Effect of Hypoxic Stress–Activated Polo-like Kinase 3 on Corneal Epithelial Wound Healing

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Purpose. Hypoxia/reoxygenation conditions can generate oxidative stresses resulting in the suppression of cell proliferation and the delay of corneal epithelial wound healing. The purpose of this study was to investigate the cellular mechanism involving the role of the stress-responsive Polo-like kinase 3 (Plk3) in hypoxic stress–induced delay of corneal epithelial wound healing.

Methods. Plk3 activities were determined by immunochemistry and immunocomplex kinase assay approaches. Corneal epithelial wound healing was evaluated by a whole-eye organ culture model and by scratch-induced wound closure assay. Corneal epithelial layer was removed by using a corneal rust-ring-remover in wild-type and $Plk3^{-/-}$ mice. Wound healing was analyzed using a confocal imaging system. Cell growth was measured by MTT assays.

Results. The effect of hypoxic stress on early stages of corneal epithelial wound healing was compared with other oxidative stresses, including UV, $CoCl_2$, and H_2O_2 treatments. Hypoxic stress–induced delay of corneal epithelial wound healing was further evaluated in human corneal epithelial cells and in the corneas of wild-type and Plk3 knockout ($Plk3^{-/-}$) mice. Hypoxic stress–induced Plk3 activation resulted in growth attenuation and delay of wound healing. Further evidence demonstrated that the increase in Plk3 activity in constitutively active Plk3-expressed cells significantly enhanced stress-induced delay of wound healing. In contrast, hypoxic stress–induced delay of wound healing was markedly diminished in the corneas of Plk3 deficient $Plk3^{-/-}$ mice.

Conclusions. These results provide for the first time important evidence that Plk3 plays a significant role in hypoxic stress–induced attenuation of cell growth and delay of corneal epithelial wound healing. (*Invest Ophthalmol Vis Sci.* 2010;51:5034–5040) DOI:10.1167/iovs.10-5501

The corneal epithelial layer acts as a physical barrier and forms a line of defense to prevent noxious agents from infecting this tissue. When injuries occur in the surface of the cornea, the corneal epithelium undergoes a wound healing process.[1,2] Environmental stresses, such as hypoxia/reoxygenation, UV irradiation, and infections can interrupt the wound healing process. Deprivation of oxygen in tissues (hypoxia) is usually caused by vascular deficiency or physical isolation from oxygen sources. Hypoxia and reoxygenation occurring in the corneal epithelium in many situations includes contact lens wear during the day and removal of the lenses at night[3,4]; vascular dysfunction in diabetes[5]; preservation of the cornea and corneal epithelial cells in storage conditions[6,7]; corneal inflammation caused by noxious agent infection[8,9]; and other extreme environmental conditions.[10–12] In general, hypoxia can be found in many corneal disease stages and is considered one of the stress stimuli that induce cellular responses directly related to cell survival and function. Hypoxia leads to pathologic conditions in the cornea, such as neovascularization, reepithelialization attenuation, and apoptosis.[8,9,13–15]

Environmental stress–induced cellular responses, including activation of signaling events and genes, are important for the control of cell cycle progression and cell survival.[8,9,13–15] These cellular responses interfere with the balance between corneal epithelial proliferation, differentiation, and death.[2,16–21] There are transcriptional changes in hypoxia-responsive genes by activator protein 1 (AP-1) and hypoxia-inducible factor 1 (Hif-1).[22–25] The transcription factor AP-1 is a member of the basic leucine zipper (bZIP) family composed of either homodimer or heterodimer protein of transcription factors, such as c-*Fos*, c-*Jun*, *ATF-2*, and *JDP-2*.[26] A recent report indicates that *ATF-2* and c-*Jun* prefer to form bZip of the heterodimer ATF-2/c-Jun and not the homodimers ATF-2/ATF-2 and c-Jun/c-Jun.[27] AP-1 regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, stress, and bacterial and viral infections. AP-1, in turn, controls a number of cellular processes including differentiation, proliferation, and apoptosis. For example, a heterodimer formed by *c-Jun* and *c-fos* is required for binding to DNA sequences (transcription response elements) in the promoter region of many genes that are involved in regulating cell fate. Recent studies suggest that hypoxic stress activates MAP kinases, including *c-Jun* N-terminal kinase (JNK), which may subsequently activate *c-Jun* and interact with *Hif-1*.[28–31] Hif-1 is a transcription factor that consists of Hif-1α and Hif-1β subunits that accumulate under hypoxic conditions and are constitutively expressed, respectively.[32,33] Under the normoxic condition, Hif-1α is hydroxylated on both 402 and 604, and 803 is catalyzed by both prolyl hydroxylase (PHD) and factor inhibiting *Hif-1* (FIH-1). Hydroxylated HIF-1α is recognized by VHL protein, a ubiquitin E3 ligase, for polyubiquitination and subsequent degradation by the proteasome.[33]

