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Albumin precursor and Hsp70 modulate corneal wound healing in an organ culture model

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ABSTRACT

In order to investigate the role of albumin precursor and Hsp70 in corneal wound healing, we have analyzed the distribution of these proteins in wounded and non-wounded corneas of rabbits and the effects of topical applications of anti-albumin precursor and anti-Hsp70 antibodies on wound healing. Anti-albumin precursor and anti-Hsp70 antibodies were topically applied in healing corneal epithelium of rabbit eyes in organ culture. Corneas were allowed to heal *in vitro* for up to 120 h in serum-free medium with 5 and 10 μg/ml or without (migrating control) anti-albumin precursor/ or anti-Hsp70 antibodies. Fibronectin (Fb) (5 μg/ml) was used as a positive control. Immunofluorescence labelling was used to detect proteins in corneal epithelium at various time intervals following an epithelial defect. Delay in wound healing ($p<0.005$) was observed with 10 μg/ml anti-albumin antibody labelling. A similar pattern was observed when anti-fibronectin antibody (5 μg/ml) alone and in combination with anti-albumin (10 μg/ml) was ectopically added with wound closure occurring at 120 h. However with anti-Hsp70 antibody (5 μg/ml) slightly delayed ($p<0.005$) wound closure was observed at 96 h and considerable retardation >120 h with 10 μg/ml. Additionally, immunofluoresence showed a strong co-localization of Hsp70 and albumin precursor during the active phase of wound healing. The presence of albumin precursor and Hsp70 in the epithelial compartment of the cornea indicates a role for these proteins in modulating cell behavior such as epithelial growth, adhesion or regeneration, thus contributing to corneal epithelial wound healing.

Introduction

The expression of the extracellular matrix proteins laminin, fibronectin (Zhao et al., 2003), integrin (Iglesia et al., 2000), F-actin and vinculin (Wu et al., 1995) has been known to play a vital role in eye development and wound repair. As yet, the cytoskeleton-related proteins that regulate cell division, and wound healing are not fully characterized. Hence, identification of various proteins that could be linked to the healing process would be important in treatment of corneal disease and repair.

Several studies have shown that albumin plays a fundamental role in maintenance of the cornea (Maurice and Watson, 1965; Gong et al., 1997; Higuchi et al., 2007). Serum albumin is the most abundant hydrophilic protein found in blood vessels (Maurice and Watson, 1965) and also is predominantly found in the corneal epithelium (Nees et al., 2003); however, the possible role of serum albumin in corneal epithelial wound healing has not yet been elucidated. We have previously described the role of albumin and Hsp70 in wound healing and also provided evidence of albumin interaction with the extracellular matrix (ECM) protein fibronectin during wound healing (Mushtaq et al., 2007).

Small subsets of heat shock proteins (Hsp) are expressed in response to metabolic stress in almost all organisms and these proteins are so named because temperature and altitude were the first described inducers for Hsp (Tanaka et al., 1996). Hsps are a most conserved group of proteins and are known to have the ability to regulate protein folding and assembly (Craig, 1993). This property is crucial for re-epithelialization, which needs such functions for the removal of disrupted proteins and transport of newly formed proteins through the cell into different cell compartments. Wound healing conditions also generate a stressful environment for the cells involved in the regeneration process and may therefore influence the expression of Hsps.

For this study, we used two technical approaches involving immunolocalization and a corneal epithelial organ culture model that have permitted a more accurate evaluation of corneal epithelium for albumin precursor and Hsp70 proteins. We report the localization of the albumin precursor of corneal epithelial proteins and Hsp70. In addition, we evaluated the contribution of the albumin precursor and Hsp70 in the wound healing process

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using an organ culture model. We believe these data indicate a new function for albumin and Hsp70 in regulating events that occur in response to an epithelial defect.

