# Expression of Phosphorylated Heat Shock Protein 27 During Corneal Epithelial Wound Healing

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## Abstract

**Purpose**—The 27-kDa heat shock protein (HSP27) has been implicated in wound healing in multiple tissues. We investigated the expression and localization of phosphorylated HSP27 during epithelial wound healing in the murine cornea.

**Methods**—Corneas of 8- to 10-week-old C57BL6 mice were wounded by epithelial debridement (n = 40). Unwounded corneas served as controls (n = 3). After 3, 7, and 14 days, phosphorylated HSP27 localization in wounded corneas was observed by confocal immunohistochemistry and double immunogold labeling transmission immunoelectron microscopy. Western blot analysis was performed to determine expression levels of phosphorylated HSP27 in scraped epithelia. Phosphorylated HSP27 localization was also separately performed with confocal immunohistochemistry 8 hours after epithelial debridement to investigate the early epithelial wound-healing process.

**Results**—In unwounded corneas, phosphorylated HSP27 was localized only to the superficial epithelium. In contrast, phosphorylated HSP27 was localized in the basal and superficial epithelia 3 days after corneal epithelial wounding. After 7 and 14 days, HSP27 localization was similar to that in unwounded controls. Expression levels of phosphorylated HSP27 were greater in wounded corneal epithelia on day 3 than in unwounded controls and on day 14. After 8 hours, phosphorylated HSP27 expression was prominent in the leading edge of migrating corneal epithelium.

**Conclusions**—Constitutive expression of phosphorylated HSP27 is limited to the superficial corneal epithelium in unwounded murine corneas. Changes in HSP27 epithelial distribution and expression levels after corneal epithelial wounding suggest that phosphorylated HSP27 plays a role in early phase of corneal epithelial wound healing.

## Keywords

heat shock protein; corneal epithelium; epithelial wound healing; cornea

Heat shock proteins (HSPs) are highly conserved and classified into 2 families according to molecular size. The larger HSP family includes HSP90, HSP70, and HSP60, and the smaller family consists of HSP27.[1] Human HSP27, also known as mouse HSP25, has various functions. Depending on expression and phosphorylation levels, HSP27 is an actin-capping protein in vitro and inhibits actin polymerization.[2,3] HSP27 also acts as a molecular chaperone to inhibit thermal denaturation and aid in the refolding of citrate synthase in vitro.[4] In vivo, HSP27 enhances pinocytosis, promotes endothelial cell migration, and provides resistance to cellular injury by stabilizing microfilaments after heat shock.[5-7] In addition, HSP27 maintains endogenous antioxidants, such as glutathione, to decrease damage induced by reactive oxygen species and also acts as an antiapoptotic mediator.[8-11] In murine inner retinal layers, HSP27 protected the retina from ischemic injury,[12] and in human corneal epithelium, HSP27 displayed rapid phosphorylation and translocation after ultraviolet-B irradiation.[13]

The biological activity of HSP27 varies depending on intracellular localization, phosphorylation status, and oligomerization level.[4-6,14,15] HSP27 phosphorylation is believed to play an important role in cell survival because it has been associated with the inhibition of Daxx-mediated apoptosis and Akt-mediated apoptosis.[16,17] Cells that overexpress phosphorylatable HSP27 are more resistant to heat and oxidative stress than are cells that express unphosphorylatable mutants.[16] In various cell types, HSP27 phosphorylation is required for cell migration, probably because of its influence on actin remodeling.[18-20]

In this study, we investigated the presence of phosphorylated HSP27 in murine corneal tissue and its role in corneal wound healing after full-thickness epithelial debridement.

## MATERIALS AND METHODS

Animals were handled in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Eight- to 10-week-old C57BL6 mice were divided into 2 groups: unwounded controls (n = 3) and those treated with epithelial debridement (n = 10, 2 per time point).

### Epithelial Debridement

Mice were anesthetized with intramuscular injection of 30 µL of 1:1 ketamine hydrochloride (100 g/mL; Hospira, Inc, Lake Forest, IL) and xylazine (20 mg/mL; X-JECT SA; Burns Veterinary Supply, Inc, Westbury, NY) and treated with topical 0.5% proparacaine eye drops (Akorn, Inc, Buffalo Grove, IL) to reduce pain. Full-thickness epithelial debridement was performed with a surgical blade after marking a 1.5-mm-diameter area in the central cornea with a 1.5-mm-diameter trephine. Two percent erythromycin ophthalmic ointment (Fougera; Fougera, Melville, NY) was applied once to the central corneal area. In the experimental group, mice were killed 0, 3, 7, and 14 days after epithelial debridement, and whole eyeballs were cryoblocked immediately. The controls were treated exactly the same as experimental subjects except for the debridement. To investigate the expression of phosphorylated HSP27 in early epithelial wound-healing process, an experimental subgroup of mice was separately killed 8 hours after epithelial debridement and their eyeballs were also cryoblocked.

