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Immunofluorescence of rabbit corneas following collagen cross-linking treatment with Riboflavin and Ultraviolet A

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Abstract

Purpose—To assess ultrastructural modifications in keratocytes and inflammatory cell response in rabbit corneas after riboflavin and ultraviolet A (UVA) exposure using immunofluorescence microscopy.

Methods—Twenty adult New Zealand albino rabbits weighing 2.0 to 3.0 kg were used in this study. Two rabbits served as controls. The animals had their epithelia removed and were cross-linked with riboflavin 0.1% solution (10mgs riboflavin-5-phosphate in 10ml 20% dextran-T-500) applied every 3 minutes for 30 minutes, and exposed to UVA (360 nm, 3 mW/cm²) for 30 minutes. Four rabbits were humanely euthanized at each time point of 1, 3 and 11 days and at 3 and 5 weeks after the procedure.

Immunohistochemistry studies of thin sections of each cornea were performed using TUNEL staining, Alpha smooth muscle actin (α -SMA), CD-3, myeloperoxidase (MPO) antibodies and DAPI counterstaining. In another experiment six additional rabbits were treated as above, and after 10 days of cross-linking, 5 μ l of lipopolysaccharide (LPS) endotoxin (1 μ g/ml) was injected in the mid stroma.

Results—Cross-linked corneas showed early stromal edema. By 5 weeks, complete resolution of the edema and a pronounced highly organized anterior 200 μ m fluorescent zone was observed. TUNEL staining showed keratocyte death by both necrosis and apoptosis between day 1 and 3 after cross-linking. At day 1 the limbal area close to the cross linking zone showed some inflammatory cells as well as α -SMA positive cells, indicative of the presence of myofibroblasts. By day 3 some myofibroblasts had migrated to the area beneath the cross linked stroma. Between day 3 and 5 weeks there was an increase in α -SMA staining in the area surrounding the cross linked stroma. The area of cross linking remained acellular up to 5 weeks.

Conclusions—Collagen cross-linking results in early edema, keratocyte apoptosis and necrosis, appearance of inflammatory cells in the surrounding area of treatment and transformation of surrounding keratocytes into myofibroblasts. Compaction of anterior stroma fibers, keratocyte loss and displacement of cell nuclei including inflammatory cells may have clinical implications in the long term risk of further corneal thinning in keratoconus and in the cross linked corneal immune response.

Keywords

Cornea; cross-linking; ectasia; myofibroblasts

INTRODUCTION

Keratoconus is a degenerative noninflammatory corneal disease that is progressive in 20% of cases and can be treated by lamellar or penetrating keratoplasty. Several structural changes have been documented in this condition including changes in collagen structure (1), organization (2), intercellular matrix alterations (3) and apoptosis and necrosis of keratocytes (4). All these findings document a structurally weakened corneal stromal tissue which is typical of ectatic diseases such as keratoconus.

Collagen cross-linking is a technique used to photopolymerize the stromal corneal fibers by the combined action of a photosensitizing substance (riboflavin) and 360 nm UV light from a solid state UVA source. The aim of the treatment is to create additional chemical bonds inside the corneal stroma while minimizing exposure to the surrounding structures of the eye (5,6). Photopolymerization increases the rigidity of the corneal collagen and its resistance to keratectasia. A similar mechanism of hardening and increased thickening of the collagen fibers is seen in the aging cornea and it's due to active glycosylation of age dependent collagen molecules (7). The method is technically simple and less invasive than other therapies proposed for keratoconus, treating essentially the underlying pathophysiological mechanism. Furthermore, this treatment is a new hope in the management of refractive surgery, corneal ulcers, stromal melting and collagenolysis and also to reduce corneal swelling in borderline endothelial function (8,9).