Materials and methods

Effects of anti-albumin precursor and anti-Hsp70 antibodies on corneal epithelial wound healing in organ culture

Preparation of corneal epithelial organ culture

The experimental protocols for the use of male albino rabbits (200–300 g) in this study were in conformity with the Declaration of Helsinki on the "Guiding Principle in Care and Use of Animals" and received institutional approval from the appropriate ethics committee. The rabbits were sacrificed and their eyes excised, including the optic nerve, using dissecting forceps and scissors. The eyes were placed immediately in cold sterile filtered normal saline. The integrity of the epithelium was first checked with fluorescein (0.1%; Sigma-Aldrich). Briefly, organ culture was carried out in a Class II type B1 laminar flow hood (The Baker Company, Sanford, Maine). Integrity of corneal epithelium was monitored by staining the corneas with 0.1% sterile fluorescein solution in phosphate buffered saline (PBS). The eyes with damaged epithelium indicated by the fluorescein-stained region were discarded. Normal eyes (i.e. with intact corneas) were used for organ culture preparations (Gipson and Kiorpes, 1982). Five sets of experiments were performed. Normal eyes ($n = 10$) were held using sterile gauze by the optic nerve and the posterior globe and the epithelial surface were demarcated with a 7-mm-diameter trephine. The epithelium within the trephined region was abraded with a ♯10 sterile scalpel blade under dissecting microscope (Olympus M081, Japan). The corneas were then excised along a 1–3 mm scleral rim designated as migrating epithelium (M); for non-migrating epithelium (N), corneas ($n = 20$) were excised without scraping the epithelium. The excised corneas were washed 3 times with Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich, St. Louis, MO) followed by a 5–7 min wash in an antibiotic–antimycotic solution (10 mg streptomycin, 1000 units penicillin, and 25 μg amphotericin B/ml in 0.9% NaCl; Sigma-Aldrich). The corneas were again rinsed in HBSS and transferred to modified serum-free supplemental hormonal epithelial media (SHEM containing EMEM:HEPES, Hams F-12, DMSO, insulin, antibiotic–antimycotic solution, epidermal growth factor (EGF) and cholera toxin (CTX))/cornea; Sigma-Aldrich) (Zieske and Gipson, 1986). The corneas were then incubated at 37 °C in a CO_2 incubator (Forma Scientific, USA) with SHEM media containing anti-albumin precursor and anti-Hsp70 antibodies (5 and 10 μg/ml, respectively), which was replaced every 24 h. For positive control ($n = 5$; 5 μg /ml), anti-fibronectin antibody was added to the SHEM media with 10 μg/ml anti-albumin precursor. Concentrations of antibodies in each group are summarized in Table 1. For immunohistochemical analysis identical SHEM media without antibodies and with 5% serum were used.

Monitoring of wound area

At 0, 24, 48, 72, 96 and 120 h, regenerated epithelia in every cornea in each group ($n = 6$) was stained with Richardson's stain (1% azure II, 1% methylene blue and 1% borax dissolved in 100 ml autoclaved water; Richardson et al., 1960).

Briefly, the corneas were removed from the SHEM media with sterile forceps, placed with the epithelial side down in 1 drop of Richardson stain for 1 min and then washed by repeated dipping in phosphate buffered saline (PBS). The wound size (Richardson stained region) was measured with a ruler and photographs were taken for records. For statistical analysis, a one-way ANOVA was applied. The results are presented as mean ± SD and a value of $p < 0.05$ was considered to be statistically significant.

Immunohistochemistry

At every 24 h in culture, eyes ($n = 6$) were processed. Immunofluorescence labeling was performed as previously published (Zieske and Gipson, 1986). Corneas were excised and frozen in Tissue Tek II OCT compound (Lab Tek Products, Naperville, IL). 6-μm-thick cryostat sections were cut and placed on gelatin-coated slides, air-dried overnight at 37 °C, rehydrated in phosphate buffered saline (PBS), and blocked in 1% bovine serum albumin (BSA) for 10 min. Sheep polyclonal antibody against albumin precursor (Sigma-Aldrich, St. Louis, MO), diluted 1:500, and mouse monoclonal antibody against Hsp70 (Abcam), diluted 1:500 were placed on the slides and incubated for 1 h in a humid chamber. The slides were rinsed for 10 min in PBS with 1% BSA. FITC-conjugated donkey anti-sheep IgG and goat anti-mouse IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA), both at 1: 5000 dilution, were applied for 1 h. Coverslips were applied, mounted with a medium consisting of PBS, glycerol and paraphenylene diamine. Negative control tissue sections (primary antibody omitted) were routinely run with non-specific anti-mouse IgG (Sigma-Aldrich, St. Louis, MO) diluted 1:500. The sections were viewed and photographed using a microscope (Nikon Eclipse E800, Japan) equipped with a digital camera (DXM 1200).