### Confocal Immunohistochemistry

Confocal immunohistochemistry was performed on murine corneas as described previously.[21] Briefly, cryosections (8 µm) from mouse corneas were fixed in paraformaldehyde for 5 minutes. After blocking with 1% bovine serum albumin (BSA) in

phosphate-buffered saline (PBS, pH 7.4), sections were incubated overnight with primary rabbit polyclonal antibodies against nonphosphorylated HSP27 (ab12351; Abcam Inc, Cambridge, MA) or primary rabbit polyclonal antibody against phosphorylated HSP27 (ab5594; hsp27 phospho S85, phosphorylated at Ser85; Abcam, Inc). After washing with PBS, sections were incubated for 30 minutes with secondary antibodies and observed using a confocal microscope (TCS 4D; Leica Microsystems, Inc, Bannockburn, IL). Primary antibody omission served as a negative control.

## Transmission Immunoelectron Microscopy

Immunogold electron microscopy was performed using a rabbit polyclonal antibody against phosphorylated HSP27 (hsp27 phospho S85; Abcam, Inc) to localize phosphorylated HSP27. Corneal tissue was excised from limbus to limbus, washed 3 times in sodium phosphate buffer (pH 7.4), and fixed in 4% (vol/vol) paraformaldehyde and 1% (vol/vol) glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.4) for 16 hours at 4°C. Next, tissue samples were washed 3 times for 10 minutes per wash in phosphate buffer and dehydrated for 105 minutes in a graded series of ethanol solutions [25%, 50%, 75%, 95%, and 100% (vol/vol) with 3 changes of each at 15-minute intervals]. Tissue infiltration continued in 2:1 ethanol:LR White resin and then in 1:1 and 1:2 relative ratios of each for 1 day. Pure LR White resin infiltration was completed over a 24-hour period with 3 changes, and samples were polymerized in 1 mL of pure LR White resin under vacuum at 50°C for 3 days. Cured blocks were trimmed with a razor blade and sectioned with a diamond knife on a Rie-chert Ultra Cut E ultramicrotome (Leica Microsystems, Inc). Sections were lifted onto 200-hex-mesh carbon/parlodion–coated Ni grids and dried. Thin sections on the Ni grids were incubated for 45 minutes at room temperature in blocking solution [0.8% (wt/vol) BSA, 0.1% (wt/vol) immunogold–silver stain–quality gelatin, 5% (wt/vol) normal goat serum in PBS, and 50 μL of 2% azide], washed twice in 0.8% BSA, 0.1% gelatin, and 0.025% (vol/vol) Tween 20 in PBS, and incubated overnight with antiphosphorylated HSP27 antibody at a concentration of 2.6 μg/mL in 0.8% BSA, 0.1% gelatin, and 1% normal goat serum in PBS. Subsequently, sections were washed 4 times in washing solution and incubated for 4 hours with 10-nm immunogold-labeled secondary antibody (goat antirabbit IgG; GE Healthcare, Piscataway, NJ) diluted 1:25. Grids were washed 6 times with washing solution for 5 minutes per wash and then twice with PBS, and the samples were fixed for 10 minutes in 2% (vol/vol) glutaraldehyde in PBS. Next, grids were washed 3 times in distilled water, dried, stained with 2% (wt/vol) aqueous uranyl acetate for 45 minutes, washed with distilled water, and dried again. Finally, grids were stained with lead citrate for 5 minutes, washed with distilled water, dried, and photographed with a JEOL 1200 EX transmission electron microscope (JEOL, Ltd, Tokyo, Japan). Seven randomly selected cells were observed at each of 3 time points; gold particles were localized by morphological criteria and counted. Cross-sectional areas of cell wall and cell interiors were assessed by weighing traced-out photographs and comparing them with equivalent traced standard areas.

For double immunogold labeling transmission electron microscopy, the above procedure was followed, with the exception that goat antimouse nonphosphorylated HSP27 (sc-1048; Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and rabbit antimouse phosphorylated HSP27 (hsp27 phosphor S85; Abcam, Inc) were used as primary antibodies, and 25-nm immunogold-labeled donkey antigoat IgG (GE Healthcare) and 10-nm immunogold-labeled donkey antirabbit IgG (GE Healthcare) were used as secondary antibodies.

