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Research Article

Hyaluronic acid-dependent protection against alkali-burned human corneal cells

Hyaluronic acid (HA) is a high-molecular-weight glycosaminoglycan and extracellular matrix component that promotes cell proliferation. This study aimed to evaluate the effects of HA on alkali-injured human corneal epithelial cells in vitro, and to elucidate the mechanisms by which HA mediates corneal cell protection. A human corneal epithelial cell line (HCE-2) was treated with sodium hydroxide before incubation with low-molecular-weight HA (LMW-HA, 127 kDa) or high-molecular-weight HA (HMW-HA, 1525 kDa). A global proteomic analysis was then performed. Our data indicated that HA treatment protects corneal epithelial cells from alkali injury, and that the molecular weight of HA is a crucial factor in determining its effects. Only HMW-HA reduced NaOH-induced cytotoxic effects in corneal cells significantly and increased their migratory and wound healing ability. Results from 2D-DIGE and MALDI-TOF/TOF MS analyses indicated that HMW-HA modulates biosynthetic pathways, cell migration, cell outgrowth, and protein degradation to stimulate wound healing and prevent cell death. To our knowledge, our study is the first to report the possible mechanisms by which HMW-HA promotes repair in alkali-injured human corneal epithelial cells.

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1 Introduction

Alkali injuries of the cornea are among the most severe ocular accidents. However, current therapeutic applications, containing healing promoters and anti-inflammatory agents, have limited success in disease management [1, 2]. Hyaluronic acid (HA) is a high-molecular-weight nonsulfated glycosaminoglycan composed of alternating β-1,4-glucuronic acid and β-1,3-*N*-acetylglucosamine. It serves as the major extracellular matrix component in connective tissues and is abundant in the vitreous body of the eye. It is biosynthesized as large 6000 kDa disaccharide chains and gradually degraded in the extracellular matrix to form intermediate-sized HA (10–100 kDa) or small fragments (<1 kDa) [3]. Several studies have

suggested that differential molecular weights of HA might exhibit different physical properties and different biological effects. The study on the correlation between molecular weight of HA and its binding ability to cell surface receptor indicated that the larger the molecular of HA, the higher the affinity between HA and its receptor [4]. Additionally, with increased concentrations and higher molecular weight of HA lead to increased viscosities and cross-over frequency of HA [5].

Different molecular weights of native or degraded HA fragments have differential abilities to induce differential chemokine gene expression [6] and angiogenesis [7]. Recent reports indicated that native-HMW-HA is an inhibitor for angiogenesis, whereas degraded HA has evidenced to stimulate cell proliferation, cell migration, and cell wound healing following activation of HA-specific receptors including CD44 and receptor for HA-mediated motility (RHAMM) [8]. Cell surface RHAMM is implicated in mediation of cell migration and cell focal adhesion in response to degraded HA activation [9]. HA also modulates

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Abbreviations: **HA**, hyaluronic acid; **HMW-HA**, high-molecular-weight hyaluronic acid; **RHAMM**, receptor for HA-mediated motility

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cell motility and cell survival through interaction with cell surface CD44. The interplay between HA and CD44 affects cell adhesion onto extracellular matrix components followed by the stimulation of cell proliferation, wound healing, migration, and angiogenesis [10]. Additionally, Takahashi et al. identified that activation of CD44 can increase the secretion of matrix metalloproteinase-2 (MMP-2) to promote cell migration, cell metastasis, and wound healing with the aid of matrix metalloproteinase-14 (MMP-14) [11]. Meanwhile, HA promotes the interaction of CD44 with PI3K through GAB1 adaptor protein providing a positive regulation of cell survival [12]. More important, the accumulation of lower molecular weight HA has been reported to stimulate the expression of cytokines such as monocyte chemoattractant protein-1 and macrophage inflammatory protein families during inflammation [13].

Proteomics is a powerful tool to rapidly distinguish protein expression alterations and better understand various complexities in which an appropriate treatment is beneficial for the corneal repair. 2DE is currently a crucial technique in proteomics to profile thousands of proteins within trace amount of biological samples and plays a complementary role to LC/MS-based proteomic analysis [14]. However, reliable quantitative comparisons between gels and gel-to-gel variations remain the primary challenge in 2DE analysis. A significant improvement in the gel-based analysis of protein quantification and detection was achieved by the introduction of 2D differential gel electrophoresis (DIGE), which can co-detect numerous samples in the same 2DE. This approach minimizes gel-to-gel variations and allows comparing the relative amount of protein features across different gels by using an internal fluorescent standard. Moreover, 2D-DIGE technique has the advantages of a broader dynamic range, higher sensitivity, and greater reproducibility than traditional 2DE [14]. This innovative technology relies on the pre-labeling of protein samples with fluorescent dyes (Cy2, Cy3, and Cy5) before electrophoresis. Each dye has a distinct fluorescent wavelength, allowing multiple experimental samples with an internal standard to be simultaneously separated in the same gel. The internal standard, which is a pool of an equal amount of the experimental protein samples, helps provide accurate normalization data and increase statistical confidence in relative quantification across gels [15–21].

