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Acceleration of Apoptosis by Extracellular Basic pH in a 3D Human Skin Equivalent System

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Abstract
Previously, we have shown that extracellular basic pH plays a significant role in both the direct and indirect regulation of cellular processes in a wound; this in turn affects the wound-healing process. Several studies have demonstrated the importance of apoptosis modulation in the wound-healing process, especially in removing inflammatory cells and in inhibiting scar formation. However, the effects of extracellular basic pH on wound healing-related skin damage are yet to be examined. Therefore, we investigated the induction of accelerated apoptosis by extracellular basic pH in skin. Apoptosis-related protein levels were measured using an array kit, target protein expression levels were detected by immunostaining, lactate dehydrogenase was analyzed spectrophotometrically, and Annexin V levels were measured by fluorescence staining. Basic pH (8.40) strongly upregulated extrinsic apoptosis proteins (Fas, high temperature requirement A, and p21) and slightly upregulated intrinsic apoptosis proteins (cytochrome c, B-cell lymphoma 2 [Bcl-2], Bcl-2-associated death promoter, and Bcl-2-like protein 4) in a 3D human skin equivalent system. Moreover, basic pH (8.40) induced heat shock protein (HSP) 60 and 70. In addition, basic pH-exposed Fas- and HSP60-knockdown cells showed significantly decreased levels of apoptosis. Taken together, these results indicate that extracellular basic pH increases early-stage apoptosis through Fas/FasL via modulation of HSP60 and HSP70.

Introduction

Wound healing is a complex and well-designed repair process that occurs following injury caused after surgical procedures or trauma [1]. The process comprises 3 serial phases: inflammation, tissue formation, and tissue remodeling [1]. Apoptosis is important for the wound-healing process, especially in removing inflammatory cells and in inhibiting scar formation [2]. The early phase of inflammation is characterized by the invasion of neutrophils, macrophages, and lymphocytes into the wound area [2]. The fibroblasts then migrate and synthesize extracellular matrix components [2]. Inflammatory cells must be removed in order to initiate the next phase of wound healing. The remodeling of granulation tissue

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during the wound-healing process is also accompanied by the apoptosis of fibroblasts [3]. Dysregulation of these apoptotic processes may result in abnormal wound healing, such as hypertrophic scars and keloid formation, or may delay wound healing [4]. Our previous studies indicate that extracellular basic pH in skin is an important effector of wound healing; we demonstrated an interruption of tissue remodeling due to an acceleration of collagen breakdown induced by an imbalance in extracellular pH in skin. In addition, Guo and DiPietro [3] have shown that both acute and chronic wounds with a basic pH exhibit lower healing rates than wounds with a more neutral pH; wound-healing progression decreases when the pH becomes basic. The environment of both acute and chronic wounds progresses from a basic to neutral pH and then to an acidic pH once healing commences [5]. Taken together, these findings reveal that the regulation of wound healing is modulated by the skin pH value. However, the effect of extracellular basic pH in the skin on apoptosis and its role in the pathogenesis of these responses are not completely understood. In particular, the association between wound healing–related apoptosis and heat shock protein (HSP) and Fas signaling is yet to be defined. In this study, we examined the acceleration of apoptosis induced by extracellular basic pH in a 3D human skin equivalent system (3HSE).

Materials and Methods

Chemical

Dulbecco's modified Eagle's medium (DMEM), opti-MEM, penicillin-streptomycin and fetal bovine serum (FBS), were purchased from Gibco (MD, USA). Hydrochloric acid, sodium hydroxide, and dimethylsulfoxide were purchased from Sigma-Aldrich (St. Louis, USA). Lipofectamine 2000 reagent was purchased from Invitrogen (Carlsbad, CA, USA). Si-RNAs (Fas Cat. No. 1051233, HSP60; Cat. No. 1071653, and p21 Cat. No. 1029367) were purchased from Bioneer Co. (Daejeon, South Korea). CytoScan™ LDH-cytotoxicity assay kit was obtained from Dojindo (MD, USA). Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis kit was obtained from Thermo Fisher Scientific (GL, USA). Biotinylated goat anti-rabbit antibody, normal goat serum, and VECTASTAIN Elite ABC Kit were purchased from Vector Labs (CA, USA).