Plk3 is a multifunctional protein that is rapidly phosphorylated in response to various stresses, including ionizing radiation (IR), reactive oxygen species (ROS), and UV irradiation involving stress-induced signaling pathways in fibroblasts, lymphoblastoids, lung carcinoma, and corneal epithelial cells.[34–36] Plk3 is predominantly localized around the nuclear membrane and involved in the regulation of cell cycle progression.[37]

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During mitosis, Plk3 appears to be localized to mitotic apparatuses such as spindle poles and mitotic spindles.[38] Expression of a constitutively active Plk3 results in rapid cell shrinkage, frequently leading to the formation of cells with an elongated, unsevered midbody. Intriguingly, ectopic expression of both constitutively active Plk3 and kinase-defective $Plk3^{K52R}$ mutant induces apparent G_2/M arrest followed by apoptosis.[39,40] A previous study[41] demonstrates that Plk3 mediates stress-induced signaling processes and AP-1 activation in corneal epithelial cells. Plk3 and *c-Jun* proteins are colocalized in the nuclei of UV irradiation and hypoxia-stimulated corneal epithelial cells. In fact, Plk3 stimulated by UV irradiation and hypoxic conditions directly phosphorylates *c-Jun*, resulting in apoptosis.[41,42] We have shown that environmental stresses including hypoxic conditions and UV irradiation activate the Polo-like-kinase 3 (Plk3) to phosphorylate *c-Jun*, resulting in corneal epithelial cell growth attenuation and apoptosis.[2,41–43] Therefore, determining how environmental stress affects corneal epithelial growth, differentiation, and apoptosis is relevant to identifying potential strategies for promoting its essential function in vision.

In the present study, the functional role of Plk3 in corneal epithelial cells was further investigated under various environmental stresses. Based on an understanding of the biochemical interactions of Plk3 and c-*Jun* in the hypoxic signaling pathway, we studied the effect of Plk3 activation on corneal epithelial wound healing. Hypoxic stress significantly delayed corneal epithelial wound healing in corneal epithelial cells and corneas of wild-type mice. However, the effect of hypoxic stress on delaying wound healing was markedly suppressed in the corneas of $Plk3^{-/-}$ knockout mice. In contrast, increased Plk3 activity in corneal epithelial cells enhanced hypoxic stress–induced suppression of proliferation and wound healing.

Materials and Methods

Culture of Corneal Epithelial Cells

Primary human corneal epithelial (PHCE) cells and human telomerase-immortalized corneal epithelial (HTCE) cells were cultured in a serum-free keratinocyte medium (Defined Keratinocyte-SFM; Invitrogen, Carlsbad, CA), and human corneal epithelial (HCE) cell line were grown in DMEM/F-12 (1:1) culture medium containing 10% FBS and 5 μg/mL insulin in an incubator supplied with 95% air and 5% CO_2 at 37°C. The medium was replaced every 2 days, and cells were subcultured by treatment of cells with 0.05% trypsin-EDTA. For hypoxia induction experiments, 90% confluent corneal epithelial cells were placed in a hypoxic incubator supplement of 1% O_2, 5% CO_2, and 94% N_2 at 37°C for 6 hours (BioSpherix, Lacona, NY). For UV irradiation experiments, corneal epithelial cells were exposed to UV-C light under a culture hold at a dosage of 42 mJ/cm^2 (Spectronics Corporation, Westbury, NY). Plk3 kinase mutant pEGFP-Plk3-PBD was constructed previously by using site-directed mutagenesis to replace the wild-type amino acid sequence with a constitutively active pEGFP-Plk3-Polo box domain (amino acids 312–652).[40,44] The cDNA encoding a full-length of pEGFP-Plk3-PBD was transfected to human corneal epithelial cells for 16 hours by using a lipofectamine-mediated method (Invitrogen).

Cell Proliferation Index Determined by MTT Assays

An established protocol for tetrazolium component (MTT) assay was performed in the laboratory.[45] The MTT cell proliferation assay used in the experiments was a colorimetric system that measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells. One hour before cell harvest, the culture medium was replaced by 1 mL fresh and serum-free medium, and 100 μL MTT solution (5 mg/mL in PBS) was added into each well. Corneal epithelial cells were incubated in a CO_2 incubator at 37°C for 1 hour. For the purpose of solubilizing the colored crystals, the medium was replaced by 0.4 mL acidic isopropanol (0.04 M HCl in absolute isopropanol). MTT was changed to blue because of the mitochondrial dehydrogenase. All samples in an ELISA plate reader (Labsystems Multiskan MCC/340; Fisher Scientific, Pittsburgh, PA) were with a spectrophotometer (DU-600; Beckman Coulter, Hialeah, FL) with a wavelength of 570 nm and a background subtraction at 650 nm. The amount of color produced normalized with the background was directly proportional to the number of viable cells and represented the proliferation index.