## Western Blot Analysis

Corneal epithelia were isolated from 10 mice (1 eye per mouse) 0, 3, and 14 days after epithelial debridement. The isolated epithelium was lysed in 100 μL of

radioimmunoprecipitation assay buffer mixed with 2 μL of protease inhibitor cocktail (Catalog No. P8340 and P7626, 1 μL each; Sigma-Aldrich, Inc, St Louis, MO) and 0.5 μL of sodium fluoride to block proteases and phosphatases and stored in refrigerator at −20°C, and the total protein concentration was estimated using the Pierce BSA Protein Assay Kit (Pierce Biotechnology, Inc, Rockford, IL). Equal amounts of protein were loaded on a sodium dodecyl sulfate–polyacrylamide gel for electrophoresis, and the electrophoresed proteins were transferred to Immobilon P membranes (Millipore, Co, Billerica, MA), blocked with 3% BSA for 60 minutes, and incubated with either rabbit anti-nonphosphorylated HSP27 antibody or rabbit antiphosphorylated HSP27 antibody. Blots were incubated with horseradish peroxidase donkey antigoat or antirabbit antibody (GE Healthcare). After washing with Tris-buffered saline–Tween-20 for 60 minutes, immunoblots were developed with an enhanced chemiluminescence reagent (PerkinElmer, Inc, Waltham, MA).

## RESULTS

### Constitutive Immunolocalization of Phosphorylated HSP27

Nonphosphorylated HSP27 was present in all epithelial layers of unwounded mouse corneas (Figs. 1A, B). Phosphorylated HSP27 was localized only in the superficial epithelium of unwounded corneas (Figs.1C, D). The immunogold electron microscopic photograph showed the presence of phosphorylated HSP27 within the superficial epithelium of unwounded central corneas (Fig. 1E).

### Immunolocalization of Phosphorylated HSP27 During Corneal Wound Healing

Phosphorylated HSP27 was not detected in wounded cornea tissue on day 0 because of the absence of epithelium (Figs. 2A, B). On day 3, the epithelialization was complete within the wounded corneas. Phosphorylated HSP27 was localized to the superficial and basal epithelial layers, and the expression intensity was similar in all epithelial layers (Figs. 2C, D). After 7 days, phosphorylated HSP27 expression in the basal epithelium was less than that in the superficial epithelium (Figs. 2E, F). After 14 days, phosphorylated HSP27 was localized only to the superficial epithelium as in unwounded corneas (Figs. 2G, H).

At 8 hours after epithelial debridement, the epithelialization was not complete, and phosphorylated HSP27 expression was prominent at the leading edge of the migrating corneal epithelium (Figs. 3A, B). Phosphorylated HSP27 in wounded central cornea was localized to the superficial and basal epithelial layers, and the staining intensity was similar in all epithelial layers (Figs. 3C, D). However, phosphorylated HSP27 in unwounded peripheral cornea was localized only to the superficial epithelium (Figs. 3E, F).

### Colocalization of Nonphosphorylated and Phosphorylated HSP27

Double immunogold labeling transmission electron microscopy revealed that the distribution of nonphosphorylated HSP27 remained unchanged in the superficial and basal epithelium over 3 time points. The distribution of phosphorylated HSP27 in the basal epithelium was sparse in unwounded corneas (Fig. 4D) and increased after 3 days (Fig. 4E) and was sparse again after 14 days (Fig. 4F), whereas that in the superficial epithelium did not differ as time went (Figs. 4A–C).

### Quantification of Nonphosphorylated and Phosphorylated HSP27

Western blot analysis demonstrated that the expression of phosphorylated HSP27 was increased in corneal epithelia at 3 days compared with that in unwounded epithelia and wounded epithelia at 14 days, whereas the expression of nonphosphorylated HSP27 was consistent at each time point (Fig. 5).

## DISCUSSION

The heat shock 27-kDa protein 1 (*HSPB1* gene) encodes the protein HSP beta-1, also called HSP27 in humans and HSP25 or HSP27 in mice. In mice, HSP25 and HSP27 are aliases for heat shock 27-kDa protein 1. The murine and human HSP27 share more than 80% primary sequence identity.[22] The similarity of murine and human HSP27 has been reported in the literature.[3,5,23,24] In this article, we have used HSP27 to denote the heat shock 27-kDa protein in consonance with published reports.