Cell Culture and 3HSE

The 3HSE was established according to previously published methods [6]. Using Neoderm®-ED purchased from TEGO Science (Seoul, South Korea), human dermal fibroblasts (HDF) were cultured in collagen matrix for 1 day. Keratinocytes were then seeded on top of the collagen matrix and co-cultured for 4 days. Next, the keratinocyte and HDF blocks were lifted and exposed to air. The skin equivalent was then treated with pH 6.40–7.70 for 10 days and the medium was changed every 2 days for 10 days. The skin equivalent was incubated at 37°C under 5% CO_2 and the cell culture system was then established according to previously published methods [6]. The HaCaT human epidermal keratinocyte cell line was donated by Dr. HS Lim at KIOM, Korea [6]. Cells were maintained in DMEM supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin under an atmosphere of 95% air and 5% CO_2 at 37°C. Human primary keratinocyte (HPK) cells were purchased from TEGO Science. These cells were maintained in keratinocyte-SFM (Gibco-BRL Inc., NY, USA) under a humidified 5% CO_2 atmosphere at 37°C. All experiments were conducted 12 h after the cells were seeded on 96- and 24-well plates at densities of 1 and 2×10^4 cells/well, respectively. According to previously published methods, cultures were exposed to the culture medium at pH 5.35–8.41 or normal culture conditions.

Measuring Apoptosis-Related Proteins and LDH Levels

Apoptosis-related proteins were determined using an apoptosis membrane array kit based on the instructions in the manual. The cells were treated and 300 μg of protein from each sample were incubated with the human apoptosis array overnight. The apoptosis array data were quantified by scanning the membrane using an LAS-4000 mini system (Fujifilm Corporation, Tokyo, Japan); analysis of the array image file was performed with image analysis software based on the manufacturer's instructions. LDH release was measured using the CytoScan LDH-cytotoxicity assay kit according to the instruction manual. Cytotoxicity induced by pH treatment was assessed by measuring LDH released into the culture medium. Following exposure to the pH treatments, the culture medium was aspirated and centrifuged at 3,000 rpm for 5 min in order to obtain a cell free supernatant and LDH activity was then determined.

Skin Immunohistochemistry and Annexin V Analysis

Skin section preparations were generated as previously described. Sections (5 μm thick) of 10% neutral formalin solution-fixed paraffin-embedded tissues were cut on silane-coated glass slides, deparaffinized 3 times with xylene, and then dehydrated through a graded alcohol bath. Next, the sections were washed with phosphate-buffered saline (PBS) prior to immunostaining and then pretreated with 1% hydrogen peroxide for 15 min to remove endogenous peroxidase activity or blocked with 0.5% bovine serum albumin for 30 min to prevent nonspecific binding of the antibodies. The sections were then incubated overnight with a primary anti-antibody (1:1,000 dilution) in PBS containing 0.3% Triton X-100 and normal serum and subsequently with a secondary antibody (1:200 dilution) for 90 min, followed by incubation in ABC solution for 1 h at room temperature. The color was developed with 3,3′-diaminobenzidine for 3 min or streptavidin for 20 min. In addition, the deparaffinized sections and fixed cells were also stained with Annexin V/or propidium iodide following the manufacturer's protocol. The slides were incubated for 10 min with 4',6-diamidino-2-phenylindole (50 μg/mL). The images were acquired using a microscope (Olympus Microscope System BX51; Olympus, Tokyo, Japan). Quantification of the effects was performed by measuring the fluorescent density of Annexin V-positive cells at ×40 magnification using ImageJ software (MD, USA); data are presented as the percent of the pH 7.40 group values.