Previous studies have shown in vitro (8,10) and in vivo (11,12) keratocyte cytotoxicity and significant changes in collagen fibers leading to pronounced organization of the anterior collagen fibers (10). Immunofluorescent staining methods have illustrated the compaction of collagen fibers. However the in vivo cellular events and the implications of the collagen and cellular changes after cross-linking have not been well clarified. The aim of the present study is to assess ultrastructural modifications in keratocytes, collagen composition and inflammatory cell response in rabbit corneas after riboflavin and ultraviolet A (UVA) exposure using immunofluorescence microscopy.

MATERIALS AND METHODS

Collagen Cross-linking

New Zealand adult albino rabbits weighing 2.0 to 3.0 kg were used in this study. Two rabbits served as controls. The study adheres to the ARVO statement for the use of animals in ophthalmology and vision research and was approved by the LSUHSC Institutional Animal Care and Use Committee. Twenty rabbits had their epithelium removed and were cross-linked with riboflavin 0.1% solution drops (10mgs riboflavin-5-phosphate in 10ml 20% dextran-T-500) applied every 3 minutes for 30 minutes, while exposed to UVA (360 nm, 3 mW/cm²) for 30 minutes. This corresponds to a total dose of 3.4 J or a total radiant exposure of 5.4 J/cm² to the cornea. After the treatment, antibiotic ointment (ofloxacin) was applied 3 times daily until complete re-epithelization was achieved. Two additional rabbits served as controls. In another experiment six additional rabbits were treated as above, and after 10 days of cross-linking, 5 µl of lipopolysaccharide (LPS) endotoxin (1µg/ml) was injected in the mid stroma.

Tissue preparation

In the initial experiment four rabbits were humanely euthanized at each time point of 1, 3 and 11 days and at 3 and 5 weeks after the procedure using an intravenous overdose of pentobarbital. In the second experiment rabbits were humanely euthanized at 2 and 4 days after the LPS injection. Eyes in both experiments were immediately enucleated and the entire corneas were excised and fixed in neutral formalin (10%) for 24 hours. The corneas were removed, bisected

and embedded in optimal cutting temperature (OCT) (Miles Inc, Elkhart, IN). Six μm cryostat sections were prepared and air-dried, then stored at -80°C until use. They were evaluated with hematoxylin and eosin (H&E) stain and by immuno-histochemical analysis.

TUNEL Staining

One of the events that follow epithelial damage is keratocyte apoptosis beneath the injury. To detect fragmentation of DNA associated with apoptosis, a fluorescent based terminal deoxynucleotyl transferase-mediated UTP-biotin-nick-end labeling (TUNEL) assay was used (Promega, Madison, WI) according to the manufacture's recommendations. For nuclear counterstaining DAPI solution was used according to the manufacture's recommendations.

Immunostaining

To stain for rabbit corneal myofibroblasts (RCM), tissue sections were incubated with the monoclonal mouse anti-alpha smooth muscle actin antibody ($\alpha\text{-SMA}$) (1:300) (Sigma, St Louis MO) for two hours at room temperature, followed by incubation with the secondary antibody fluorescein conjugated goat anti-mouse IgG for 1 hr at room temperature.

To study the postoperative inflammatory cell infiltration, tissue sections were stained with H&E. Additionally tissue sections were incubated with myeloperoxidase (MPO) and CD-3 antibodies (Sigma, St Louis MO) for 2 hours at room temperature. The sections were then incubated with the secondary antibody fluorescein conjugated goat anti-mouse IgG for 1 hour at room temperature.

DAPI nuclear counterstaining was performed for 30 minutes at room temperature in all tissue sections. Negative controls consisted of secondary antibody alone and irrelevant isotype-matched antibodies. All sections were viewed and photographed with a Nikon Eclipse TE 200 fluorescence microscope equipped with a Nikon digital camera DXM 1200 (Nikon Inc, Melville, NY).

RESULTS

A cytotoxic effect of combined riboflavin/UVA treatment was observed in the anterior 250 μm corneal stroma. TUNEL positive cells were observed in the cross linked area at day 1 and day 3 after treatment (Figure 1). By day 10 no TUNEL positive cells were observed. No keratocyte damage was observed beneath the treated area. Finally, no endothelial toxicity was evidenced in any rabbits studied.