Previous studies have identified endogenous HA in various diseased human corneas, including alkali-wounded corneas [1, 22]. Studies have also described that high-molecular-weight hyaluronic acid (HMW-HA) decreases benzalkonium chloride- and UVB-induced apoptosis in human corneal epithelial cells [3, 23], and can accelerate wound healing in the corneal epithelium of diabetic rats [24]. However, the effects of various forms of HA on corneal wound healing following alkali injury have yet to be elucidated. In this study, we evaluated the effects of LMW-HA (127 kDa) and HMW-HA (1525 kDa) on alkali-injured human corneal epithelial cells using cell viability, cell migration, wound healing assays, and proteomic analysis. Our data indicated that

HA could have potential use in the treatment of alkali injury, and that the molecular weight of HA is a crucial factor in determining its corneal cell protective ability.

2 Materials and methods

2.1 Chemicals and reagents

Generic chemicals were purchased from Sigma-Aldrich (St. Louis, USA), while reagents for 2D-DIGE were purchased from GE Healthcare (Uppsala, Sweden). All primary antibodies were purchased from GeneTex (Hsinchu, Taiwan) and anti-mouse and anti-rabbit secondary antibodies were purchased from GE Healthcare. The LMW-HA (127 260 ± 7014 Da with a PDI 1.273) and HMW-HA (1 525 000 ± 8000 Da with a PDI 1.053) used in this study were both medical grade (listed in European Pharmacopoeia) and purchased from Shiseido Co. (Tokyo, Japan). These HAs contain less than 1% protein content and less than 0.0003 IU/mg bacterial endotoxins. All the chemicals and biochemicals used in this study were of analytical grade.

2.2 Cell line and cell cultures

The human corneal epithelial cell line HCE-2 was a gift from National Taiwan University Hospital, Taiwan. The cell line was maintained in Dulbecco's Modified Eagle's medium and F-12 medium (DMEM/F-12) supplemented with 10% FCS, L-glutamine (2 mM), streptomycin (100 μg per mL), penicillin (100 IU per mL), epidermal growth factor (20 ng per mL) (all from Gibco-Invitrogen, UK), insulin (10 μg per mL) (Sigma) and hydrocortisone (0.5 μg per mL) (Sigma). Cells were incubated at 37°C and 5% CO_2.

2.3 MTT cell viability assay

HCE-2 cells growing exponentially were trypsinized and seeded at a density of 5000 cells per well into 96-well plates. After washing with PBS three times, the HCE-2 cells were transiently treated with indicated concentrations of NaOH for 1 min or left untreated followed by incubation in serum-free medium containing indicated concentrations of either LMW-HA (127 kDa) or HMW-HA (1525 kDa) for 24 h. The detailed MTT procedure has been described in our previous publication [25].

2.4 Cell migration assay

For cell migration assay, the outside surface of 12-well culture plate bottom was marked with the outer circumference of a cloning ring to allow for precise positioning of the ring inside the well before cell seeding for an accurate photography of cell outgrowth later on. Subsequently, a cloning ring

was placed onto the marked circumference inside each well and the HCE-2 cell suspensions were carefully applied into the cloning ring for an overnight incubation at 37°C before the rings were lifted. The cell monolayers were washed once with PBS and the experiment was initiated by the addition of 0.012 N of NaOH for 1 min or left untreated followed by PBS washings. The damaged HCE-2 monolayers were immediately supplied with serum-free medium containing indicated concentrations of HA for 24 h before staining with crystal violet for the measurement of migration areas.

2.5 Scratch wound healing assay

Exponentially growing HCE-2 cells were trypsinized and seeded at a density of 30 000 cells per well into 24-well plates for 24-h incubation (∼90% confluence). The scratch wounds were made by a sterile 200-μL pipette tip through a pre-marked line. After removal of the resulting debris from five lineal scratches, HCE-2 monolayer was subsequently treated with 0.012 N NaOH for 1 min and rinsed three times with PBS followed by incubation in serum-free medium containing indicated concentrations of HA for 0, 4, 8, 12, and 24 h. The wound areas were displayed by taking images just above the interchanges between scratched wound areas and pre-marked lines.