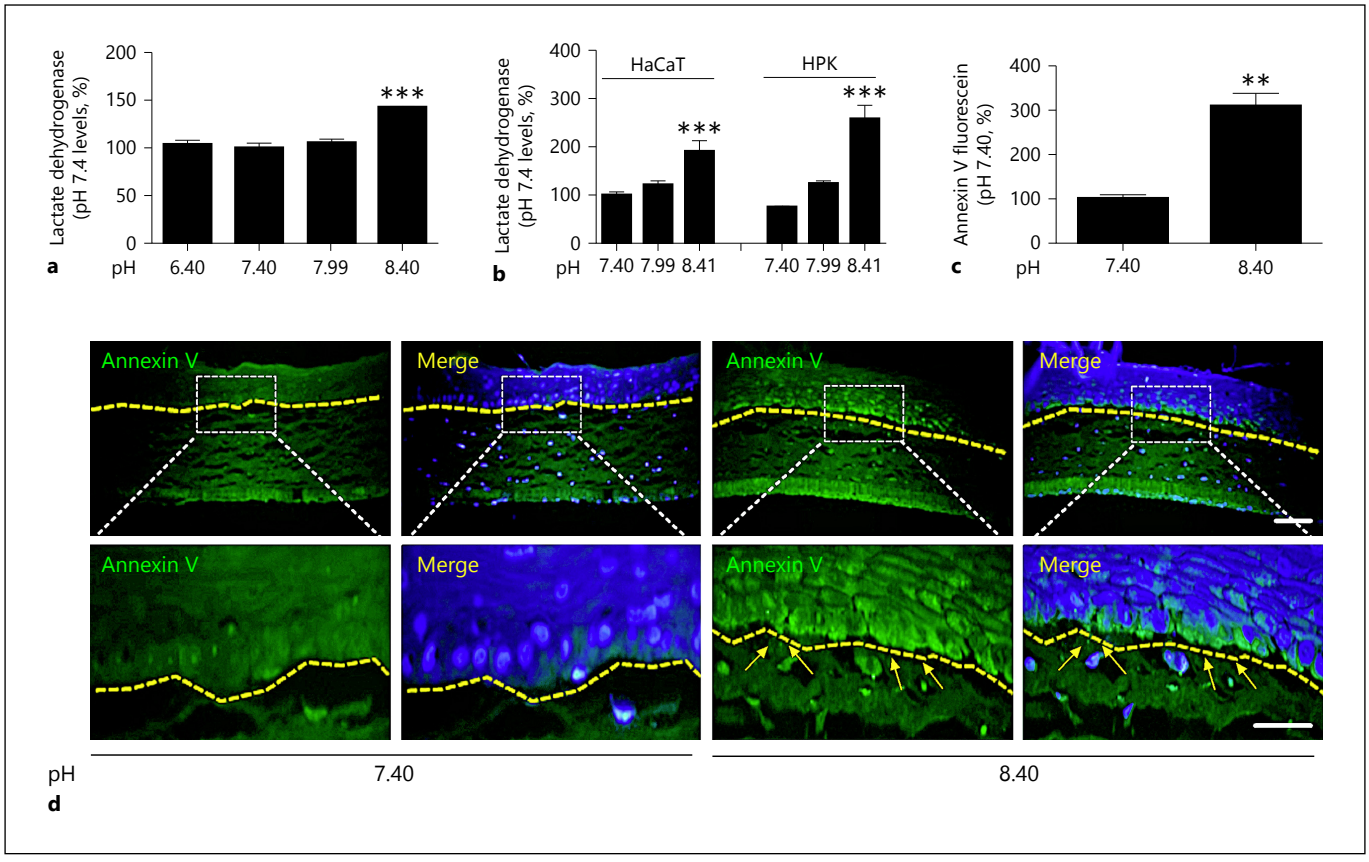


Fig. 1. Effect of basic pH on cell death or apoptosis in a 3HSE. 3HSE (**a**), HaCaT (**b**), and HPK toxicity under basic conditions were assessed using the LDH assay. **c** Annexin V levels were measured using Kit. **d** Representative photomicrographs of the 3HSE; scale bar = 100 μm. Values are presented as mean ± SEM. ** $p < 0.01$ and *** $p < 0.001$; one-way analysis of variance followed by Tukey's post hoc test was performed using the GraphPad Prism software.

Small Interfering RNA Transfection

Transfection of preparations was generated as previously described [7]. HaCaT cells were used at a confluence of 85–90% in 60-mm dish or 24-well plates. Cells were transfected with Stealth small interfering RNA (siRNA) using Lipofectamine 2000 (Invitrogen). Lipofectamine 2000 (25 μL) was mixed with 25 μM siRNA solution (an equimolar mix of Fas, HSP60, or p21 siRNA and scramble siRNA) and 2.5 mL of Opti-MEM medium Gibco (MD, USA). Following incubation for 3 h at room temperature, 300 μL of the mix were added to 300 μL of serum-free DMEM in each dish and incubated for 24 h.

