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Heat shock protein 27 phosphorylation is involved in epithelial cell apoptosis as well as epithelial migration during corneal epithelial wound healing☆

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ABSTRACT

We reported the expression of phosphorylated HSP27 during epithelial wound healing in murine corneas (Jain et al., 2012) in July of 2012. This *in vivo* investigation demonstrated that the expression levels of phosphorylated HSP27 were greater in wounded corneal epithelial cells than in unwounded controls and that the localization of phosphorylated HSP27 was in the basal and superficial epithelia three days following corneal epithelial wounding. We suggested that phosphorylated HSP27 had a role in the early phase of corneal epithelial wound healing. The purpose of this study was to investigate the exact role of heat shock protein 27 (HSP27) phosphorylation for the wound healing of cultured human corneal epithelial cells (HCECs). HSP27-specific siRNAs and control-siRNAs, with no known homologous targets in HCECs, were created. The cultured HCECs were divided into two groups: Scrambled control-siRNA-transfected group vs. HSP27-specific siRNA-transfected group. The scratch-induced directional wounding assay, Western blotting, using antibodies against non-phosphorylated and phosphorylated HSP27, non-phosphorylated and phosphorylated Akt, and Bcl-2–associated X protein (Bax), immunofluorescence staining to determine the filament actin, flow cytometry to measure apoptosis, and proliferation assay were performed to determine the role of HSP27. Western blot assay showed that the expression of phosphorylated HSP27 significantly increased at 5, 10, and 30 min after scratch wounding, compared with those in unwounded HCECs (all $p < 0.05$). Western blot assay also showed HSP27-specific siRNAs effectively blocked the expression of non-phosphorylated HSP27. The HSP27-specific siRNA-transfected group had more Bax expression, less phosphorylated Akt expression, and less non-phosphorylated and phosphorylated HSP27 expression (all $p < 0.05$). The scratch-induced directional wounding assay showed the HSP27-specific siRNA-transfected group with a less migrating cell number than the control-siRNA-transfected group ($p < 0.05$). Immunofluorescence staining showed that reorganization of actin cytoskeleton prominently decreased in the HSP27-specific siRNA-transfected group, compared with the control siRNA-tranfected group. Flow cytometry revealed that the HSP27-specific siRNA-transfected group had more HCEC apoptosis. Proliferation assay showed no difference between the two groups. In conclusion, the role of HSP27 in corneal epithelial wound healing can be epithelial cell apoptosis, as well as epithelial migration. HSP27 is involved in HCEC migration by the reorganization of actin cytoskeleton.

1. Introduction

The corneal surface is the most specialized part of the body's surface. Corneal epithelial cells are continuously shed into the tear film and are simultaneously replaced by cells from the limbus and basal layers of the epithelium (Dua et al., 1994). Ultraviolet radiation, hypoxia, or mechanical stress can induce apoptosis and desquamation of corneal epithelial cells (Estil et al., 2000).

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Heat shock protein 27 (HSP27) has a strong protective activity against a number of cytotoxic agents, including heat shock, oxidative stress, and chemotherapeutic agents (Huot et al., 1996). Cells that over-express phosphorylatable HSP27 are more resistant to heat and oxidative stress than cells that express unphosphorylatable mutants (Charette et al., 2000). A phosphorylation of HSP27 modifies the equilibrium in favor of polymerized actin, thereby contributing to the maintenance of the microfilament network and could thus affect cellular physiology, such as cell-to-cell interaction and cell migration, proliferation, and secretion (Guay et al., 1997). HSP27 phosphorylation is believed to have an important role for cell survival, because it has been associated with the inhibition of Daxx-mediated apoptosis and Akt-mediated apoptosis (Charette et al., 2000; Rane et al., 2003). In July of 2012, the authors reported the expression of phosphorylated HSP27 during epithelial wound healing in murine corneas (Jain et al., 2012). This *in vivo* investigation demonstrated that the expression levels of phosphorylated HSP27 were greater in wounded corneal epithelial cells than in unwounded controls, and that the localization of phosphorylated HSP27 was in basal and superficial epithelia three days following corneal epithelial wounding. The authors suggested that phosphorylated HSP27 had a role in the early phase of corneal epithelial wound healing. Therefore, *in vitro*, we attempted to demonstrate the potential role of HSP27 phosphorylation in cell migration, proliferation, and apoptosis inhibition in human corneal epithelial wound healing.

2. Methods

2.1. Cell line

Telomerase-immortalized human corneal epithelial cells (HCECs) were kindly given to us by Dr. James V. Jester, Gavin Herbert Eye Institute, University of California Irvine, CA. The cells were serum-starved for cell cycle synchronization and cultured at 37 °C in a 5% CO_2 atmosphere in bronchial epithelium growth medium (BEGM; Lonza, Walkersville, MD) containing human corneal growth supplement. They were sub-cultured with 0.25% trypsin-EDTA every three to four days and were then used in this study.