2.6 Sample preparation and 2D-DIGE-based proteomic analysis

The detailed experimental procedures have been described in our previous publications [16]. Briefly, HCE-2 cells in normal growth medium at approximately 80% confluence were used for proteomic analysis. HCE-2 cells with various treatments (NaOH treatment for 1 min, NaOH pretreatment for 1 min followed by treatment with 0.05% HMW HA and NaOH pretreatment for 1 min followed by treatment with 0.3% HMW HA) or left without treatment were lysed with 2DE lysis buffer. Before performing 2D-DIGE, protein samples were labeled with *N*-hydroxy succinimidyl ester-derivatives of the cyanine dyes Cy2, Cy3, and Cy5 according to the sample arrangement shown in Fig. 3A. A total of 150 μg of protein sample was minimally labeled with 375 pmol of either Cy3 or Cy5 for comparison on the same 2DE. In contrast, to facilitate image matching and cross-gel statistical comparison, a pool of all samples was also prepared and labeled with Cy2 at a molar ratio of 2.5 pmol Cy2 per μg of protein as an internal standard for all gels. Thus, the triplicate samples and the internal standard could be run and quantify on multiple 2DE. Afterward, the fluorescence 2DE was scanned directly between the low fluorescent glass plates using an Ettan DIGE Imager and gel analysis was performed using DeCyder 2D Differential Analysis Software v7.0 (GE Healthcare) to co-detect, normalize, and quantify the protein features in the images. Features detected from nonprotein sources (e.g. dust particles and dirty backgrounds) were filtered out. Spots displaying a 1.3 average-fold increase or decrease in abundance with a *p*-value <0.05 were selected for protein identification.

2.7 Protein staining, in-gel digestion, and MALDI-TOF MS analysis

Colloidal coomassie blue G-250 staining was used to visualize CyDye-labeled protein features in 2DE followed by excised interested poststained gel pieces for MALDI-TOF MS identification. The detailed procedures for protein staining, in-gel digestion, MALDI-TOF MS analysis and the algorithm used for data processing were described in our previous publication [16]. The spectrometer was also calibrated with a peptide calibration standard (Bruker Daltonics) and internal calibration was performed using trypsin autolysis peaks at *m/z* 842.51 and *m/z* 2211.10. Peaks in the mass range of *m/z* 800–3000 were used to generate a peptide mass fingerprint that was searched against the Swiss-Prot/TrEMBL database (v57.12) with 513877 entries using Mascot software v2.2.06 (Matrix Science, London, UK). The following parameters were used for the search: *Homo sapiens*; tryptic digest with a maximum of 1 missed cleavage; carbamidomethylation of cysteine, partial protein *N*-terminal acetylation, partial methionine oxidation, and partial modification of glutamine to pyroglutamate and a mass tolerance of 50 ppm. Identification was accepted based on significant MASCOT Mowse scores (*p* <0.05), spectrum annotation and observed versus expected molecular weight and p*I* on 2DE.

2.8 Immunofluorescence

The detailed experimental procedures for immunofluorescence analysis were described in our previous reports [16].

3 Results

3.1 Hyaluronic acid increases the viability of NaOH-injured HCE-2 cells

To investigate the effects of NaOH on HCE-2 cells, we exposed the cells to NaOH (0 to 0.04 N) for 1 min. We washed the NaOH-exposed cells twice in a PBS and incubated them in various concentrations of a FCS (0, 1, 5 and 10%) for 24 h. We then performed MTT assay to evaluate the NaOH-induced cell damage. We observed dose-dependent loss of viability in HCE-2 cells exposed to NaOH (Supporting Information Fig. S1). At an NaOH concentration of 0.012 N, we observed significant loss (50%) of cell viability after 24 h. When we varied the FCS concentrations, cell viability remained unchanged, which suggested that serum concentration does not influence NaOH-induced changes in cell viability. To investigate the effects of HA on repair of HCE-2 cells from NaOH-induced damage, we exposed HCE-2 cells to 0.012 N NaOH for 1 min, and then incubated them in the indicated concentrations of LMW-HA or HMW-HA for 24 h before evaluating cell
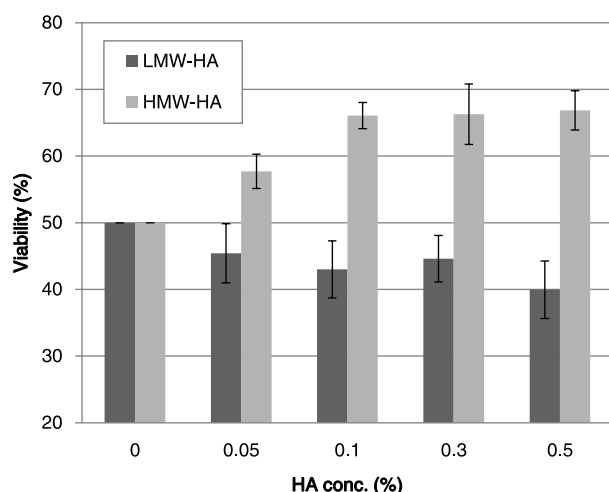
Figure 1. Effect of LMW-HA and HMW-HA on HCE-2 cell viability after NaOH treatment. HCE-2 cells were incubated in serum-free medium and treated with 0.012 N NaOH for 1 min. After removal of NaOH, HCE-2 were incubated in serum-free medium containing indicated concentrations of HA or left untreated for 24 h. Cell viability was subsequently determined by MTT cell viability assay. Data are mean ± SD of six independent experiments.

viability. Our data indicated that HMW-HA increased cell viability significantly in a dose-dependent manner. However, LMW-HA induced insignificant changes in cell viability (Fig. 1). These results indicated that HMW-HA, but not LMW-HA, has the ability to increase the viability of HCE-2 cells following NaOH injury.