Results

Wound healing analysis following topical application of anti-albumin precursor and anti-Hsp70 antibodies

In order to investigate the potential link between the albumin precursor and Hsp70 expression and progression of healing, we monitored the effects of anti-albumin and anti-Hsp70 antibodies (5 and 10 μg/ml) in serum-free media in a time-dependent manner (0–120 h). We observed wound closure at 96 and 120 h with 5 and 10 μg/ml anti-albumin antibody, respectively (Figs. 1B, C). A low concentration (5 μg/ml) of anti-albumin antibody inhibits cell migration without altering the wound closure time,

Table 1
Concentrations of antibodies in each group.

	No. of corneas	Antibodies in SHEM	Quantity (μg/ml)
Group 1	10	Anti-albumin precursor	5
Group 2	10	Anti-albumin precursor	10
Group 3	10	Anti-Hsp70	5
Group 4	10	Anti-Hsp70	10
Group 5 (positive control)	5	Anti-fibronectin and anti-albumin precursor	5 and 10

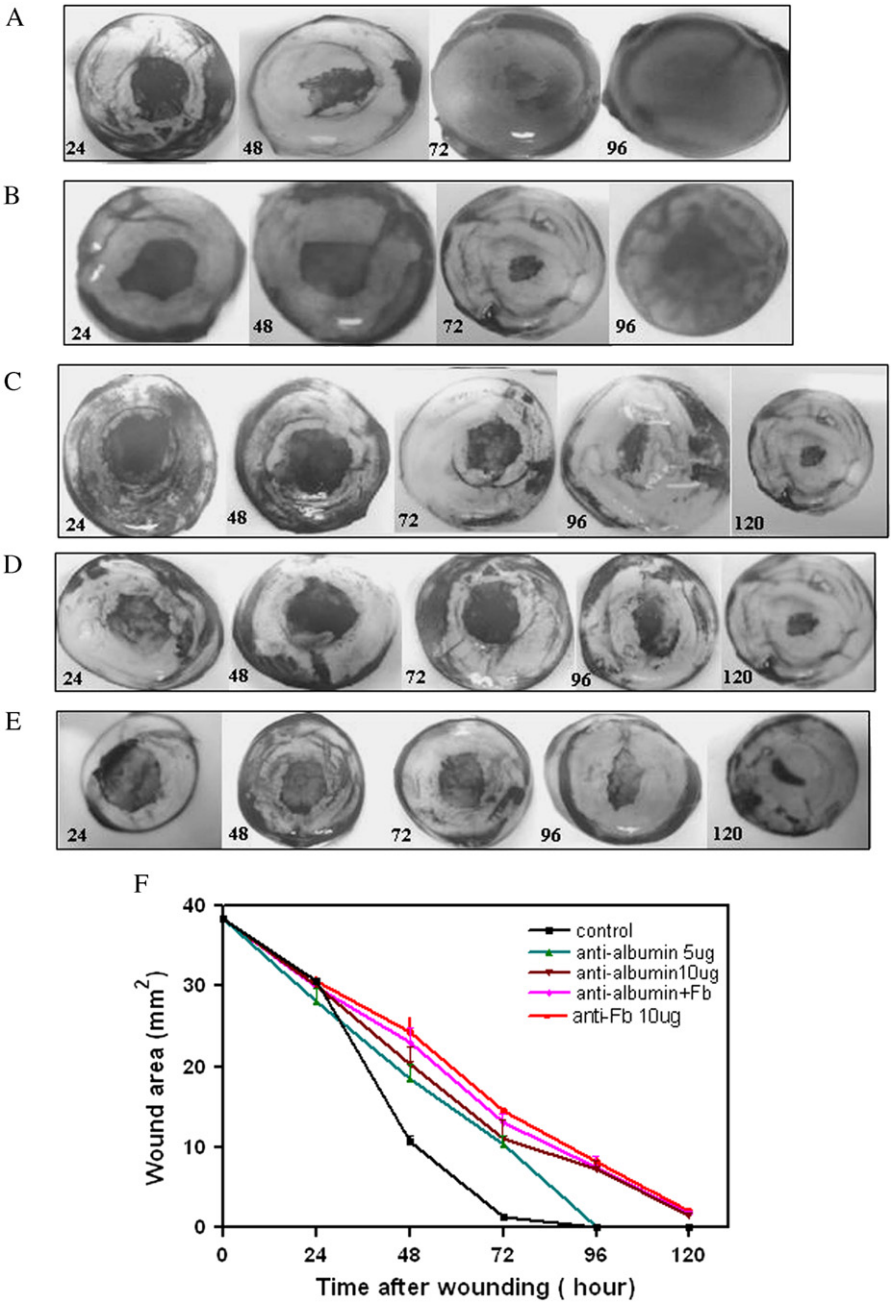

Fig. 1. (A) Rabbit corneal epithelial wound healing at different time intervals 24, 48, 72 and 96 h after wounding in serum free media without anti-albumin and anti-Hsp70 antibodies (control), wound was closed at 72 h, (B) with 5 μg/ml anti-albumin antibody, wound was closed at 96 h, (C) with 10 μg/ml anti-albumin antibody, wound was closed after 120 h, (D) 10 μg /ml anti-albumin antibody and 5 μg anti-fibronectin antibody wound was closed after 120 h, and (E) only 5 μg/ml anti-fibronectin antibody was applied as ECM protein positive control, wound was closed after 120 h. (F) The effect of anti-albumin precursor and anti-fibronectin (Fb) antibodies on the rate