Results and Discussion

Previous studies have shown that extracellular basic pH in skin accelerates collagen breakdown, which is an important effector of wound healing. Apoptosis signaling is a key factor in the wound-healing process that can be utilized to improve treatment strategies. Moreover, a number of studies have already demonstrated that apoptosis signaling correlates with wound healing under basic pH skin conditions [5]. In this study, we examined accelerated apoptosis induced by extracellular basic pH in a 3HSE.

To evaluate the effect of basic pH on cell death in the 3HSE, we performed an LDH assay. The LDH assay results demonstrate that the cytotoxicity of the 3HSE was not affected by treatment at pH 6.40–7.99. However, pH 8.40 caused increased cytotoxicity (Fig. 1a; 142.47 ± 3.81% of the corresponding pH 7.40 values). In addition, we confirmed that human immortal keratinocyte HaCaT cells (Fig. 1b; 190.52 ± 25.04% of the corresponding pH 7.40 values) and HPK cells (Fig. 1b; 257.44 ± 32.57% of the corresponding pH 7.40 values) responded to increased toxicity at pH 8.41. Similarly, our previous study showed that mitogen-activated protein kinase signaling and ROS generation were significantly increased by pH values >7.64 in human fibroblast skin cells or 3HSE [6].

In addition, human fibroblast HS68 cells responded to increased pH toxicity by inducing NADH dehydrogenase activity [6]. To assess whether basic pH increases apoptosis, we measured Annexin V levels. In normal live cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However, in early apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. Annexin V, a Ca^{2+}-dependent phospholipid-binding protein, has high affinity for PS and fluorochrome-labeled Annexin V can be used to detect exposed PS [8, 9]. In this study, 3HSE exposed to pH 8.40 exhibited significantly induced FITC-labeled Annexin V values (Fig. 1c, d; 309.10 ± 36.23% of the corresponding pH 7.40 values).