3.2 High-molecular-weight HA facilitates migration and wound healing in alkali-injured HCE-2 cells

We then investigated the effects of HA on corneal epithelial cell proliferation and migration. Results indicated that LMW-HA and HMW-HA both increased HCE-2 cell migration postexposure to an IC_{50} concentration of NaOH (0.012 N). Following the treatment of alkali-injured HCE-2 cells with HMW-HA, we observed areas of cell migration and wound healing (Supporting Information Fig. S2). We then evaluated the effects of various doses of LMW-HA and HMW-HA on wound closure in HCE-2 cells. Figure 2 shows the changes in epithelial cells exposed to 0.012 N NaOH and then treated with a serum-free medium containing 0, 0.05, or 0.3% LMW-HA, or 0.3% HMW-HA. Results indicated that minimal epithelial wound healing occurred within 8 h of wounding. However, 8–24 h postinjury, wound healing accelerated. Of the tested conditions, we observed that 0.3% HMW-HA was most effective at promoting healing in alkali-injured HCE-2 cells. In HCE-2 cells treated with 0.05% LMW-HA, 0.3% LMW-HA, and 0.05% HMW-HA, wound healing occurred to a significantly lesser extent than in 0.3% HMW-HA-treated cells. These results indicated that 0.3% HMW-HA exerts potent healing promoting effects on alkali-injured corneal epithelial cells.

3.3 Profilin and cofilin play key roles in HMW-HA-dependent protection against alkali injury in HCE-2 cells

The assembly and disassembly of actin networks are major driving forces for cell movements. Profilin can recycle actin monomers to form ATP-actin for further assembly, whereas cofilin causes depolymerization at the minus end of actin microfilaments to regulate actin dynamics and cell migration. In this study, we used immunostaining to identify that treatment of HCE-2 cells with 0.012 N NaOH for 1 min induced the downregulation of profilin-1 and upregulation of cofilin-1, and disruption of cytoskeletal polarity at the leading edge of cells (Supporting Information Fig. S3). However, treatment of NaOH-exposed cells with 0.3% HMW-HA for 30 min protected F-actin polarity and recovered profilin-1 and cofilin-1 expression to control levels (Supporting Information Fig. S3). Overall, our findings indicated that 0.3% HMW-HA induces the polymerization of profilin-1 and depolymerization of cofilin-1 to modulate peripheral actin organization. This leads to the efficient regulation of protrusion dynamics and wound healing in alkali-injured corneal cells.