Immunocytochemistry Experiments

Immunoprecipitation and kinase assays were carried out with Plk3 antibodies and *c-Jun* substrate protein. Cells (5×10^7) were incubated in 1 mL lysis buffer (20 mM Tris (pH 7.5), 137 mM NaCl, 1.5 mM, 2 mM EDTA, 10 mM sodium pyrophosphate, 25 mM glycerophosphate, 10% glycerol, 1% Triton X-100, 1 mM Na-vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL leupeptin) on ice for 10 minutes. The immunocomplex was recovered by incubation with 50 mL of 10% protein A Sepharose (expanded and stocked at 0.1 mg/mL in 20 mM HEPES, pH 7.5). A two-step immunocomplex kinase assay was applied to separate JNK and Plk3 proteins in immunoprecipitation experiments. First, JNK-1 proteins in cell lysates from hypoxia- and UV-induced cells were removed by immunoprecipitation. Second, Plk3 was further immunoprecipitated from the cell extracts in the JNK-1 protein that had been depleted. Kinase assays were carried out by incubation of *c-Jun* substrate 20 μM ATP, 10 μCi [^{32}P]-ATP, and immunocomplex in a 30-μL kinase buffer for 10 minutes at room temperature (RT). Samples were boiled for 5 minutes and loaded onto 5% to 15% SDS-PAGE, and the gel was run at the 120 V. After drying, the gels were exposed on X-ray film for approximately 5 minutes. Western blot analysis was performed by lysing corneal epithelial cells (2×10^5) in sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) sample buffer that contains 62.5 mM Tris-HCl, pH 6.8, 2% wt/vol SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% wt/vol bromophenol blue or phenol red. Proteins in cell lysates were denatured by boiling for 5 minutes and size-fractionated in 10% PAGE gels. Proteins in PAGE gels were electrotransferred to polyvinylidene difluoride (PVDF) membranes by using a semidry gel-transferring apparatus (Bio-Rad, Hercules, CA). The PVDF membranes were exposed to the blocking buffer containing 5% nonfat milk in TBS-0.1% Tween 20 (TBS-T) for 1 hour at RT and then were incubated with the primary antibodies at 4°C overnight. Horseradish peroxidase–conjugated secondary antibody was applied in TBS-T buffer for 1 hour at RT. Western blot analysis was developed by enhanced chemiluminescence (ECL Plus System; Santa Cruz Biotechnology, Santa Cruz, CA) and visualized by exposure of X-ray films.

Corneal Wound Healing Assays

Corneal epithelial wound healing assays were performed by two protocols. One was the scratch-induced directional wound healing assay. This assay protocol was performed in HCE cells grown in DMEM-F12 medium. Cross-stripe scrapes were made at 700 μm apart using a yellow pipette tip. Wound closure was measured using an inverted Nikon microscope with a fine measuring scale after a time course. The second was a whole-eye organ culture model: this organ level protocol was performed to measure corneal epithelial wound healing using cultured mouse corneas. Under a dissecting microscope, the corneal epithelial layer was debrided without damaging the Bowman's membrane using a corneal rust ring remover with a 0.5-mm burr (Algerbrush; The Alger Company, Inc., Lago Vista, TX). The whole eye was dissected and placed in culture wells (the corneas facing up) with the medium containing 10% fetal bovine serum and 1% antibiotic/antimycotic solution at 37°C and 5% CO_2 in a humidified incubator. The rate of epithelial healing in whole-eye organ culture was measured imme-

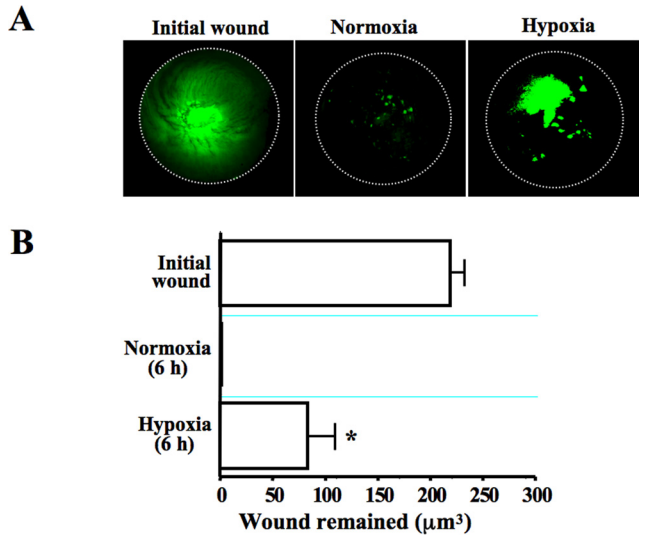

FIGURE 1. Hypoxic stress–induced delay of wound healing. (**A**) Corneal epithelial wound healing determined in normoxic and hypoxic conditions. (**B**) Significant delay of wound healing in hypoxic stress–induced corneal epithelium. Corneal epithelial débridements were made in the defined area of the mouse cornea by using a corneal lesion with an Algerbrush corneal rust ring remover and a 0.5-mm burr and then were stained by fluorescein (1.0% wt/vol). Delayed wound healing of mouse corneal epithelia was determined by comparing control and stress-treated groups 6 hours after wounding. Values represent mean ± SEM from four paired mouse corneas. $^*P < 0.01$, Student's *t*-test.

diately after wounding. The eyeballs were taken from wild-type or Plk3-knockout mice and allowed to heal in the culture condition. The mouse corneal epithelial layer was removed in an area 0.5 mm in diameter near the central cornea. Wounded corneas were incubated for 6 hours in the normal and stress-induced conditions. Lesions of the eyes were topically stained with fluorescein (fluorescein sodium 1.0% wt/vol) and photographed with a confocal microscope (Leica). All animals used in our experiments were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, using protocols approved and monitored by the Animal Care Committee of LABioMed at the University of California School of Medicine.