of re-epithelialization of corneal wounds. Corneal re-epithelialization was observed in serum-free media with and without anti-albumin precursor antibody at 5 and 10 μg/ml and at same concentration with anti-fibronectin antibody. Only anti-fibronectin 5 μg/ml used as positive control. Regenerated epithelium was measured with Richardson stain at 0, 24, 48, 72, 96 and 120 h post-wounding. Mean wound area was plotted. Bar indicates SEM. $P<0.005$.

which was achieved at 96 h similar to that of the control (Figs. 1A, B). However, a strong inhibition of cell migration with delay in wound closure time (120 h) was observed with 10 μg/ml anti-albumin antibody (Fig. 1C). We further explored the relationship between albumin and cell adhesion molecule to establish its role in healing mechanism. We used anti-fibronectin antibody 5 μg/ml in combination with 10 μg/ml anti-albumin antibody (Fig. 1D).

A strong inhibition of cell migration with delay in wound closure time (120 h) was observed with 10 μg/ml anti-albumin antibody labeling; a similar pattern was observed when anti-fibronectin antibody (5 μg/ml) alone and in combination with anti-albumin (10 μg/ml) was ectopically added (Figs. 1D, E). These data indicate that anti-albumin antibody and/or fibronectin can retard wound healing (Fig. 1F). An anti-Hsp70 antibody applied ectopically also retarded corneal epithelial wound healing with 10 μg/ml, and showed further retardation in wound closure compared to 5 μg/ml (Figs. 2A, B). Slightly delayed ($p<0.005$) wound closure was observed at 96 h with 5 μg/ml and considerable retardation >120 h with 10 μg/ml (Fig. 2C).