3.4 Global analysis of protein expression in untreated or NaOH-treated HCE-2 cells with or without HMW-HA treatment

We performed 2D-DIGE analysis to evaluate changes in protein expression in untreated HCE-2 cells, and in cells treated with NaOH, and NaOH plus 0.05 or 0.3% HMW-HA. The analysis revealed more than 80 protein features that displayed differential expression ($>$1.3-fold changes; $p < 0.05$) among the four treatment conditions (Fig. 3). We identified proteins in 42 of these features using MALDI-TOF MS and MS/MS analysis (Supporting Information Table S1). The differentially expressed proteins predominantly function in cytoskeleton regulation, protein biosynthesis, metabolic regulation, and protein folding. Nine of the 42 identified proteins showed NaOH-dependent changes that were partially reversed by HA treatment (including alpha-enolase, aspartyl-tRNA synthetase, calcium-binding mitochondrial carrier protein, heat shock 70 kDa protein, cytoskeletal 18, NEDD8-conjugating enzyme Ubc12, proteasome activator complex subunit 2, rab5 GDP/GTP exchange factor, ribonuclease inhibitor, and SUMO-conjugating enzyme UBC9). For example, protein spot 1543 (NEDD8-conjugating enzyme Ubc12) showed a 1.7-fold downregulation in response to NaOH treatment, and a 1.60- and 2.22-fold upregulation following treatment with 0.05 and 0.3% HMW-HA, respectively (Supporting Information Fig. S4). Protein spot 549, identified as alpha-enolase, showed a 1.69-fold downregulation in response to NaOH treatment, and a 2.11- and 1.41-fold upregulation following treatment with 0.05 and 0.3% HMW-HA, respectively (Supporting Information Table S1). These data confirmed that HMW-HA partially rescues HCE-2 cells from NaOH-induced cell death signaling, and promotes corneal repair
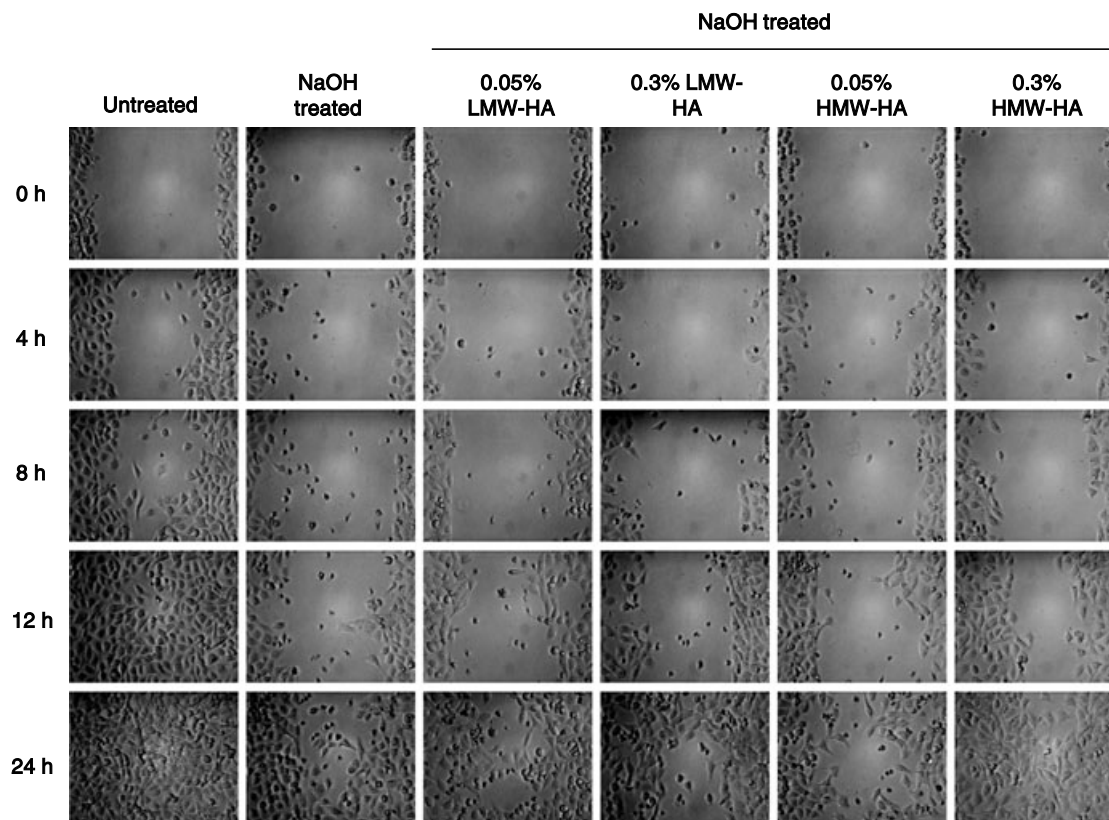
Figure 2. Effect of HA on NaOH-treated corneal epithelial wound closure in HCE-2 cells. The wound corneal epithelial cells (HCE-2) were treated with indicated concentrations of LMW-HA or HMW-HA in serum-free medium or with serum-free medium alone after the HCE-2 cells were transiently treated with 0.012 N NaOH for 1 min. The wound healing of HCE-2 cells was photographed at the times indicated. Each treatment condition has been performed at least three times.

processes such as cell survival, cell migration, and wound healing.

4 Discussion

The eye is one of the most sensitive organs to toxic materials [3]. The cornea is located on the outer surface of the eye and, therefore, more commonly injured by toxicants from the environment than other eye parts. Sodium hydroxide is a component in several household cleaning agents [26]. Because of the invasive nature of alkalis, corneal alkali burns are severe ocular disorders with limited effective therapies. Greater understanding of appropriate treatments for alkali-induced injury could, therefore, potentially improve corneal healing. In this study, we exposed HCE-2 cells to 0.012 N NaOH for a short incubation period (1 min) to induce cell damage. Our results showed that NaOH was significantly cytotoxic to HCE-2 cells and markedly reduced viability (confirmed by at least three independent MTT assays), migratory ability, and wound healing rate. Our proteomic analyses identified that these effects were associated with decreased carbohydrate metabolism (such as alpha-enolase) and increased expression of cellular stress response proteins (such as heat shock 70 kDa protein), degradation proteins (such as SUMO-conjugating enzyme), and redox regulatory proteins (such as peroxiredoxin-6). Treatment of the HCE-2 cells with HA reversed the NaOH-induced changes significantly. HA is a viscous biopolymer located on the outer surface of the plasma membrane and reportedly neutralizes reactive oxygen species and cytotoxins. However, its biological functions are dependent on the number of disaccharides it contains [23,24,27]. In this study, we evaluated the association between HA molecular weight and its ability to negate NaOH-induced corneal epithelial cell damage. First, we evaluated cell viability in response to treatment with various concentrations of HMW-HA (1525 kDa) or LMW-HA (127 kDa). After 4–8 h incubation, we observed increased wound healing (Supporting Information Fig. S5). After 24-h treatment, we observed a mild increase in cell proliferation. We then aimed to clarify the effects of HA on NaOH-treated HCE-2 cells. Results showed that HMW-HA, but not LMW-HA, reduced NaOH-induced toxic effects and recovered the cells' wound healing and cell migratory abilities. Using proteomic analysis, we identified that HMW-HA stimulated the expression of biosynthesis proteins (such as aspartyl-tRNA synthetase) and migration-regulated proteins (such as cytoskeletal 18, septin-11, and shootin-1) in alkali-damaged cells. High-molecular-weight HA also