Statistical Analysis

For Western blot analysis, signals in the films were scanned digitally, and the optical densities were quantified with Image Calculator software. Data were shown as fractions of original values in MTT assay, the wound closure in the culture dishes, or 3-dimensional volumes of the measured wounding region (mean ± SD). Significant differences between the control group and the treated groups were determined by one-way ANOVA and Student's *t*-test at $P < 0.05$.

RESULTS

Hypoxic Stress–Induced Delay of Corneal Epithelial Wound Healing

We investigated hypoxic stress–induced effects on mouse corneal epithelial wound healing. Debridement of corneal epithelial cells was made in an area of 0.5 mm in diameter near the center of the cornea. The corneas were incubated for 6 hours in the absence (for controls) and presence of hypoxic stress. There was delayed healing in corneas that were exposed to hypoxic conditions containing 1% oxygen at 37°C (Fig. 1A). In control corneas, wounding areas were completely healed in 6 hours. However, there was average 30% remained staining area in hypoxia-treated corneas (Fig. 1B). The results demonstrate that environmental stresses such as hypoxia and UV irradiation affect the corneal epithelial wound healing process in the cultured mouse cornea.

Effect of Hypoxic Stress on Wound Closure

The effect of hypoxic stress on wound closure was measured by comparing cells grown in normoxic and hypoxic conditions. There were markedly hypoxic stress–induced delays of wound closure observed in 6 hours for HCE and HPCE cells and in 12 hours for slow-growing HTCE cells (Fig. 2). Time courses of various stress (hypoxia, UV irradiation, $CoCl_2$, and H_2O_2)-induced wound closures were also determined in HCE cells. Hypoxic conditions significantly inhibited cell migration within 2 hours after wounding (Fig. 3A). In UV-exposed HCE cells, different dosages of UV irradiation suppressed cell migration within 3 hours after wounding (Fig. 3B). In the meantime, treating HCE cells with $CoCl_2$ and H_2O_2 induced dose-dependent responses in the suppression of wound closures, respectively (Figs. 3C, 3D). These results indicate that the corneal

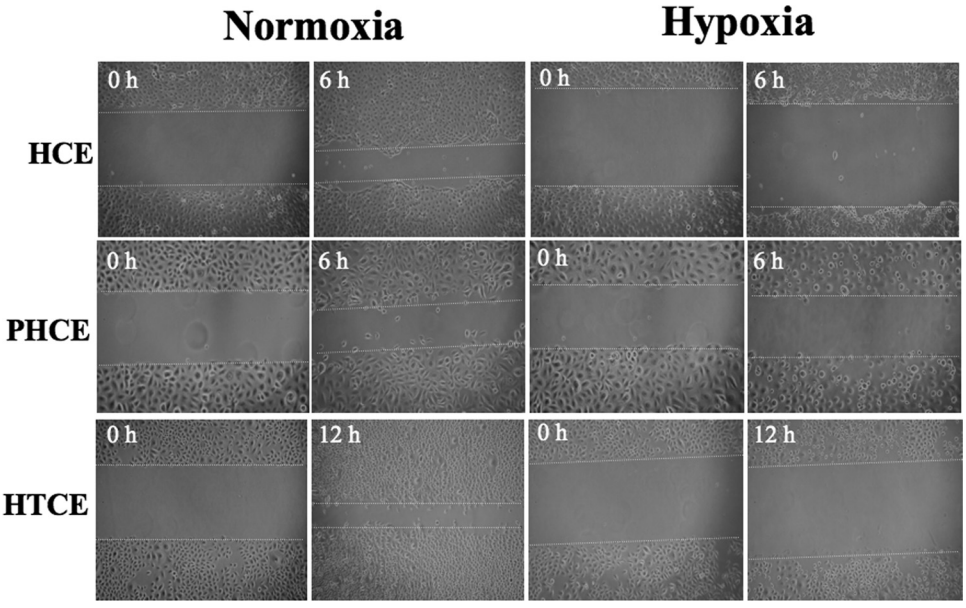

FIGURE 2. Effect of hypoxic stress on wound closure of corneal epithelial cells. Three types of cultured human corneal epithelial cells were studied in the absence (normoxia) and presence of hypoxic stress. Corneal epithelial cells were grown in their respective normal culture conditions for 48 hours to reach 90% confluence. Wounds were made by linear scrape of an average 700 μm in width using a 200-μL yellow pipette tip. The cells were washed with PBS to move debris and were refed with the fresh medium. The width of wounding areas was measured at 6 hours for PHCE cells and the HCE cell line and at 12 hours for slow-growing HTCE cells. Pictures were taken under an inverted microscope (×20).