The biological activity of HSP27 varies with intracellular localization, phosphorylation status, and oligomerization level. Under unstressed conditions, HSP27 levels are usually low, and the protein exists as a large oligomeric unit of up to 800 kDa.[20] Under stress, HSP27 phosphorylation precedes an increase in HSP27 levels and changes the quaternary structure of HSP27 from a large 600- to 800-kDa homotypic multimer to dimers and monomers.[4,25] HSP27 phosphorylation occurs at several residues, Ser-15, Ser-78, Ser-82, Ser-85, and Thr-143[20,26-28] and is dependent on various kinases such as mitogen-activated protein kinase–activated protein kinase 2/3, protein kinase C, cyclic guanosine monophosphate-dependent protein kinase, and protein kinase D.[27,29-33] In this study, we postulated that a direct correlation exists between corneal epithelial wound healing and HSP27 phosphorylation in wild-type mouse cornea. HSP27 phosphorylation and upregulation are involved in the migration of canine tracheal myocytes, human airway epithelial cells, and bovine arterial endothelial cells.[7,19,34] Contractile proteins, such as actin, myosin, and microtubule, are important in cytoarchitectural remodeling and cell migration during wound healing. Serum-starved fibroblastic cells showed a dramatic decrease in filamentous actin (F-actin) content after exiting the growth cycle, and after readdition of serum or specific growth factors, microfilaments rapidly reformed beneath the membrane and reorganized into stress fibers that extended through the cytoplasm.[35,36] Actin polymerization/depolymerization occurs predominantly at the leading edge of fibroblastic cells and affects plasma membrane activities, such as ruffling, pinocytosis, extension of lamellipodia (a site of actin polymerization in fibroblastic cells), and cell motility.[35-38] This dynamic process is controlled in vivo by an actin-capping protein, HSP27, which inhibits actin polymerization. HSP27 phosphorylation also alters actin dynamics. Nonphosphorylated HSP27 monomers have been reported to inhibit the polymerization of actin.[2,3] Interestingly, the F-actin modulating activity of HSP27 is inhibited by HSP27 phosphorylation.[3] The phosphorylation of HSP27 also affects the generation of lamellipodia microfilaments structures that aid in cellular migration.[3,7,19] HPS27 phosphorylation-mediated microfilament reorganization plays a role in filament stabilization.[6,18,39] However, hydrogen peroxide–inducible clone (Hic-5), a binding protein of HSP27, also binds the focal adhesion structural protein vinculin and focal adhesion kinase.[40-42] Hic-5 reduces integrin-mediated cell migration by competing with paxillin in binding to sites on paxillin-binding proteins and also by inhibiting paxillin tyrosine phosphorylation.[43] HSP27 phosphorylation and expression affect cellular adhesion by controlling the availability of Hic-5.[44]

In our experiments, we showed that phosphorylated HSP27 was localized to the superficial epithelial layer of unwounded mouse corneas. Interestingly, increased HSP27 phosphorylation was observed 3 days after injury. This finding is consistent with that of Hirano et al[45] regarding the rat full-thickness skin defect model. Similar to the corneal wound, fibroblasts and myofibroblasts that are involved in skin wound healing are believed to be the source of a contractile force.[45,46] Myofibroblasts are observed in actively contracting tissues, such as granulation tissue and hypertrophic scar tissue.[47] HSP27 is highly phosphorylated in granulation tissue and wound edges.[45]

The expression pattern of phosphorylated HSP27 during corneal epithelial wound healing was noticeable. Phosphorylated HSP27 was expressed only in the superficial epithelium of unwounded corneas. In contrast, in wounded corneas after 3 days, phosphorylated HSP27 was localized to the superficial and the basal epithelial layer. After 7 days, phosphorylated HSP27 expression remained only in the superficial epithelium, and after 14 days, phosphorylated HSP27 was localized only to the superficial cell layers similar to its expression in unwounded corneas. At the early corneal epithelial wound-healing process (8 hours after epithelial debridement), phosphorylated HSP27 expression was prominent in the leading edge of migrating central corneal epithelium. During wound healing, HSP27 phosphorylation may play a role in cytoarchitectural remodeling and cell migration.

Stratified squamous epithelial cells assemble specialized protective surface barriers that are exposed to the environment and termed the cornified envelope.[48] The corneal epithelium is stratified, but unlike skin, it does not have a water-impermeable layer. Like skin, various envelope precursors are expressed in human corneal epithelium.[48] In this article, we showed that phosphorylated HSP27 is constitutively expressed in superficial epithelial cells of corneas that are most exposed to the environment. Given that phosphorylated HSP27 is known to possess antiapoptotic properties, its superficial localization suggests that it contributes to an innate corneal defense and corneal barrier. HSP27 is known to play a cytoprotective role through its antioxidative, antiapoptotic, and actin-stabilizing properties during cell stress. Further experimentation is required to determine the functional significance of HSP27 in ocular surface disorders and corneal epithelial wound healing. Moreover, constitutive expression of phosphorylated HSP27 could be a result of cellular stress from continuous insults from the environment.

In conclusion, constitutive expression of phosphorylated HSP27 was limited to the superficial corneal epithelium in unwounded murine corneas. Changes in HSP27 epithelial distribution and expression levels after corneal epithelial wounding proposed that phosphorylated HSP27 played a role in early phase of corneal epithelial wound healing.