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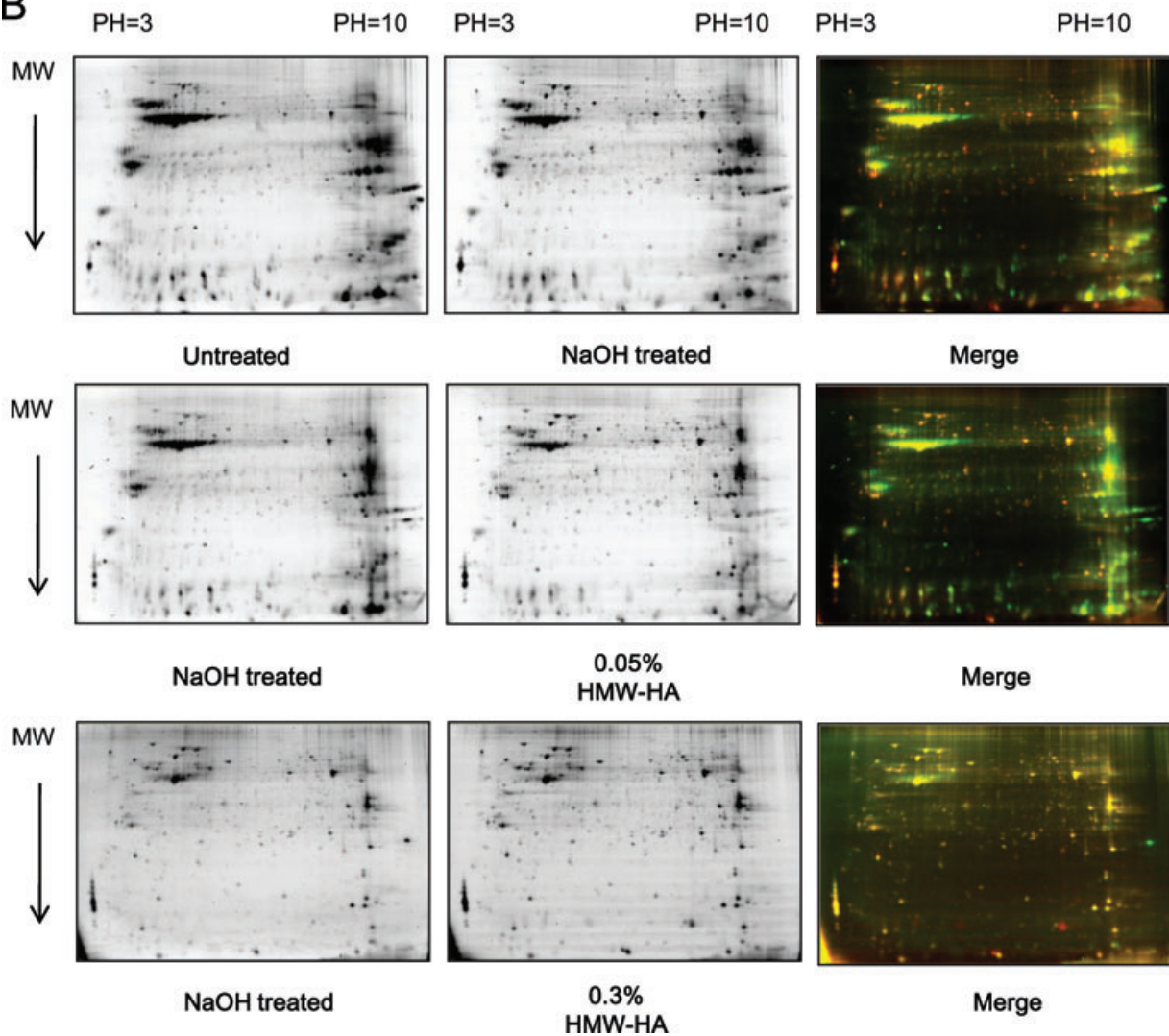|	Cy2 (150mg)	Cy3 (150mg)	Cy5 (150mg)
Gel 1	Pool	Untreated	NaOH treated
Gel 2	Pool	0.05% HMW-HA	0.3% HMW-HA
Gel 3	Pool	NaOH treated	0.05% HMW-HA
Gel 4	Pool	0.3% HMW-HA	Untreated
Gel 5	Pool	NaOH treated	0.3% HMW-HA
Gel 6	Pool	Untreated	0.05% HMW-HA