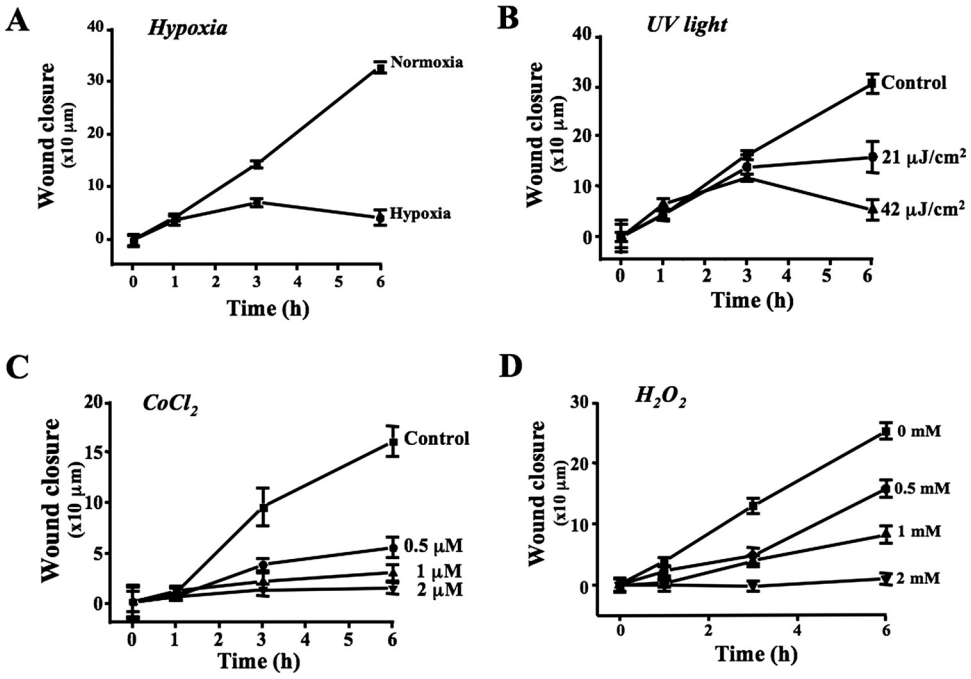

FIGURE 3. Effect of various stress stimuli on corneal epithelial cell wound closure. (**A**) Effect of hypoxic stress on wound closure. (**B**) Effect of UV irradiation on wound closure. (**C**) Effect of $CoCl_2$-induced hypoxia on wound closure. (**D**) Effect of H_2O_2 on wound closure. HCE cells were grown to reach 90% confluence, and wounds were made by linear scrape of an average 700 μm in width. Cells were washed with PBS to remove debris and were refed with the fresh medium. The width of wounding areas was measured at the indicated time. Control and wounded cells were exposed to hypoxic stress with 1% oxygen, UV light of various dosages (21–42 $\mu J/cm^2$), $CoCl_2$ (0.5–2.0 μM), and H_2O_2 (0.5–2.0 mM), respectively. Data are plotted as mean ± SD (in triplicate, repeated three times).

epithelial wound healing process is interrupted by hypoxia and other oxidative stresses.

Hypoxic Stress–Induced Activation of Plk3 and Growth Attenuation

A previous study demonstrates that the hypoxic stress–induced phosphorylation of *c-Jun* is mediated by the activation of Plk3 in addition to the JNK signaling pathway.[41] However, the functional role of Plk3 activation on corneal epithelial wound healing in response to hypoxic stress has not been revealed. In the present study, we focused on the role of Plk3 in corneal epithelial wound healing in response to oxidative stress. The effect of hypoxic stress–induced Plk3 activation was determined by a two-step immunocomplex kinase assay. Activities of the precipitated Plk3 from the extracts were determined by immunocomplex kinase assays, and c-Jun fusion protein was used as a substrate. We found that hypoxic and UV stresses were able to activate Plk3, resulting in c-Jun phosphorylation without interference from JNK activity (Figs. 4A, 4B). Plk3 activity was also found to be significantly increased in $CoCl_2$- and H_2O_2-treated HCE cells although $CoCl_2$ and H_2O_2 treatments had weaker effects on Plk3 activation compared with effects of hypoxic and UV stimulation (Fig. 3C). In addition, oxidative stresses induced significant attenuations of HCE cell