In order to further investigate the mechanism by which extracellular basic pH induces apoptosis, the profiles of various apoptosis-related proteins were examined using apoptosis array analysis. The apoptotic pathways associated with skin apoptosis involve several mechanisms; the "extrinsic" pathway is triggered by the binding of the Fas ligand or tumor necrosis factor (TNF) to membrane death receptors (DRs) that recruit adapter molecules leading to the activation of caspase-8 [10]. The "intrinsic" pathway includes mitochondrial release of cytochrome c and, along with the cofactor apoptotic protease activating factor-1, the formation of an activated caspase-9 apoptosome [10]. Mitochondria might also trigger apoptosis through the release of a second mitochondria-derived activator of the caspase/Diablo homolog, which blocks the inhibition of apoptosis, or via an apoptosis-inducing factor that mediates caspase-independent apoptosis [11]. Both pathways end in DNA cleavage followed by the formation of apoptotic bodies and phagocytosis by neighboring cells [11]. Apoptosis is controlled by B-cell lymphoma 2 (Bcl-2) family proteins, several of which (Bcl-2 and Bcl-extra large [Bcl-xL]) may block apoptosis, whereas others such as Bcl-2-like protein 4 (Bax), Bcl-2 homologous antagonist/killer (Bak), and BH3 interacting-domain death agonist (Bid) stimulate apoptotic process [11]. The array results demonstrate that the expression of DRs-related protein Fas, serine protease (HtrA), and inhibitors of the cell cycle protein p21 (red zone) were induced (Fig. 2a, b). Moreover, the expression of HSP70 and 60 proteins (red zone) was dramatically induced (Fig. 2a, c). Additionally, the expression levels of Bcl-2-associated death promoter (Bad), caspase 8, CD40 ligand, cytochrome c, Fas ligand, Insulin-like growth factor (IGF) 2, IGF-binding protein (IGRBP) 5, IGRBP6, p53, second mitochondria-derived activator of caspases, TNF-related apoptosis-inducing ligand (TRAIL) R2, and X-linked inhibitor of apoptosis protein (orange zone) were slightly increased by basic pH (Fig. 2a). In contrast, the expression levels of Bid, DR 6, IGF-1, IGFBP1, IGFBP3, IGFBP4, IGF 1sR, Livin, Survivin, soluble TNF receptor 1, TNF-α, and TRAILR4 (white zone) were not affected by basic pH (Fig. 2a). Fas (CD95/Apo-1) is a 45 kDa cell-surface receptor belonging to the TNF/nerve growth factor receptor family, which is highly expressed in activated lymphocytes and a variety of cells of lymphoid or non-lymphoid origin as well as tissues and tumor cells. Fas ligand (FasL/CD95L) is a membrane protein belonging to the TNF family [12]. Although the expression of FasL was originally thought to be restricted to activated T cells and natural killer cells, it is now known that FasL is widely expressed and functional in many tissues [12]. The binding of 3 Fas molecules to a FasL homotrimer leads to the subsequent binding of Fas-associated death domain (FADD) and procaspase-8. The formation of this complex, known as the death-inducing signaling complex, triggers a cascade of caspase activation, including caspase-3, leading to cell death [13]. In addition to the recruitment of FADD, the Fas-induced apoptosis pathway can also be mediated by a receptor-interacting protein (RIP), RIP associated ICH/CED-3-homologous protein with a death domain, and procaspase-2 [13]. Fas-mediated apoptosis of keratinocytes has multiple functions and has been implicated in various conditions such as allergic contact dermatitis, wound healing, lupus erythematosus, toxic epidermal necrolysis, and the formation of sunburn cells by UV radiation [14]. Recent studies have demonstrated Fas expression associated with apoptotic keratinocytes in aging human epidermis [15]. In particular, Hosono-Nishiyama et al. [16] showed occasional premature apoptotic cells in the spinous and basal layers, pointing to possible age-related changes in the rate of apoptosis. Thus, based on the Annexin V results, we chose to focus on early stage-related apoptotic proteins, such as Fas/FasL and further examined the expression of Fas proteins by immunohistochemical analysis. We found that the expression of Fas in the epidermal layer was dramatically increased in 3HSE (Fig. 2d). In addition, LDH levels in cells with significant Fas siRNA transfection efficiency and cells with slight p21 siRNA transfection efficiency were significantly decreased by pH 8.41, compared with siRNA-control-transfected cells treated with pH 8.41 (Fig. 2e; 50.02 ± 6.67 and 82.19 ± 19.52%). Moreover, FITC-labeled Annexin V values decreased in Fas and slightly p21 siRNA-transfected cells treated with pH 8.40 compared with siR-