## Acknowledgments

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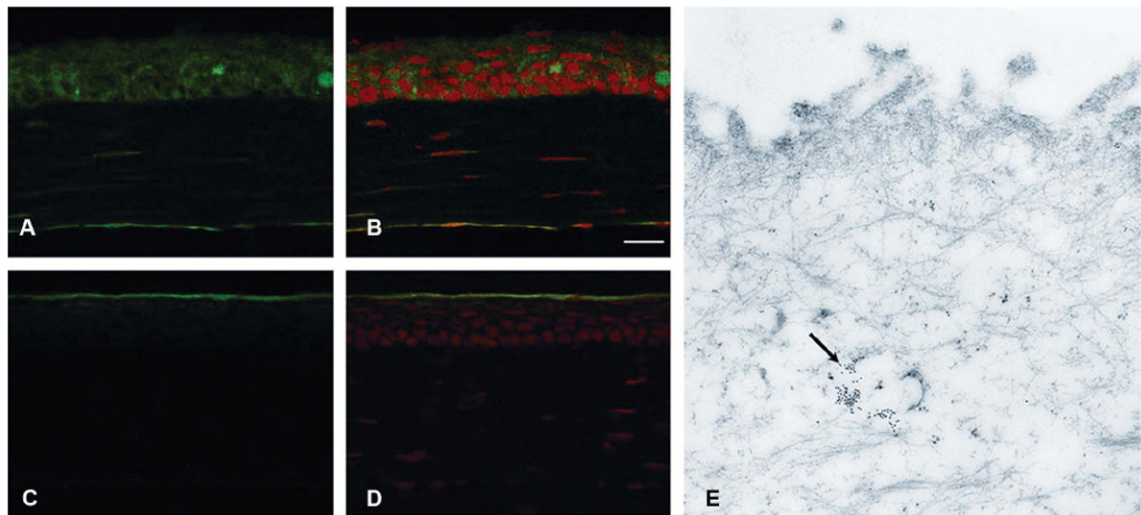
**FIGURE 1.**
Constitutive immunolocalization of nonphosphorylated HSP27 in unwounded corneas (A) and merged image with PI staining (B). Localization of phosphorylated HSP27 in unwounded corneas (C) and merged image with propidium iodide staining (D). Localization of phosphorylated HSP27 in unwounded corneas by immunogold electron microscopy (E, indicated with black arrow). Nonphosphorylated HSP27 was present in all epithelial layers of unwounded mouse corneas (A and B), whereas phosphorylated HSP27 was localized only in the superficial epithelium of unwounded corneas (C–E).
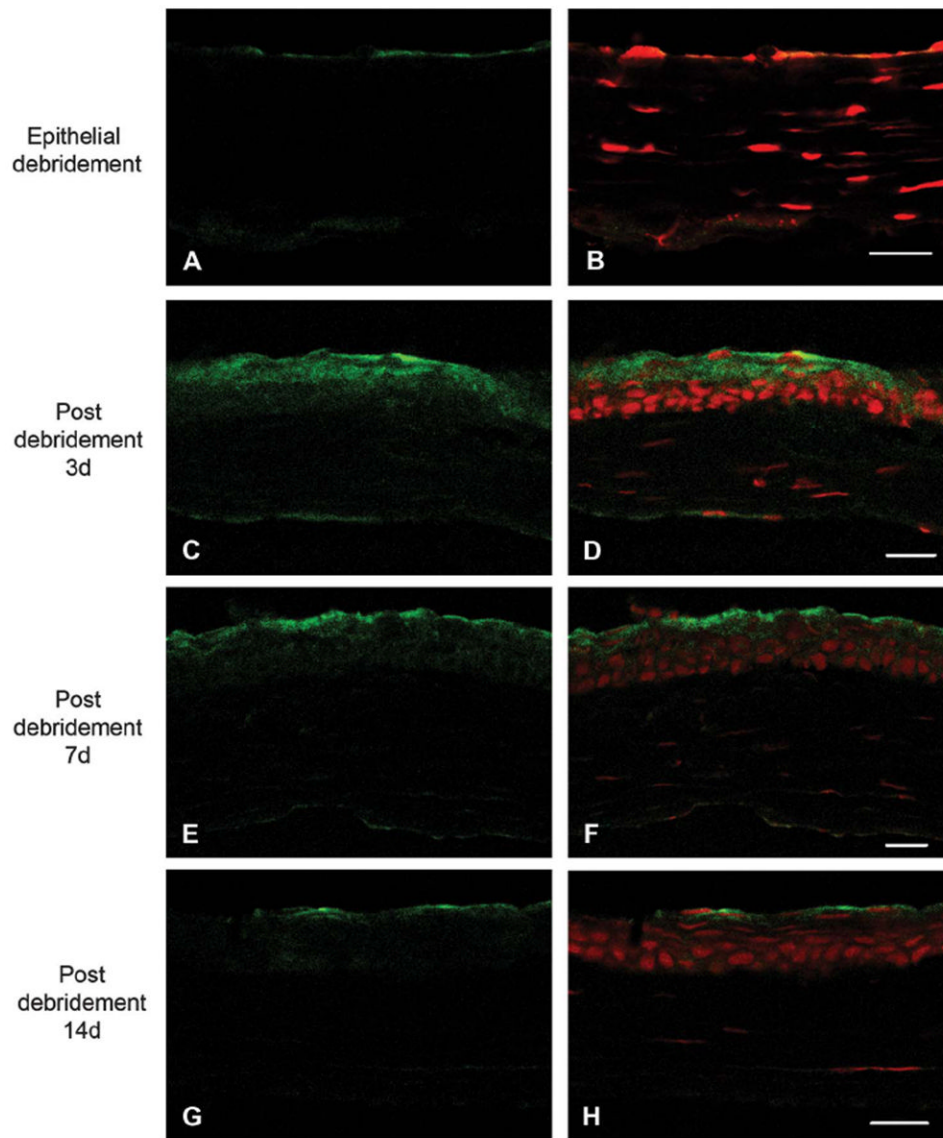
