of topical saline. Incubation with 35S-methionine and electrophoresis was done as noted under Materials and Methods and in Figure 1. The autoradiograph represents a typical set of patterns. The general patterns obtained were essentially similar with the various topical treatments. The pattern obtained with cortexolone (C) shows five new prominent bands in the molecular weight regions 17K, 18K, 22K, 35K, and 44K, as indicated by arrows. B, A scan of the autoradiograph up to 43K shown in A. Both P and P + C show the presence of a band at about 13K.

sponsible for the release of arachidonic acid from the membrane phospholipids. Arachidonic acid is metabolized into potent inflammatory and chemotactic products. In the present study, we have clearly demonstrated that extracts of prednisolone-treated conjunctival epithelium enhanced the anti-phospholipase activity, and that this enhanced anti-phospholipase A2 activity was reversed by the corticosteroid antagonist, cortexolone. Interestingly, we found that there was some residual phospholipase A2 inhibitory activity in the control (saline-treated) migrating conjunctival epithelium. Recent studies similarly reported that dexamethasone enhanced anti-phospholipase activity in a variety of cells which was due to the induction of antiphospholipase proteins, such as macrocortin, lipo-modulin and renocortin.

Changes in protein synthetic patterns in migrating corneal and conjunctival epithelium of rabbit have been reported by Kinoshita et al, who studied keratin-like protein synthetic bands (36K–63K) in migrating corneal and conjunctival epithelium of rabbit. Normal conjunctival and corneal epithelium had different keratin-like protein patterns which were retained for up to 10 days during regeneration. However, new bands were detected during the early stages of healing (1 day) within this region in both migrating corneal epithelial cells and conjunctival epithelial cells. In the present study, the protein bands that lie between the region
43k and 68k M.W. probably represent cytoskeletal elements in which keratin-like proteins may also be found. A comparison of saline-treated, prednisolone-treated, cortexolone-treated, and prednisolone plus cortexolone-treated columns did not show significant alterations in the protein synthetic patterns in this region, although limitations imposed by our technique would make it impossible to detect subtle changes in the protein synthetic patterns.

Since crude extracts of migrating conjunctival epithelium contained some anti-phospholipase activity which was enhanced in the extracts of prednisone-treated migrating epithelial cells, and since glucocorticoid antagonist (cortexolone treatment) inhibited steroid-induced, anti-phospholipase activity, we investigated the effect of prednisolone and cortexolone on protein synthesis in normal, uninjured corneal and migrating conjunctival epithelium after complete corneal deepithelialization. The effect of prednisolone and cortexolone on protein synthetic patterns in uninjured corneal epithelium were similar in that they both induced the synthesis of two new protein bands (approximately 15K and 40K), while such new protein bands were not observed in saline-treated tissues. Indeed, proteins of similar molecular weights (lipomodulin, 40K; macrocortin, 15K; renocortin, 15K and 30K) have been shown to be induced by glucocorticoids in other types of cells.5-7

The effects of prednisolone and cortexolone were quite different in migrating conjunctival epithelium. Prednisolone treatment did not appreciably change the protein band pattern except for the presence of a new 13K band. Cortexolone treatment induced the synthesis of five additional new protein bands, with M.W. ranging from 17K to 44K. To our knowledge, this is the first report of a corticosteroid antagonist inducing the synthesis of new proteins in migrating epithelial cells in vivo. However, when migrating conjunctival epithelium was treated with prednisolone and cortexolone together, the protein band pattern, as expected, was similar to that of saline-treated cells, except for a new 13K band. It must be emphasized that this 13K band may be composed of proteins that are different from the proteins of the 13K band induced by prednisolone. Although, in this study, we demonstrate the induction of new protein bands by prednisolone and cortexolone treatment in uninjured corneal and migrating conjunctival epithelium, we do not know which of the protein bands affect phospholipase activity. These results indicate that cortexolone, a known antagonist of glucocorticoids, may, under certain circumstances, initiate specific cellular protein synthesis.

This effect of cortexolone on conjunctival protein synthesis cannot readily be explained by glucocorticoid receptor affinity and partial agonist activity, since prednisolone and cortexolone have different effects on protein synthetic band formation in migrating conjunctival epithelium. On the other hand, it is likely that cortexolone acts independently of glucocorticoid receptors to initiate cellular protein synthesis which is markedly different from that produced by prednisolone. Thus, our previous observation² that cortexolone reversed steroid-induced inhibition of migrating conjunctival epithelium may possibly be due to the action of these new proteins, which could antagonize glucocorticoid effects at the receptor or postreceptor level.

Key words: glucocorticoids, prednisolone, cortexolone, antagonist, protein synthesis, cornea, conjunctival epithelium

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References