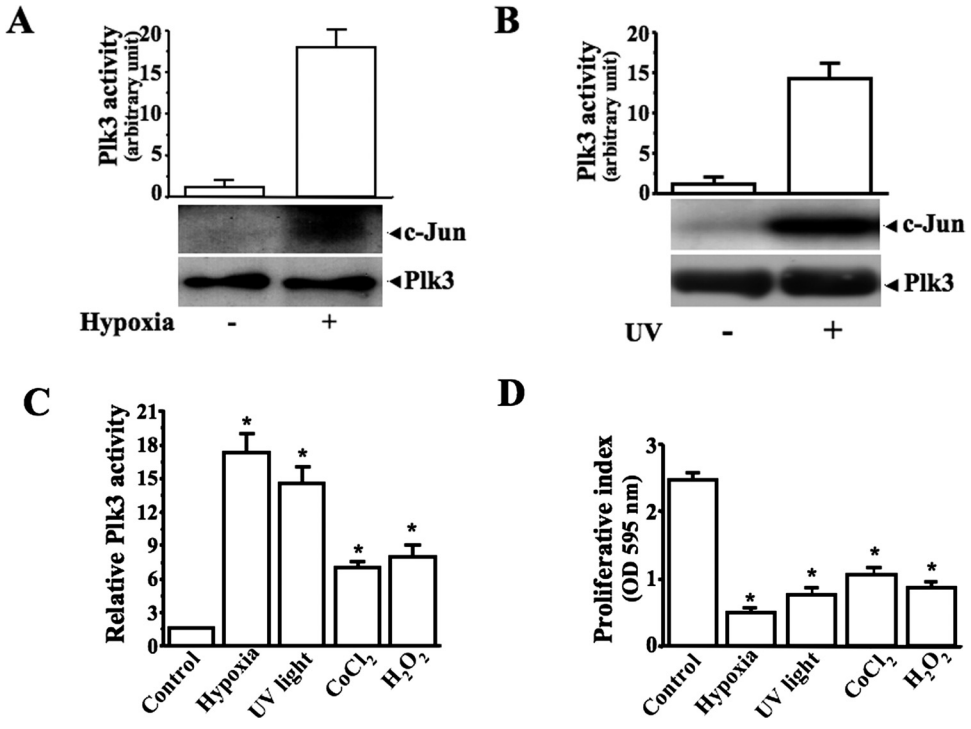

FIGURE 4. Stress-induced Plk3 activation and inhibition of proliferation. (**A**) Hypoxic stress–induced Plk3 activation and c-Jun phosphorylation. (**B**) UV stress–induced Plk3 activation and c-Jun phosphorylation. (**C**) Comparing effects of hypoxic, UV, $CoCl_2$, and H_2O_2 stresses on Plk3 activation. (**D**) Effects of various stress stimuli on proliferation. HCE cells were stimulated with hypoxic stress (1% oxygen), UV irradiation (42 J/cm^2), $CoCl_2$ (1 mM), and H_2O_2 (1 mM), respectively. Plk3 activity was measured by immunocomplex assay, and cell proliferation index was determined by MTT assays. Data are presented as mean ± SD. $^*P < 0.01$ ($n = 4$).

proliferation measured by MTT assay (Fig. 3D). Cell growth attenuation in response to stress stimulation in parallel to stress-induced activation of Plk3 suggests that Plk3 is likely to play an important role in corneal epithelial wound healing. In particular, it has been shown that Plk3 involves cell cycle regulation in other cell types.

Effect of Plk3 Activation on Hypoxia-Induced Inhibition of Wound Healing

Although Plk3 activated by stress stimulation was parallel to inhibitions of proliferation and wound closure, it is necessary to know whether Plk3 is functionally associated with the hypoxic stress–induced inhibition of proliferation and wound closure. In constitutively active Plk3 (Plk3-PBD) overexpressed HCE cells, we found that overexpression of Plk3-PBD not only suppressed wound closure under normoxic conditions but also effectively enhanced hypoxia-induced inhibition of wound closure (Fig. 5A). In addition, the overexpression of constitutively active Plk3 markedly suppressed proliferation in the normoxic condition (the control group), and increased Plk3 activity significantly enhanced the effect of hypoxia on the inhibition of cell proliferation (Fig. 5B). These results strongly support the initial observation that hypoxic stress–induced Plk3 activation is directly involved in the regulation of the corneal epithelial cell wound healing process.

Effect of Plk3 Knockout on Corneal Epithelial Wound Healing

We demonstrated that Plk3 was activated in response to hypoxic stress, resulting in a delay of corneal epithelial cell wound closure. To further investigate the role of Plk3 in corneal epithelial wound healing, the corneal epithelial layer in wild-type and Plk3 knockout ($Plk3^{-/-}$) mouse corneas was debrided with a defined corneal lesion using Algerbrush corneal rust ring remover with a 0.5-mm burr and was then stained by fluorescein (1.0% wt/vol). Mouse eyeballs were cultured for 4 hours in normoxic and hypoxic conditions in the presence of 1% O_2 concentration. A confocal microscope was used to observe and to determine the wounded area in the cornea. Hypoxic stimulation induced significant retardation of corneal wound healing in wild-type mice with three-dimensional confocal microscopic views compared with the control cornea measured within 4 hours after wounding. However, accelerated corneal epithelial wound healing was observed in both control and hypoxia-stimulated corneas of $Plk3^{-/-}$ mice (Fig. 6A). Statistical analysis revealed that there were significantly fast rates and improvements in corneal epithelial wound healing in $Plk3^{-/-}$ mice compared with their wild-type counterparts (Fig. 6B). The results of a comparison of the corneal epithelial wound healing rate between wild-type and $Plk3^{-/-}$ mice were consistent with the observation that Plk3 plays a role in hypoxic stress–induced corneal epithelial cell proliferation and wound closure.