**FIGURE 2.**
Immunolocalization of phosphorylated HSP27 in wounded central cornea at 0 days (A), 3 days (C), 7 days (E), and 14 days (G) after epithelial debridement and merged images with propidium iodide staining (B, D, F, and H), respectively. Phosphorylated HSP27 was not detected on day 0 because of the absence of epithelium (A and B). On day 3, phosphorylated HSP27 was localized to the superficial and basal epithelial layers, and the expression intensity was similar in all epithelial layers (C and D). After 7 days, phosphorylated HSP27 expression in the basal epithelium was less than that in the superficial epithelium (E and F). After 14 days, phosphorylated HSP27 was localized only to the superficial epithelium as in unwounded corneas (G and H). Scale bar = 20 μm.
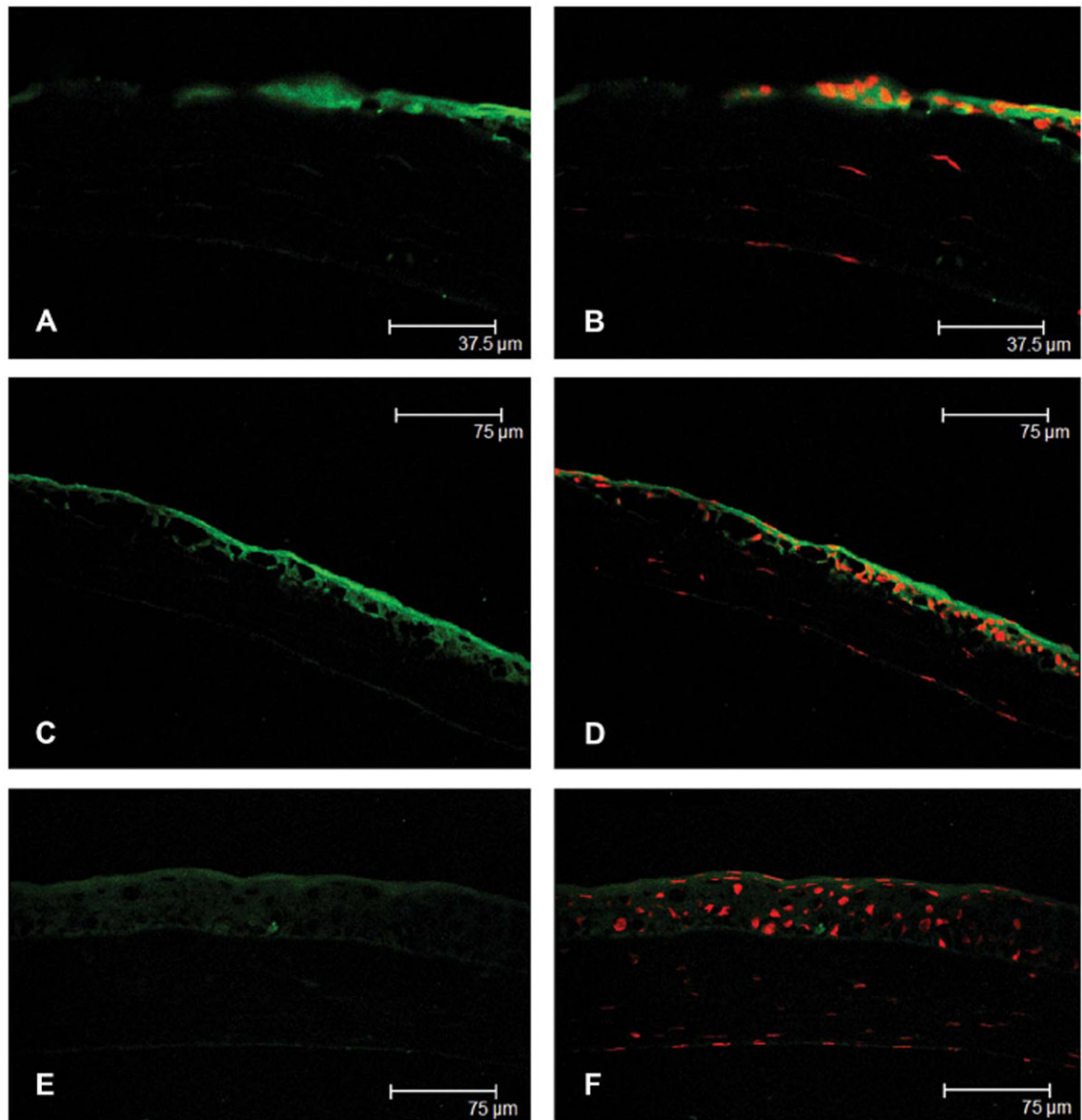