Corneas treated with riboflavin/UVA showed an absence of MPO positive cells in the area adjacent to the treatment including the anterior 250 μm of the cornea stroma. However, numerous MPO positive cells are seen in the transition area peripheral to the treatment zone. The inflammatory reaction was seen at 1 and 3 days after treatment. No MPO positive cells were observed 11 days after treatment (Figure 2). In order to better characterize the inflammatory cell infiltration a pan-T cell marker was used (CD-3). Significant number of T cells were present in the transition corneal stroma surrounding the treated cornea (Figure 3).

Alpha- SMA positive cells were observed up to five weeks after treatment in the area below the cross-linked stroma and surrounding the treated area (Figure 4). By 10 weeks some cells start to migrate to the treated area from the surrounding stroma.

Ten days after cross-linking, 6 rabbit corneas received an intrastromal injection of LPS. Corneas were stained with H&E at 2 and 4 days after the injection. There was an intense infiltration of inflammatory cells in the anterior corneal stroma in non-treated corneas while

no inflammatory cell infiltration was observed in the cross-linked corneas. Additionally, very few inflammatory cells were found in the stroma beyond the treated area (Figure 5).

DISCUSSION

Collagen cross linking is the only approach to progressive keratoconus and post-LASIK ectasia based on the pathophysiology of the disease that can delay or arrest their progression reducing the demand for keratoplasties. Unlike other invasive methods such as intrastromal rings (INTACS) and laser surgery which mainly treat the refractive effects of the disease, cross linking attempts to modify the structurally weakened corneal tissue typical of keratoconus. Collagen cross linking has been shown to increase biomechanical rigidity in human corneas by up to 328.9% (13). Recent studies performed in rabbits have shown an increase in the Young's modulus by 78.4–87.4%, an increase in ultimate stress by 69.7–106% and a decrease in ultimate strain by 0.57–78.4% after treatment that remained stable over time (14).

Previous in vitro studies have shown that the combined riboflavin/UVA-treatment leads to a 10 fold lower threshold for keratocyte cytotoxicity at 0.5 mW/cm² compared to 5 mW/cm² after UVA irradiation alone (10). Our study showed that collagen cross-linking results in early edema and massive keratocyte damage down to a depth of 250 µm using 3 mW/cm² surface irradiance. Additionally, we observed late compaction of anterior stroma fibers, and displacement of cell nuclei including inflammatory cells. The clinical implications of this keratocyte loss and the long term risk of corneal thinning are still unknown. Keratocyte apoptosis of variable degrees have been well documented in other corneal procedures such as LASIK, PRK or epithelial injury. In most cases keratocyte deficiencies can be overcome rapidly from adjacent migrating keratocytes. Previous studies have noted that the corneal thinning after cross linking is not permanent and after 6 months may reach its original value (15). However in keratoconus, treatment-induced keratocyte loss might be an issue, due to the presumptive role of keratocyte apoptosis in the pathogenesis of keratoconus. Due to the relatively short observation period (up to 10 weeks), our study did not include corneal thickness measurements.

Wand et al using a mouse model showed a cell-free zone in the stroma produced by UVA/riboflavin irradiation in vitro and suggested that this technique may be employed to prevent or reduce immunological reactions and graft failure by pre-treating donor corneas (11). Wollensak et al observed however that after initial massive keratocyte apoptosis there is a gradual repopulation process of the anterior corneal stroma (12). They found a normal keratocyte density after 6 weeks of treatment using a rabbit model. Our study found massive keratocyte apoptosis in the treated area but persistence of keratocytes below the treatment area as opposed to Wollensak study in which all layers of the cornea were involved. We observed migration of activated keratocytes 10 weeks after treatment. Keratocyte apoptosis is assumed to initiate the corneal wound healing response and start the complex cytokine cascade. Probably a more limited damage, restricted to the anterior corneal stroma, may produce a more delayed repopulation response seen in our study. Interestingly in both studies the repopulation process started in the posterior stroma.