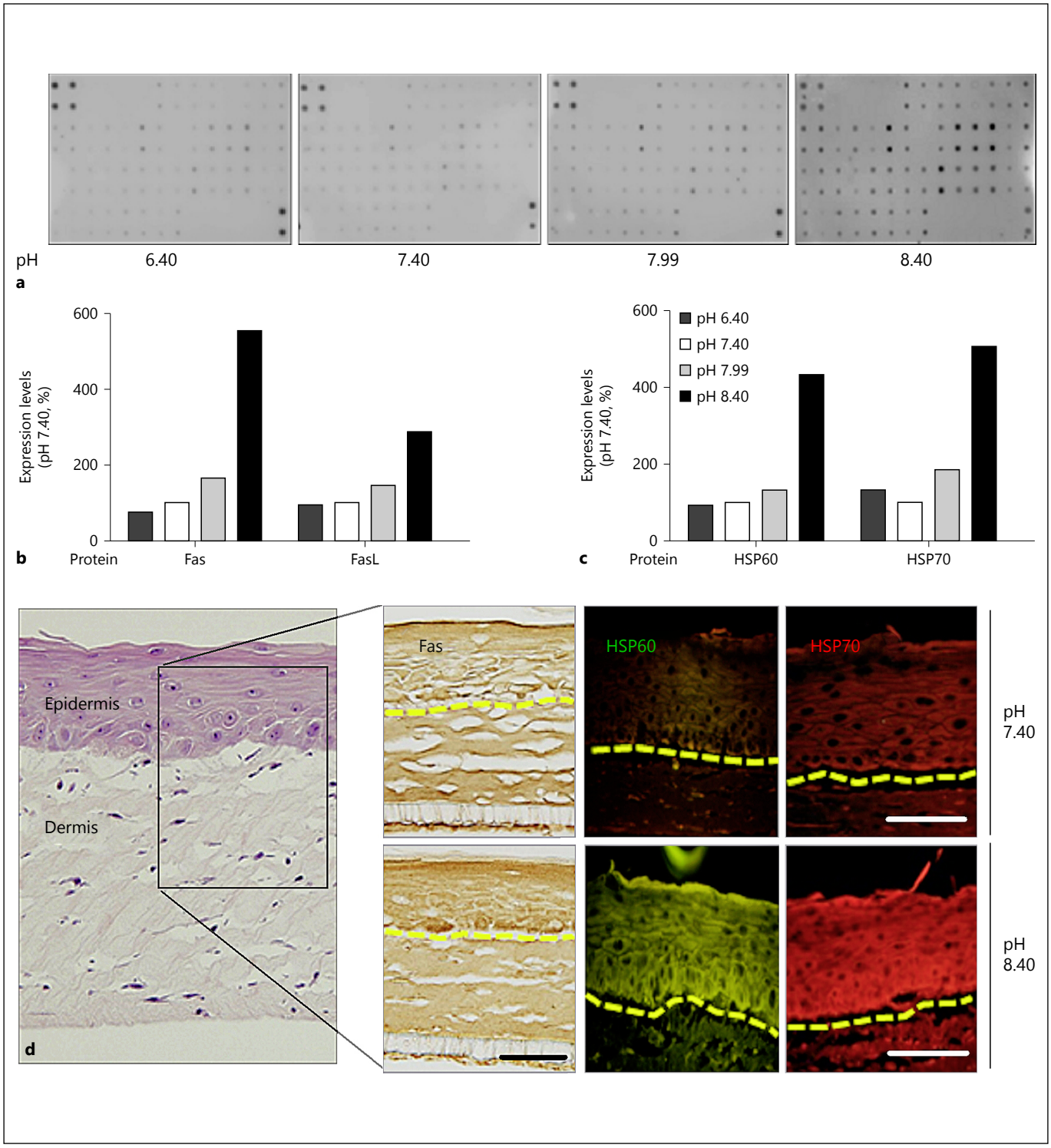
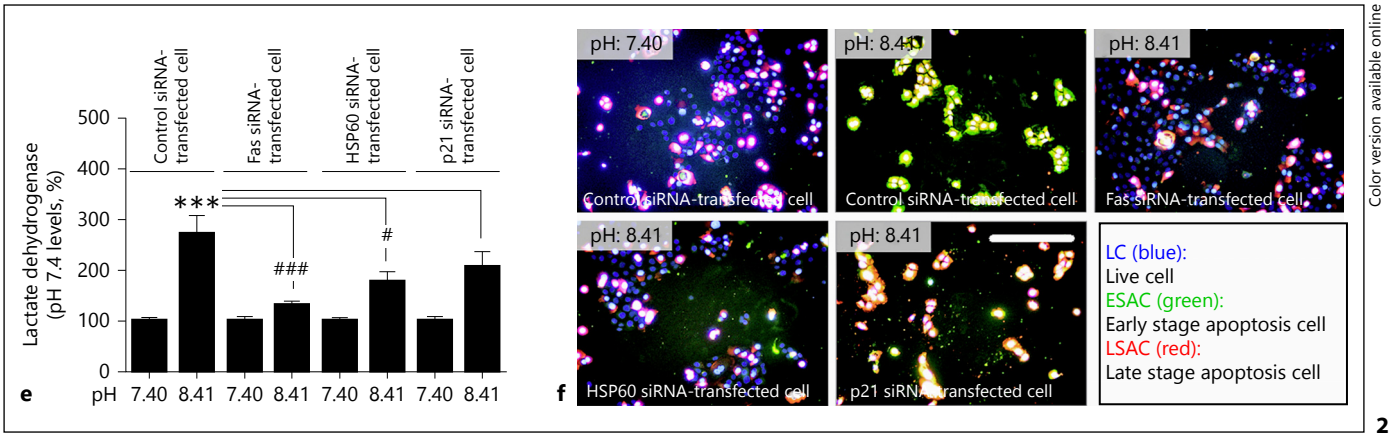