**FIGURE 3.**
Immunolocalization of phosphorylated HSP27 from the wounded central cornea (A and C) to the unwounded peripheral cornea (E) at 8 hours after epithelial debridement and merged images with propidium iodide staining (B, D, and F), respectively. The epithelialization was not complete, and phosphorylated HSP27 expression was prominent at the leading edge of the healing central corneal epithelium (A and B). Phosphorylated HSP27 in wounded central cornea was localized to the superficial and basal epithelial layers, and the staining intensity was similar in all epithelial layers (C and D). However, phosphorylated HSP27 in unwounded peripheral corneal was localized only to the superficial epithelium (E and F).
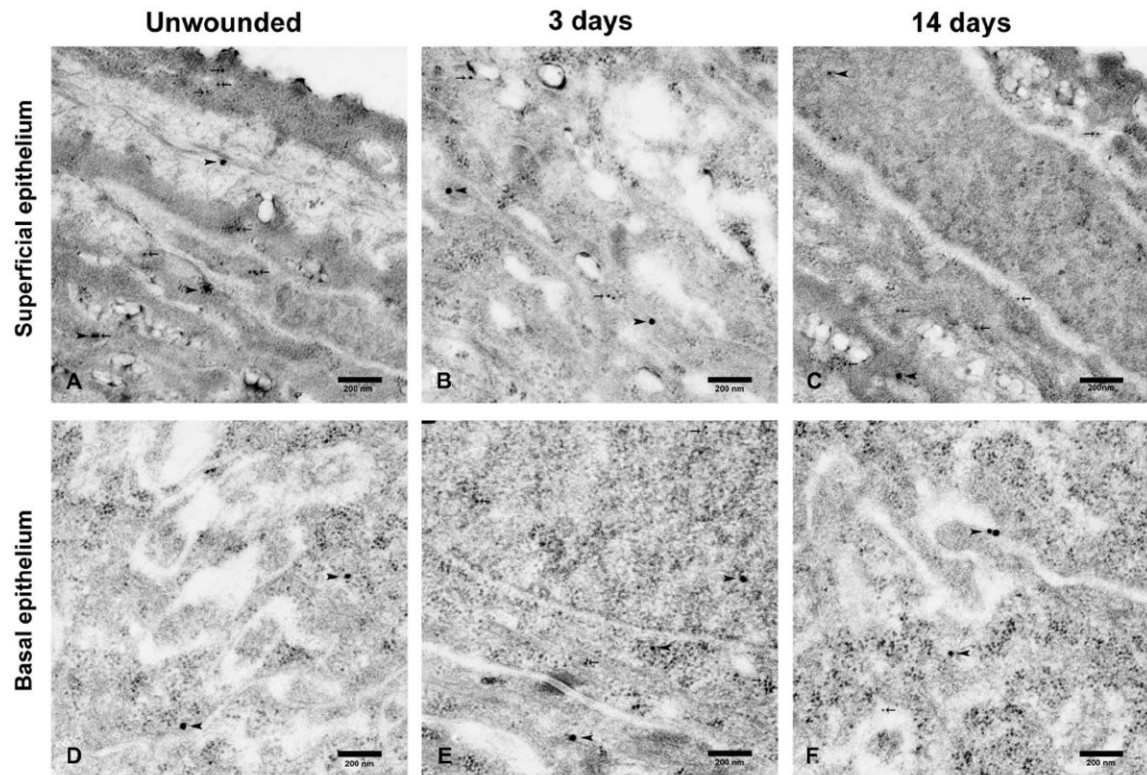
**FIGURE 4.**