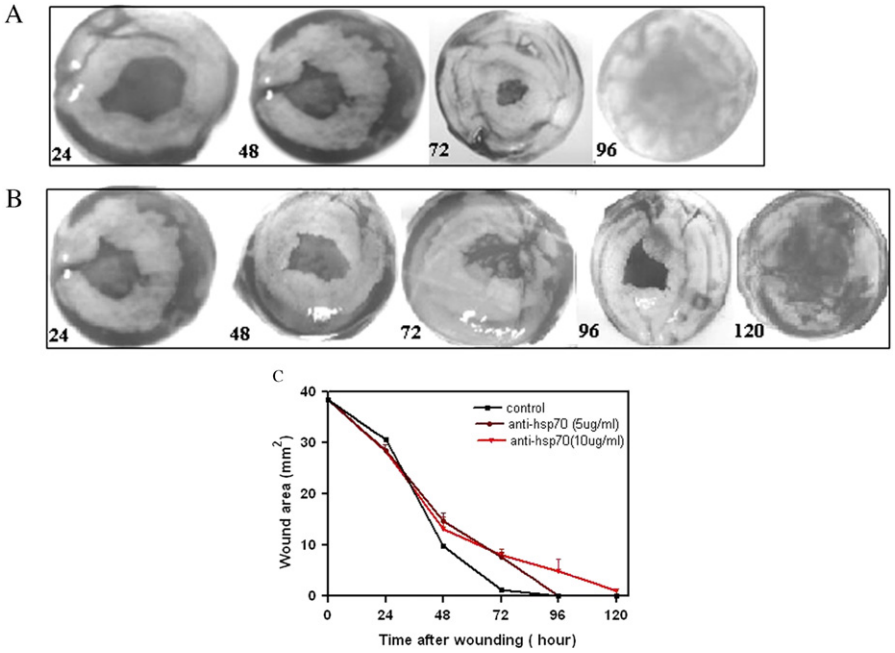

Fig. 2. Rabbit corneal epithelial wound healing in response to topical administration of anti-Hsp70 antibody (5 and 10 μg/ml) at 24, 48, 72, 96 and 120 h after wounding. (A) With 5 μg/ml anti-Hsp70 antibody, the wound was closed at 96 h (B) With 10 μg/ml anti-Hsp70 antibody, wound was closed at 120 h. (C) The effect of anti-Hsp70 antibody on the acceleration of re-epithelialization of corneal wounds. Time course of re-epithelialization in wounded corneas was observed in serum-free media with and without anti-Hsp70 antibody. Regenerated epithelium was measured with Richardson stain at 0, 24, 48, 72, 96 and 120 h post-wounding. Mean wound area was plotted. Bar indicates SEM ($p < 0.005$).

Localization of albumin precursor and Hsp70

To obtain more insight into the molecular mechanisms of albumin function with respect to corneal epithelial wound healing, we focused on the immunolocalisation of albumin at different time points. A role of albumin in corneal healing has been highlighted by 48 h following debridement, showing up-regulation. Albumin was not only immunolocalised in the epithelial layer but was strongly immunolocalised in the superficial layer and basal cells of the ocular surface epithelium as a linear labelling (Fig. 3). A time-dependent study showed no detectable albumin precursor at the leading edge of the wound at 0 h (Fig. 3b); a thin layer of epithelium resurfaced the defect at 24 h, exhibiting very weak immunofluoresence for albumin compared to control non-migrating epithelium (Fig. 3a). However, the first immunopositivity of albumin was shown in the posterior stroma at 24 h and the anterior stromal layer under the epithelium showed elongated patches of albumin labelling, which then spread to the entire stroma (Fig. 3c). We further noticed albumin immunoreactivity at 72 h post-wounding; faint albumin labelling was observed mainly in the superficial layer of regenerated epithelium, while in non-wounded epithelium labelling becomes gradually more diffused and appeared discontinuous at the leading edge of the epithelium (Fig. 3e), whereas negative control using IgG showed no cross-reactivity (Fig. 3f).

Hsp70 distribution was assessed by immunofluoresence using anti-Hsp70 antibody. Localization of Hsp70 in normal (non-wounded) cornea was observed primarily in the epithelial layer with some labelling in the stromal cells (Fig. 4a). Interestingly, there was no detectable Hsp70 in the cells at the leading edge (Fig. 4d), suggesting a link between Hsp 70 up-regulation and cell migration. The scraped wound showed resurfacing of epithelium and at 0 h after injury there was no detectable Hsp70 in the cells at the leading edge (Fig. 4b), although it appeared in the regenerated epithelia, which gradually diffuses at different time points between 24 and 72 h (Figs. 4c, d, e). Negative controls, without anti-Hsp70 antibody, did not show any cross-reactivity (Fig. 4f).