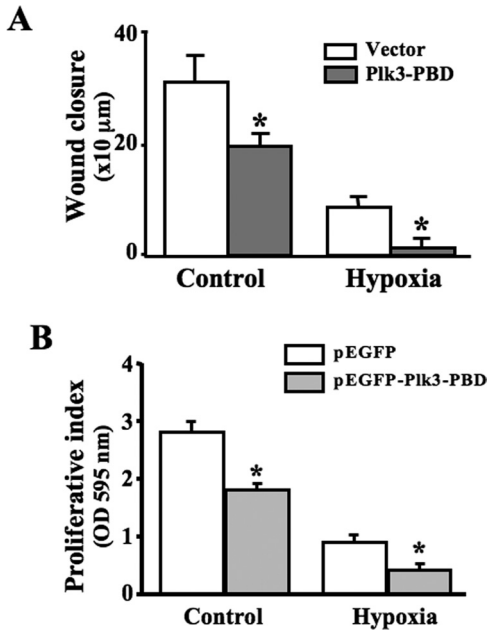

FIGURE 5. Effect of enhanced Plk3 activity on hypoxic stress–induced delay of wound closure. (**A**) Effect of constitutive active Plk3 (Plk-PBD) overexpression on hypoxic stress–induced delay of wound closure. (**B**) Effect of constitutive active Plk3 overexpression on hypoxic stress–induced inhibition of proliferation. HCE cells were transfected with constitutive active Plk3 (Plk3-PBD) 48 hours before stress stimulation. Cell proliferation was examined by MTT assay. Data are presented as mean ± SD. $^*P < 0.01$ ($n = 4$).

DISCUSSION

Oxidative stress constitutes a major insult to all exposed tissues of the body, including the cornea. The common oxidative stresses to the cornea are hypoxia/reoxygenation, UV irradiation, and other ROS. Cellular responses induced by oxidative stresses in various cells are complex processes at early stages to activate cellular signaling pathways that are distinct from the later responses occurring in the nuclei.[46] We explored in the present study hypoxic stress–induced cellular responses and their effect on corneal epithelial wound healing. Hypoxic stress delayed corneal epithelial wound healing in the corneal organ culture system with a small wounding area (0.5 mm in diameter) in the cornea by using corneal epithelial debridement methods. This small area wound healing model requires a short time course to repair within 6 hours (Fig. 1), which is comparable to the time course of the scratch-induced wound closure assay performed in cultured PHCE and HCE cells (Fig. 2). In the cultured corneal epithelial cells, we observed the effect of hypoxic stress on suppressing the essential process of wound healing. Hypoxia induced a significant delay in wound closure rates in the cultured PHCE, HCE, and HTCE cells. The similar effect of hypoxic stress on suppressing corneal epithelial cell wound closure was also observed in other stressor stimulation, such as $CoCl_2$ (mimic hypoxic effects) and H_2O_2. These stressors can also attenuate proliferation and damage corneal epithelial wound closure. We believe that these stressors are capable of suppressing both corneal epithelial cell proliferation and delayed corneal epithelial wound healing in the mouse eye. In the cultured corneal epithelial cells, hypoxic conditions showed a stronger effect on the inhibition of wound healing in HCE and HTCE cells than the effect observed in PHCE cells (Fig. 2). The wound width in HCE cells at 6 hours and HTCE cells at 12 hours was larger than the initial wounding time (at 0 hours) under hypoxic conditions. Contrary to this, wound width remained unchanged in PHCE cells under hypoxic conditions, which suggests that PHCE cells were able to recover early from hypoxic stress and to initiate the wound healing process, suggesting that there may be a different tolerance to hypoxic stress for PHCE cells than for transformed cell lines. The advantage of using corneal epithelial cell lines in eye research is that these cells have a much longer life expectancy than primary cells in culture. PHCE cells obtained directly from the corneal tissue are functionally differentiated cells and have a limited lifespan, though additives to the culture medium and components of the extracellular matrix have been optimized to maintain the best viability of the primary cells. The