Fig. 2. Protein arrays of the 3HSE. **a** Representative images of the apoptotic protein array at pH 6.40–8.40; the grid details the proteins represented by each dot in the array. Densitometric ratios of the arrays showed differences in the apoptotic markers Fas and FasL (**b**) and HSP60 and HSP70 (**c**). **d** Immunohistochemical analysis of Fas, HSP60, and HSP70 protein levels in a 3HSE block treated with basic pH 8.40. Moreover, Fas, HSP60, and p21 knockdown reduced the levels of LDH (**e**) and Annexin V (**f**) induced by basic pH 8.40 in siRNA transfected HaCaT cells. Scale bar = 100 or 50 μm. Values are presented as mean ± SEM. *** $p < 0.001$, # $p < 0.05$, and ### $p < 0.001$; one-way analysis of variance followed by Tukey's post hoc test was performed using the GraphPad Prism software.

(*For figure 2e, f see next page.*)



NA-control-transfected cells treated with pH 8.40 (Fig. 2f). Because HSP-dependent pathways are involved in apoptosis regulation, we next examined HSP70 expression levels. As mentioned above, the array results demonstrated that the expression of HSP70 and 60 proteins (red zone) was dramatically induced by basic pH. Moreover, immunohistochemistry analysis revealed that the expression of HSP60 and HSP70 was dramatically increased in the epidermal layer. Further, LDH levels were significantly decreased in HSP60 siRNA-transfected cells treated with pH 8.41 compared with siRNA-control-transfected cells exposed to pH 8.41 (Fig. 2e; 50.02 ± 6.67, 69.45 ± 14.59, and 82.19 ± 19.52%). Moreover, FITC-labeled Annexin V values decreased significantly in HSP60 siRNA-transfected cells treated with pH 8.40, compared with siRNA-control cells exposed to pH 8.40 (Fig. 2f). Recent findings indicate that HSPs may exert pro- and anti-apoptotic functions; HSP27 and HSP70 are anti-apoptotic, while HSP60 is pro-apoptotic [17]. HSPs function at multiple points in both the intrinsic and extrinsic apoptotic signaling pathways [17]. Modulation of the intrinsic pathway involves the binding of HSP27 to cytochrome c and HSP70 to apoptotic protease activating factor-1, resulting in the inhibition of apoptosome formation and thus the prevention of caspase-9 maturation [18]. In this part, expression levels of cytochrome c are induced by basic pH via non-binding HSP27 proteins by its protein downregulation [19]. In contrast, HSP60 can directly promote the proteolytic maturation of caspase-3. Modulation of the extrinsic pathway involves HSP27 interacting with and inhibiting the death-associated protein, 6 apoptotic pathway, while HSP70 binds to JNK 1 mitogen-activated protein kinases, resulting in the inhibition of JNK activation [19]. Interestingly, our previous study indicated that skin fibroblast cells and 3HSE exposed to an acidic pH of 6.04–6.48 showed a slight but significant elevation in p38 mitogen-activated protein kinase levels at pH 6.48; ERK and JNK protein levels remained unchanged. In contrast, skin fibroblast cells and 3HSE exposed to a basic pH of 7.64–7.99 exhibited significantly increased ERK, JNK, and p38 protein levels. These findings indicate that HSP70 does not interrupt JNK activation induced by extracellular basic pH skin conditions. However, the pathogenic mechanisms underlying these responses are not completely understood. The results of our current study suggest that basic pH conditions increase early-stage apoptosis through Fas/FasL via the modulation of HSP27, HSP60, and HSP70. Taken together, the findings of the present study using 3HSE systems suggest that apoptosis is accelerated in a highly basic pH environment. Additional basic and clinical studies will be required to further elucidate the mechanism(s) underlying this phenomenon. Importantly, the effect of pH imbalance on the apoptosis processes involved in skin diseases including wound healing and atopy dermatitis suggests that the development of pH-regulating or maintenance materials may lead to the formulation of effective therapeutic strategies for maintaining good skin health.

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Disclosure Statement

The authors declare no conflicts of interest.

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