Discussion

We recently reported the role of albumin precursor and Hsp70 interaction with fibronectin during wound healing (Mushtaq et al., 2007). Our results showed that in organ culture debridement wounding, production and localization of the albumin precursor and Hsp70 proteins in stratified squamous epithelium of the cornea were dramatically altered. These findings also indicate that albumin precursor and Hsp70 are involved in the healing mechanism. We therefore considered the possibility that fibronectin, an extracellular matrix (ECM) protein, was required for the interaction of albumin and analyzed whether albumin was interacting with fibronectin, and this proved to be the case. It is well documented that ECM proteins play a vital role in re-epithelialization, in addition to cell proliferation, migration, and interaction with integrin and other ECMs (Watanabe et al., 1987; Saika et al., 2000; Filenius et al., 2003). In the present study, in order to examine the effect of topical applications of anti-albumin precursor and anti-Hsp70 antibodies on corneal epithelial wound healing, we used a rabbit corneal epithelial organ culture model, which has previously been shown to demonstrate corneal epithelial wound healing (Mushtaq et al., 2007). We found that in our organ culture model of corneal epithelium, 5 μg/ml anti-albumin precursor and anti-Hsp70 did not significantly influence corneal re-epithelialization, whereas 10 μg/ml of these antibodies caused a retardation of wound healing.

Antibodies against fibronectin were found to retard wound healing when we used them with anti-albumin precursor, indicating that a fibronectin and albumin-dependent mechanism is involved (Fig. 1D, E). We used anti-fibronectin as a positive control because this protein, 1 h after wounding, forms a