2.2. Western blot analysis to measure the expression of HSP27 after wounding

The confluently cultured HCECs were wounded by dragging a sterile 200 μl pipette tip across the surface of well. Four scratch wounds were created per dish and the wounded monolayers were washed three times with BEGM media. And then 2.0 mL of BEGM media was added to each culture. At 1, 5, 10, 30, 60, and 120 min after the corneal epithelial wounding, HCECs were extracted using ice cold lysis buffer (10 mM Tris, 10 mM NaCl, 2 mM EDTA, 25 mM NaF, 2 mM Na3VO4, 1 mM PMSF, protease inhibitors, 0.5% Triton X-100, and pH 7). They were mixed with 2 mL of protease inhibitor cocktail (Catalog No. P8340 and P7626; 1 mL each; Sigma–Aldrich, Inc, St. Louis, MO) and 0.5 mL of sodium fluoride to block proteases and phosphatases. Cell lysates were rocked on ice for 30 min and then pelleted at 13,000 rpm for 15 min. The supernatant was aliquoted and stored at −80 °C. Protein concentrations in the cell lysates were determined using the Bradford protein assay (BioRad Laboratories, Hercules, CA). For Western blot assay, samples with equal amounts of cell proteins were loaded into a 10% or 12% acrylamide gel and SDS–PAGE and were electrophoretically transferred to nitrocellulose filters (Amersham, Little Chalfont, UK). After 1-h blocking in 5% non-fat dry milk and 0.1% Tween 20, the blots were treated with primary rabbit polyclonal antibodies against primary rabbit polyclonal antibodies against non-phosphorylated HSP27 (1:1000 dilution; ab12351; Abcam Inc, Cambridge, MA) or primary rabbit polyclonal antibody against phosphorylated HSP27 (1:1000; ab5594; hsp27 phospho S85, phosphorylated at Ser85; Abcam, Inc). After three washes with Tris-buffered saline with 0.1% Tween-20 for 10 min each wash, the membranes were incubated with horseradish peroxidase-conjugated anti-IgGs (1:10,000). The specific bands were visualized using enhanced chemiluminescence reagents (Santa Cruz Biotechnology, Inc., Dallas, TX).

2.3. siRNA transfection assay

The HSP27-specific siRNA and a control-siRNA with no known homologous targets in HCECs were designed by Invitrogen, Corp., Carlsbad, CA. The sequence for HSP27-specific siRNA was 5′-AAA-TACCCGACTGGAGGAGCATAAA-3′. The final concentration of siRNA used was determined according to that described in the siRNA Transfection Kit (Invitrogen, Corp.). The HCECs were plated in six-well plates at 5×10^5 cells/well and were transfected with 10 nM and 50 nM of HSP27-specific siRNAs and control-siRNAs using 2.5 μl and 7.5 μl lipofectamine RNAiMax (Invitrogen, Corp.) in OPTI-MEM. After four hours, the cells were changed in complete BEGM media. At 48 h post-transfection, silencing was confirmed by HSP27 protein expression.

2.4. Western blot assay for siRNA-transfected cells

The HSP27-specific siRNA-transfected and control siRNA-transfected HCECs were extracted as described above. The blots

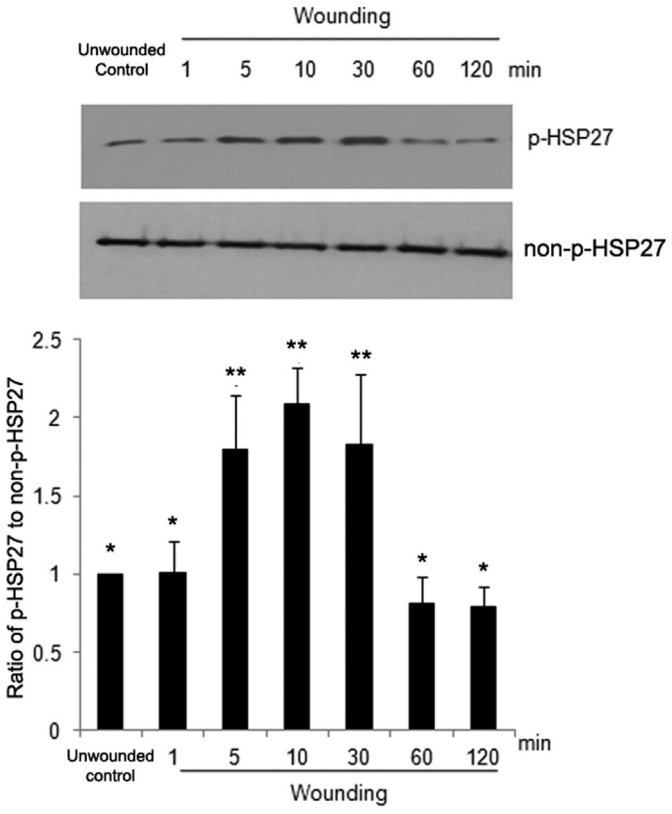

Fig. 1. Western blot analysis with antibodies against phosphorylated and non-phosphorylated HSP27 at 1, 5, 10, 30, 60, and 120 min after the corneal epithelial wounding. The expression of phosphorylated HSP27 significantly increased at 5, 10, and 30 min after wounding, compared with those in unwounded HCECs (all $p < 0.05$). The HSP27 phosphorylation increased at 5 min after wounding, peaked at 10 min, and returned to normal at 60 min. However, the expression of non-phosphorylated HSP27 did not change for 60 min. **, *: a statistically significant difference among groups ($p < 0.05$).