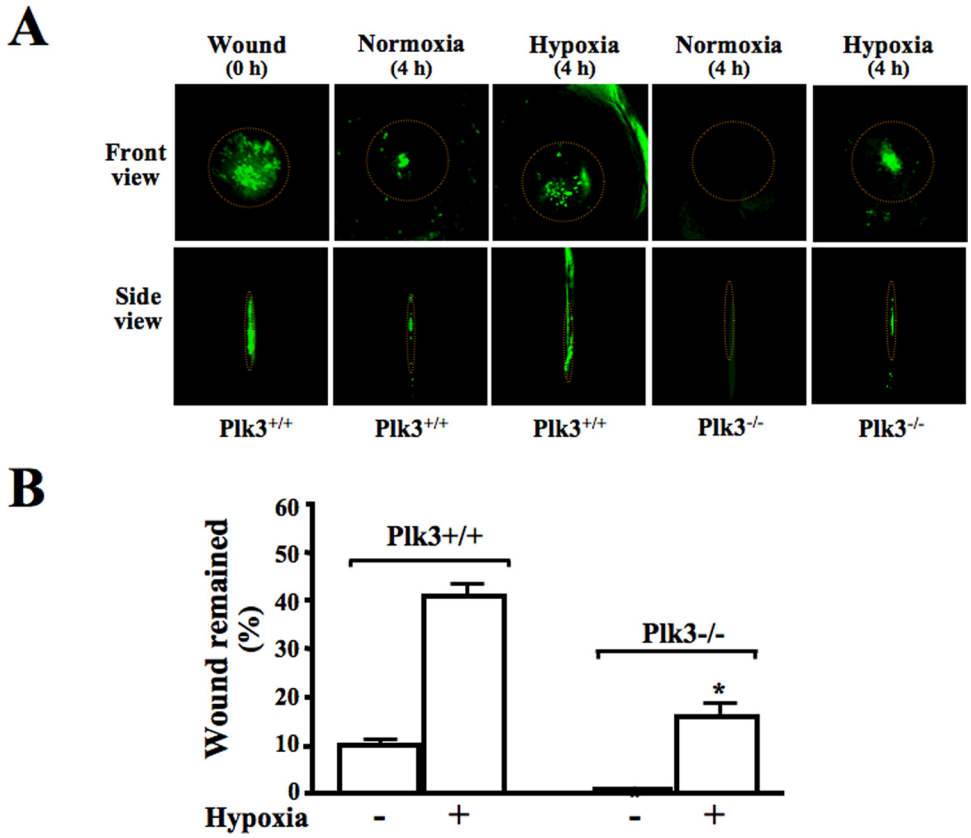

FIGURE 6. Effect of Plk3 knockout on hypoxia-induced delay of wound healing. (**A**) Effect of hypoxic stress on corneal epithelial wound healing in the eyes of wild-type and Plk3$^{-/-}$ mice. The denuded surfaces of the cornea were topically stained with fluorescein. Wounding areas were displayed by a fluorescent confocal microscope and analyzed. Representative images of the front and side views of corneal epithelial wounding were presented immediately and 4 hours after wounding under normoxic and hypoxic conditions. (**B**) Quantitative analysis of corneal epithelial wound healing by comparing corneas obtained from three pairs (the same litters) of wild-type and Plk3$^{-/-}$ mice. The significance of rescuing hypoxia-induced delay of corneal epithelial wound healing by knocking out Plk3 was determined by Student's *t*-test ($P < 0.01$; three pairs of mice, six corneas).

early study in our laboratory indicates that SV40-transformed corneal epithelial cells preserve many characteristics of primary corneal epithelial cells, such as expression of specific keratin 3 and 12, serum and growth factor dependence of cell cycle regulation, and intact signal transduction mechanisms.[47] It is unlikely that the effect of hypoxia on the wound healing response of HCE and HTCE cells is fundamentally different from that of primary cultured PHCE cells. However, we observed in the present study that PHCE cells were able to recover quickly from hypoxic stress stimulation, suggesting that HCE and HTCE cell lines may have their limitations to replace the primary cells for hypoxic time course studies of corneal epithelial wound healing. The reason for the delayed wound healing processes of HCE and HTCE cells in hypoxic conditions may be the lower degree of differentiation and the greater sensitivity to hypoxic stress than in PHCE cells. The detailed mechanism behind this discrepancy requires further investigation.

We have identified hypoxic stress–activated Plk3 in corneal epithelial cells as a key player in the signaling pathway to regulate AP-1 (*c-Jun*) activity and its later effect on apoptosis.[42] Results of the study further demonstrate that hypoxic stress–induced Plk3 activation activates AP-1 components that are able to switch kinase cascade signals to genetic responses and to affect corneal epithelial wound healing in both cultured cells and mouse corneas. In fact, our data are consistent with the previous results, indicating that Plk3 is a multifunctional protein and that it involves stress-induced signaling pathways in fibroblasts, lymphoblastoid, lung carcinoma, and epithelial cells.[34–36] When cells are exposed to reagents that damage DNA, such as IR, ROS, or methyl methane sulfonate, Plk3 is rapidly phosphorylated. Plk3 protein levels remain constant throughout the cell cycle, but protein kinase activity of Plk3 becomes activated after stress stimulation (Fig. 3). Previous studies demonstrate in different cell types that Plk3 activated by ROS-induced DNA damage interacts with Chk_2 and phosphorylates the tumor suppressor protein p53.[48,49] However, the effect of Plk3 activation on wound healing has not been well understood. In the present study, we found that Plk3 activation in response to hypoxic stress affected corneal epithelial wound healing with prominent evidence. In HCE cells, constitutively active Plk3 significantly enhanced hypoxic stress–induced suppression of proliferation and wound closure (Fig. 4). In contrast, knocking down Plk3 in the eye of Plk3$^{-/-}$ mice markedly promoted corneal epithelial wound healing in the presence of hypoxic stimulation (Fig. 5). We believe that our results revealed an important role of Plk3 in hypoxic stress–induced signaling events involving the inhibition of corneal epithelial growth, contributing to the effect of oxidative stresses on corneal epithelial wound healing.

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