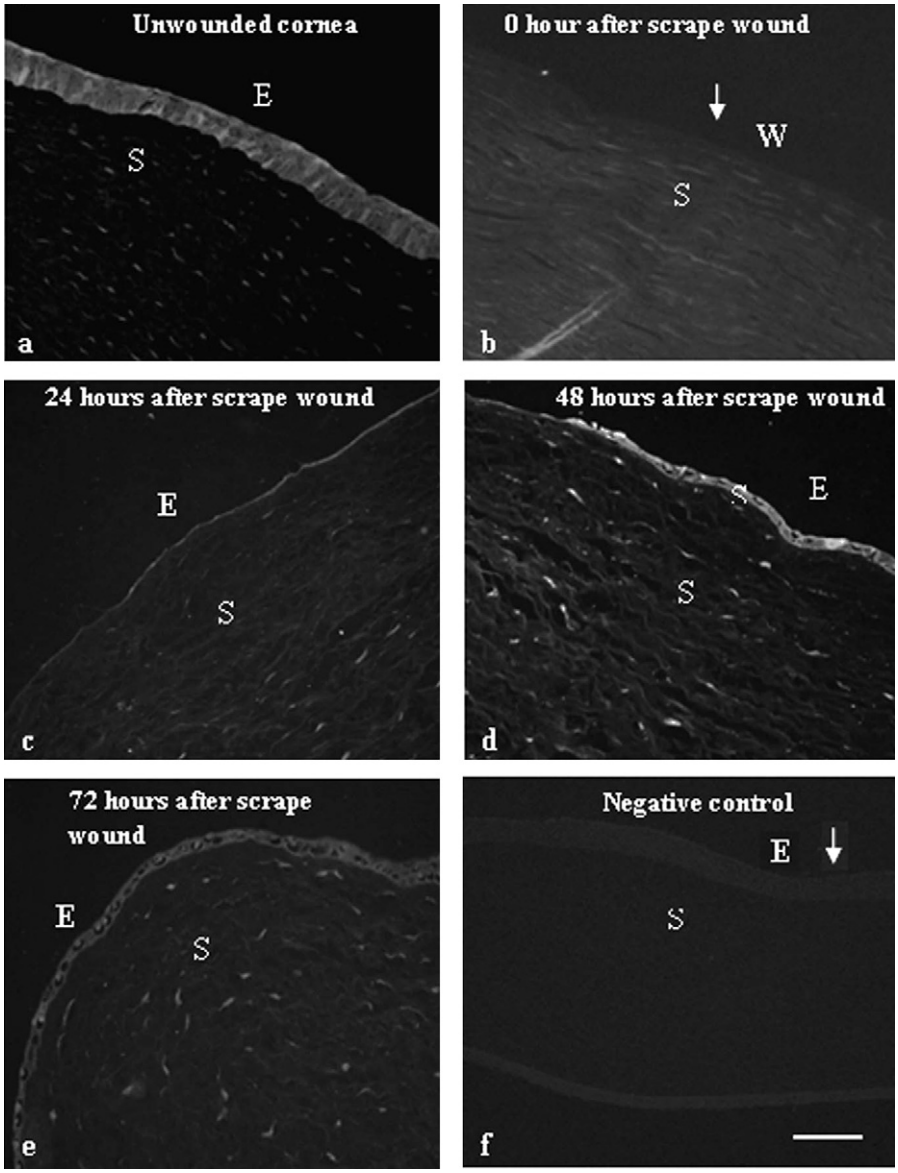

Fig. 3. Micrographs showing immunofluorescence in rabbit cornea after scrape wound stained for albumin precursor. (a) Normal epithelium (unwounded), (b) at 0, (c) 24, (d) 48, (e) 72 h after wounding and (f) negative control. Scale bar 20 μm. E, epithelium; W, wound; S, stroma.

temporary matrix for cell migration, accelerates re-epithelialization by supporting epithelial cell migration, and decreases after re-epithelialization is completed (Saika et al., 2002). We therefore considered the possibility that fibronectin is required for ECM re-modulation during corneal wound healing. Other provisional ECMs, e.g., lumican, perlecan and fibrin, also modulate epithelial wound healing (Drew et al., 2000; Saika, et al., 2000; Sta Iglesia and Stepp, 2000). The cornea provides an ideal model to evaluate interactions of epithelial cells and the ECM proteins underlying the basal lamina during wound healing because epithelial injuries of the avascular corneal tissues heal in a bloodless wound field. Various proteins such as vinculin (Zieske et al., 1989), keratins (Yu et al., 1995) and gelatinase are up-regulated and modulate cell adhesion and migration during wound healing. Chung et al. (1995) and Stepp and Zhu (1997) have previously shown that larger debridement wounds that require cell proliferation to generate the cells necessary for sheet movement result in a dramatic stimulation of proteins and their mRNA during both migration and regeneration (Chung et al., 1995; Stepp and Zhu, 1997). This provides persuasive support for our finding that albumin precursor and Hsp70 may be one of the proteins expressed during cell migration and involved in cell matrix adhesion in forming epithelium and play a role in cell–cell interaction while changes in protein expressions are regulated at the level of transcription (Mushtaq et al., 2007). In this study we demonstrated localization of albumin and Hsp70 proteins in corneal epithelium in squamous epithelium and at sites of cell–cell interaction. This distribution changed little during the active phase of migration in the *in vitro* organ culture model at different time intervals post-wounding (0, 24, 48 and 72 h).

Therefore, the presence of Hsp70 may be associated with folding, activation or inactivation and conformational changes of proteins that would be required during migration or proliferation (Langer and Neupert, 1991; Hendrick, et al., 1993). During wound healing, epithelial sheet movement requires that entire sheet endure the forces generated by the dramatic cell shape changes occurring in the basal layer at leading edge. Because of the distribution and presence of albumin and Hsp70 at squamous epithelium and at the site of cell–cell interaction and interaction with ECM component fibronectin, albumin and Hsp70 are likely to