Early studies using confocal microscopy have shown transient corneal opacity similar to haze in five of 44 patients treated (16). Confocal analysis showed hyperactivated keratocyte nuclei in the anterior stroma. Our study showed early myofibroblasts transformation beneath the treated area as a keratocyte wound healing response, but these cells do not migrate to the treated area up to 10 weeks after treatment. In some instances we found increase density of the extracellular stromal matrix beneath the treated area along with persistence of myofibroblasts. Our study is in agreement with previous studies performed in hen corneas that showed significant decrease in forward light scattering after cross linking (17). These findings may

explain the mild corneal haze seen clinically in some patients after treatment. Another interesting observation regarding myofibroblast transformation is that even the most severe cases resolved spontaneously after 10 weeks after the original surgery that triggered the haze response (18). Presumably, these cells undergo apoptosis when the epithelial and basement membrane integrity is restored and the level of TGF- β derived by the epithelium falls to a level that no longer allows maintaining myofibroblast viability. Finally, repopulating keratocytes should reabsorb abnormal collagen and other matrix abnormal materials deposited by myofibroblasts allowing the cornea to restore its clarity as observed in our animal study.

We also observed proliferation of inflammatory cells in the transition cornea outside the treated area, probably secondary to massive cytokine release produced by the keratocyte damage after cross-linking. These cells probably play an important phagocytic role of cell debris. Our study showed intense inflammatory cell infiltration during the first 5 days after treatment and gradual disappearance after day 10. This finding evidences that collagen cross linking induces both massive cell apoptosis and necrosis of keratocytes in the treated area.

In order to assess the capacity of inflammatory response after injury in previously cross linked corneas, LPS treatment was administered 10 days after the initial treatment. We observed an attenuated inflammatory reaction in cross-linked corneas after LPS treatment as compared to controls, suggesting a milder inflammatory and immune response. These may be due to reduced chemiotactic stimulus produced by the absence of cell bodies in the anterior corneal stroma or the effect of difficulty of migration of the inflammatory cells produced by stromal compaction. If our assumption is correct there may be a reduced cell response after injury early after treatment whose implications in the corneal host defense response need to be further studied.

Finally, long term studies involving in vivo confocal microscopy in humans after riboflavin/UVA treatment should evaluate depth of keratocyte loss, the repopulation process and exclude the development of treatment related stromal haze, scarring and thinning due to cell loss and impaired immune response to injury in humans.

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Figure 1. Early apoptosis after collagen cross-linking

TUNEL positive cells (arrows) are observed in the cross-linked area at day 1 and day 3 after treatment. No cells are noted at day 11. A). x10 magnification. B). x20 magnification.

Figure 2. Absence of inflammatory cell infiltration in the cross-linked area

Cross-linked corneas showed an absence of MPO positive cells in the cross-linked area.

However, multiple MPO positive cells (arrows) are observed in the transition zone peripheral to the treatment zone. No MPO positive cells were observed 11 days after treatment.

Figure 3. CD-3 immunostaining in corneas after cross-linking

A). Absence of CD-3 positive cells are noted in the cross-linked area at all times studied. B). Presence of CD-3 positive cells corresponding to activated T cells are noted in the transition corneal stroma surrounding the treated area (arrows).

Figure 4. Presence of myofibroblasts in corneas after cross-linking

Alpha SMA positive cells are observed up to 5 weeks after treatment in the area below the cross-linked stroma and surrounding the treatment area (arrows). By 10 weeks most cells have disappeared and some have migrated to the treatment area.

Figure 5. Decreased inflammatory reaction to endotoxin in cross-linked corneas

Intense inflammatory cell infiltration in control corneas after endotoxin intrastromal injection (arrows). Few inflammatory cells are observed however in the stroma beneath the treatment area (arrows) and no inflammatory cell infiltration in the treated stroma of previously cross-linked corneas (asterisk).