Figure 3. 2D-DIGE analysis of NaOH-induced differential protein expression profiles in human corneal epithelial cells were post-treated by HMW-HA or left untreated. Total cellular proteins (100 μg each) purified from PBS buffer containing 0.012 N NaOH or PBS buffer alone treated HCE-2 cells before treated with indicated concentrations of HMW-HA for 24 h were labeled with Cy-dyes and separated using 24 cm, pH 3–10 nonlinear IPG strips followed by resolved with 12.5% SDS-PAGE. 2D-DIGE images of untreated HCE-2 cells and NaOH-treated HCE-2 cells followed by incubation with various concentrations of HMW-HA at appropriate excitation and emission wavelengths were pseudo-colored and overlaid with ImageQuant Tool (GE Healthcare).

modulated the expression of proteins involved in degradation, such as proteasome activator complex subunit 2, NEDD8-conjugating enzyme Ubc12, and SUMO-conjugating enzyme UBC9.

NEDD8-conjugating enzyme Ubc12 is an E2-conjugating enzyme that plays a central role in protein degradation and is involved in many cellular functions. Read et al. reported that NEDD8-conjugating enzyme Ubc12 is required for optimal ubiquitination of IκB [28]. The IκB protein binds to the transcription factor NF-κB in cytosol and inhibits its translocation to the nucleus for activation of genes involved in survival [29]. In our proteomic study, NEDD8-conjugating enzyme Ubc12 was upregulated in NaOH-injured HMW-HA-treated HCE-2 cells. This indicated that HMW-HA induces IκB degradation and, therefore, promotes NF-κB-stimulated cell survival in alkali-damaged HCE-2 cells. Another function of NEDD8-conjugating enzyme Ubc12 enzyme is mediation of the degradation of the cyclin-dependent kinase (CDK) inhibitor p27 during cell cycle progression [30]. High expression of NEDD8-conjugating enzyme Ubc12 in alkali-injured HMW-HA-treated HCE-2 cells might, therefore, induce cell cycle progression through the degradation of p27 (Fig. 4).

Small ubiquitin-like modifier SUMO is another post-translational modifier of proteins and is covalently attached to the lysine residues of target proteins by SUMO activation enzyme (E1), SUMO-conjugating enzyme (E2), and SUMO ligase (E3). The SUMO-conjugating enzyme UBC9 is the only E2-conjugating enzyme that negatively regulates the activity of most transcription factors including AP-1, AP-2, and NF-κB [31, 32]. According to our proteomic data, SUMO-conjugating enzyme UBC9 is downregulated in response to HMW-HA treatment in NaOH-injured HCE-2 cells. These results suggest that HMW-HA reduces SUMO-conjugating enzyme UBC9 expression, inhibits the sumoylation of transcription factors, and further induces cell growth (Fig. 4).

In our proteomic analyses, we identified changes in proteins responsible for the folding/unfolding of other proteins (such as heat shock 70 kDa protein 1, and heat shock cognate 71 kDa protein). Heat shock protein 70 is a molecular chaperone that accelerates cell recovery from harmful conditions by reducing the concentrations of unfolded or denatured proteins [33]. We observed the upregulation of heat shock 70 kDa protein 1 in NaOH-treated HCE-2 cells and its downregulation in response to HMW-HA treatment. This indicated that NaOH induces cellular stress in HCE-2 cells, whereas HMW-HA reduces alkali-induced cellular stress. Heat shock 70 kDa protein 1 expression was downregulated in control cells (Fig. 4).

Rab proteins are small GTPases that play essential roles in protein trafficking by modulating vesicle budding, transport, and fusion [34]. Rab5 is involved in early endosome fusion, endosome motility, actin remodeling, and growth factor signaling [35–38]. In this study, we identified high expression of Rab5 GDP/GTP exchange factor in HCE-2 cells in response to NaOH treatment, and its downregulation following treatment with HMW-HA. These results suggest that HMW-HA

might protect HCE-2 cells from alkali injury by regulating Rab5 GDP/GTP exchange factor expression (Fig. 4).

Our proteomic analyses also identified the upregulation of proteins involved in cell cycle progression (such as cell division protein kinase 4), protease inhibition (such as cystatin-B), biosynthesis (such as elongation factor 1-gamma and L-aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase), cytoskeleton regulation (such as plastin-2), cell movement (such as septin and shootin-1), and RNAase inhibition (such as ribonuclease inhibitor). We observed the downregulation of proteins involved in gene silencing (such as trinucleotide repeat-containing gene 6B protein) and catabolism (such as alpha-enolase, glyceraldehyde-3-phosphate dehydrogenase). Overall, these results suggest that HMW-HA stimulates biosynthetic pathways, cell cycle progression, cell migration, cell outgrowth, wound healing, and gene expression to maintain the correct folding of intracellular proteins, and inhibits protein degradation and catabolism to protect alkali-injured corneal epithelial cells from cell death.