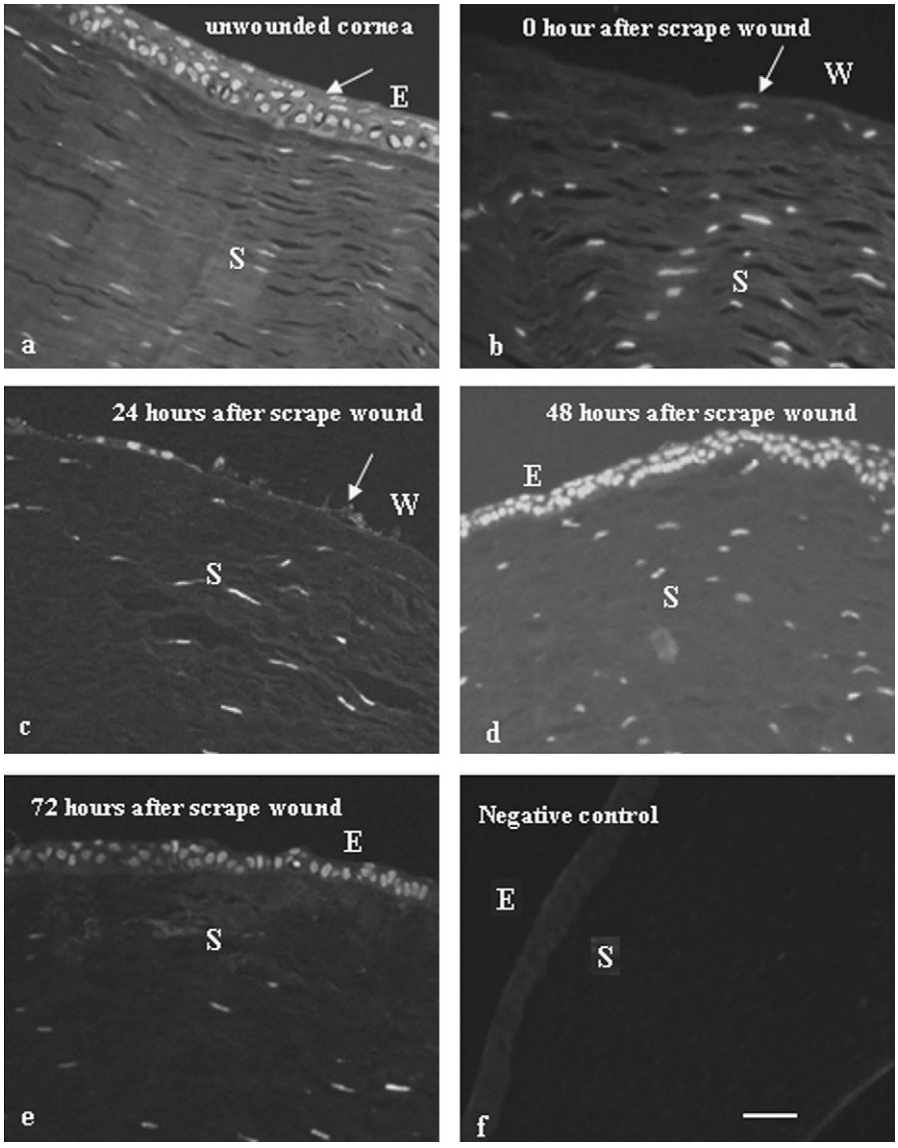

Fig. 4. Micrographs showing immunofluorescence in rabbit cornea after scrape wound stained for Hsp70. (a) Normal epithelium (unwounded), (b) at 0, (c) 24, (d) 48, (e) 72 h after wounding and (f) no immunopositivity was seen in the negative control. Scale bar, 20 μm. E, epithelium; W, wound; S, stroma.

be the important components of several aspects of epithelial cell migration. This also includes the cell shape changes, migration and transient adhesion of the sheet to underlying ECM, the ability of the sheet to remain intact and cohesive as it stretches and thins after migration is complete.

We believe that Hhsp70 appeared in response to wound healing because of its fundamental role in protein function like protein folding, degradation, assembly and transport. Among HSP27, HSP60 and Hsp70 family, only Hsp70 shows high expression in wound healing tissue (Oberringer et al., 1995) and is closely related to cell proliferation (Pechan, 1991) most probably by interacting with proteins needed for proliferation process or by modifying the activity of certain receptors. The presence of detectable levels of Hsp70 in corneal epithelium is also associated with cell stress because any stress response would alter the levels of Hsp70. It is likely that this characteristic may be related to the strong healing ability of the corneal epithelial cells, which can repair a wounded area in 72 h.

This study also supported our data, as an obvious accumulation of albumin precursor and Hsp70 proteins occurs in the superficial layer and basal cells of wounded epithelia at 48 h. This observation is consistent with notion that these proteins are secreted by epithelial cells and their strong localization observed in later stages of re-epithelialization indicates a novel role in desmosomal cytoskeleton during migration.

Cumulatively, this study also marks these proteins as putative targets for therapeutics in corneal epithelial regeneration.

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