Detection of nonphosphorylated HSP27, indicated by arrow head, and phosphorylated HSP27, marked by arrow, by double immunogold labeling transmission electron microscopy in unwounded superficial (A) and basal (D) epithelial layers, superficial epithelial layers of wounded corneas after 3 days (B) and 14 days (C), and basal epithelial layers of wounded corneas after 3 days (E) and 14 days (F). The distribution of nonphosphorylated HSP27 remained unchanged in the superficial and basal epithelium over 3 time points. The distribution of phosphorylated HSP27 in the basal epithelium was sparse in unwounded corneas (D) and increased after 3 days (E) and was sparse again after 14 days (F), whereas that in the superficial epithelium did not differ as time went (A–C).
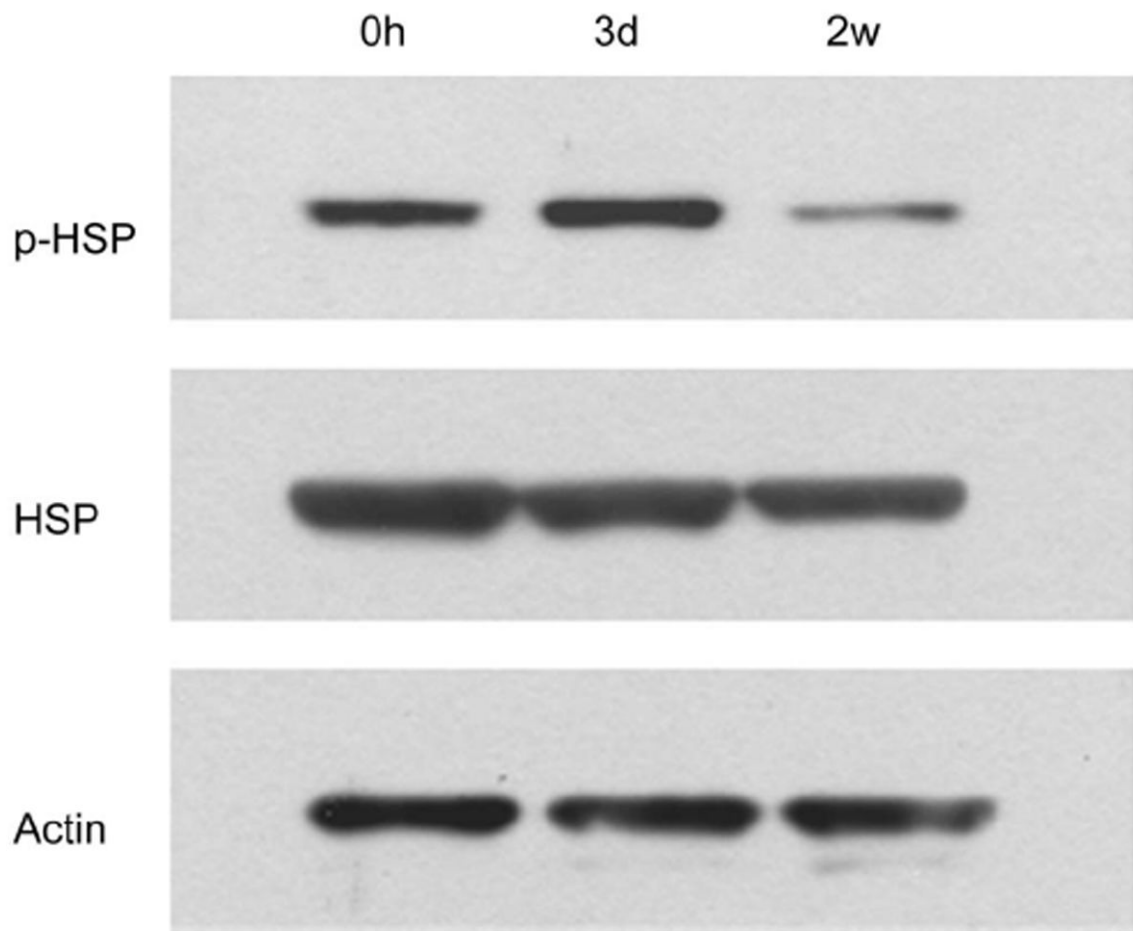
**FIGURE 5.**
Western blot analysis with antibodies against phosphorylated HSP27 and nonphosphorylated HSP27 in unwounded corneal epithelia and epithelia at 3 days and 14 days after epithelial debridement. The expression of phosphorylated HSP27 was increased in corneal epithelia at 3 days compared with that in unwounded epithelia and wounded epithelia at 14 days, whereas the expression of non-phosphorylated HSP27 was consistent at each time point.