Our data indicated that HA might also modulate the levels of profilin and cofilin expression, which are essential actin-binding proteins that play key roles in the regulation of actin dynamics and actin-based motility processes. Profilin specifically binds ATP-G-actin, contributes to filament assembly at the plus ends of actin filaments, and reduces the steady-state concentration of ATP-G-actin-cofilin. In contrast, cofilin mediates the dissociation of ADF-bound F-actin from the minus ends of actin filaments, resulting in actin filament disassembly [39]. In our study, we identified that NaOH downregulated profilin-1 expression and upregulated cofilin-1 expression in HCE-2 cells. Treatment of the cells with 0.3% HMW-HA reversed these changes, indicating that HMW-HA might contribute to cell migration and wound healing through profilin- and cofilin-modulated actin dynamics and actin-based motility.

HA has been reported to specifically interact with the cell surface receptor CD44 and RHAMM. Thus, HA might strongly bind to the plasma membrane through CD44/RHAMM and play roles in maintaining the integrity of corneal cell plasma membranes following alkali damage. Our analyses indicated that HA molecular weight and concentration are critical factors in determining its protective effects against NaOH. We observed that 0.3% HMW-HA was the optimal concentration for protection of corneal epithelial cells from NaOH-induced damage and induction of cell proliferation. At this concentration, HMW-HA might form a biopolymer structure with high cross-over frequency that coats the outer surface of plasma membranes through linkage with CD44/RHAMM receptors. The CD44/RHAMM receptors bound HA might then promote cell survival signals as well as stimulate cell migration and wound healing or prevent activation of death receptors to maintain cell function and survival [40, 41]. However, at HMW-HA concentrations higher than 0.5%, we observed marked decreases in cell viability and protection (data not shown). This suggested that high HA
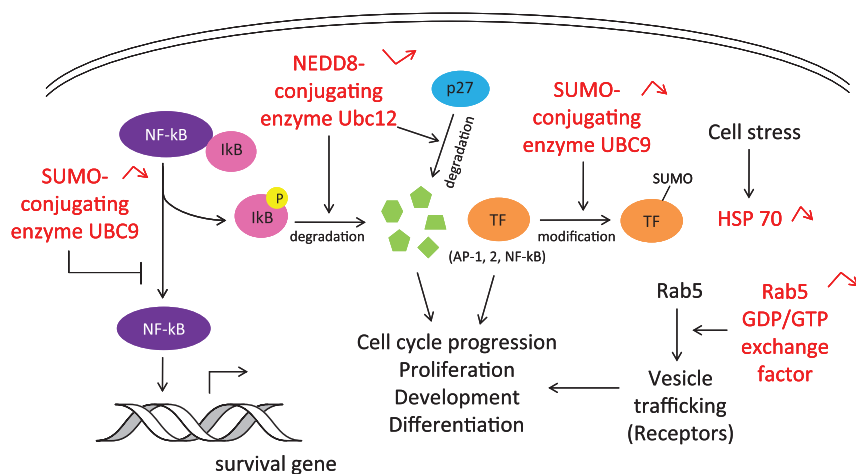
Figure 4. The hypothetical mechanisms of identified proteins with HA-protection responses through expression proteomics in NaOH-damaged HCE-2 cells compared with untreated cells. The differentially expressed proteins identified by expression proteomic study are shown as red color. Red arrows indicate the levels of the identified proteins. The swerve arrows indicate the trends of these identified proteins.

concentrations might interfere with nutrients, metabolism, and gas exchanges. Importantly, numerous studies demonstrated that LMW-HA but not HMW-HA is able to stimulate cell proliferation, cell migration, and cell wound healing following HA activation (see Introduction). These observations are inconsistent to our experimental results suggesting LMW-HA- or HMW-HA-mediated cell survival, cell migration, and wound healing might depend on cell type. Additionally, the combination of molecular weight of HA and concentration differences might result in significant differences in solution viscosity, viscoelasticity, and colloid osmotic pressure. Since corneal cells are sensitive to the mechanical properties of their surroundings, it is difficult to clarify whether HA and cell surface protein interaction or HA and mechanical properties contribute more to the cell behaviors such as cell migration ability during wound healing and cell viability under HA treatment. These mechanisms warrant further investigation.

In conclusion, our study results showed that HMW-HA (1525 kDa) and LMW-HA (127 kDa) do not display detectable toxic effects on the human corneal epithelial cell line HCE-2. However, only HMW-HA reduces NaOH-induced cytotoxic effects on corneal cells significantly and increases cell migratory and wound healing ability. Our findings could potentially facilitate the development of eye drops for repair of alkali-injured ocular surfaces, or the design of biomaterials to accelerate wound healing postinjury.

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