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Human Corneal Epithelial Cells Produce Antimicrobial Peptides LL-37 and β-Defensins in Response to Heat-Killed *Candida albicans*

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Abstract

Aims—To explore the innate response of human corneal epithelial cells (HCECs) exposed to fungus by producing antimicrobial peptides LL-37 and β-defensins.

Methods—Primary HCECs were treated with heat-killed *Candida albicans* (HKCA) at different doses (10^3 –10^6 cells/ml) for 2–48 h. The cells were subjected to total RNA extraction, reverse transcription and quantitative real-time PCR for mRNA expression. Cells treated for 48 h were used for immunofluorescent staining and ELISA.

Results—Human LL-37 and β-defensins (hBDs) 1–4 were detected in normal HCECs. The mRNA expression of LL-37, hBD2, and hBD3 was dose-dependently induced by HKCA with their peak levels at 4 h. HKCA (10^6 cells/ml) stimulated the mRNA of LL-37, hBD2, and hBD3 4.33 ± 1.81, 3.75 ± 1.31, and 4.91 ± 1.09 fold, respectively, in HCECs. The stimulated production of LL-37, hBD2, and hBD3 by HKCA was confirmed at protein levels by immunofluorescent staining and ELISA. The protein production of LL-37, hBD2, and hBD3 significantly increased to 109.1 ± 18.2 pg/ml, 4.33 ± 1.67 ng/ ml, and 296.9 ± 81.8 pg/ml, respectively, in culture medium of HCECs exposed to HKCA (10^6 cells/ml) compared to untreated HCECs.

Conclusions—HCECs produce antimicrobial peptides, LL-37, hBD2 and hBD3, in response to stimulation of HKCA, which suggests a novel innate immune mechanism of the ocular surface in defense against fungal invasion.

Keywords

Ocular surface; Epithelium; Antimicrobial peptides; LL-37; Defensin; Fungus; Innate immunity

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Disclosure Statement

None of the authors have any financial and commercial conflicts of interests.

Introduction

In addition to functioning as a physical barrier, the epithelia of skin and mucosal tissues, such as the ocular surface, have been recognized to be an important immune organ with various defense mechanisms, including natural epithelial defense, innate immunity, and antigen-elicited adaptive immunity. The innate immune cells, represented primarily by monocytes, macrophages, dendritic cells, granulocytes, and epithelial cells, are the first line of host defense and are responsible for immediate recognition and control of microbial invasion see review articles [1–4]. In addition, antimicrobial peptides (AMPs) are innate host defense molecules that have a direct effect on bacteria, fungi, and enveloped viruses [5–7] .

AMPs are small cationic host defense peptides secreted by a variety of cell types. Throughout the animal and plant kingdoms, genetically encoded endogenous AMPs have been shown to be key elements in the response to epithelial compromise and microbial invasion. In mammals, a variety of such peptides have been identified, including the well-characterized defensins and cathelicidins [8, 9] . AMPs have also been recognized to play an important role in host defense against ocular surface infection [6, 10–13] . At the ocular surface, AMPs are expressed strongly by neutrophils and epithelial cells in combating infection that may result from environmental challenges such dust, trauma, or wearing contact lenses [14] . AMPs are widely present on the ocular surface, and include cathelicidin (LL-37) [15], human β-defensins (hBDs) 1–4 [15] and hBD9 (gene analog of defensin β109) [16], liver-expressed antimicrobial peptides (LEAP)-1 and LEAP-2, and RNase-7 [17] . However, few studies reported AMP production by ocular surface epithelium in response to fungal infection.

Fungal keratitis is a leading cause of ocular morbidity. The incidence of fungal keratitis is on the rise in the densely populated continents of Asia and Africa, whereas it is relatively less common in developed countries [18, 19]. Fungal infections of the cornea are frequently caused by species of *Fusarium*, *Aspergillus*, *Curvularia*, and *Candida*, with trauma being the most important predisposing cause [20, 21] . *Candida albicans* is a commensal fungus of the normal flora, but it causes opportunistic infections of the cornea, especially after trauma or surgery, during periods of immunosuppression (such as prolonged corticosteroid use) and in topical anesthetic abuse [20, 22] . *C. albicans* breaches the eye's defenses and penetrates the stroma when organisms change into filamentous forms [23] . Hyphal invasion elicits host responses that may cause corneal inflammation and ulceration [24] . Innate immunity and acute inflammation actively participate in the pathogenesis of fungal keratitis. The cornea detects the invading *C. albicans* by Toll-like receptors and other pathogen-recognition molecules [25] . Corneal epithelial cells, keratocytes, and phagocytes are involved in distinguishing pathogen-associated molecular patterns, and this interaction activates innate responses.

Factors that might enable otherwise vulnerable epithelial cells to form a resistant barrier in vivo include extra-epithelial molecules (e.g., factors in tear fluid or the basement membrane) or epithelial cell-derived factors differentially expressed in the in vivo environment. Potential candidates include secretory IgA [26], surfactant proteins [27], mucin

glycoproteins [28], and epithelium-derived AMPs, which can inhibit or kill microbes [5–7] . This study set out to explore the stimulated expression and production of cathelicidin LL-37 and hBD1–4 by human corneal epithelial cells (HCECs) in response to heat-killed *C. albicans* (HKCA).

Materials and Methods

Materials and Reagents

Dry HKCA was purchased from Invitrogen (Carlsbad, Calif., USA). Cell culture dishes, plates, centrifuge tubes, and other plastic ware were purchased from Becton Dickinson Co. (Franklin Lakes, N.J., USA). Nunc Lab-Tec II 8-chamber slides were from Nalge Nunc International Corp. (Naperville, Ill., USA). Dulbec-co's modified Eagle's medium, Ham F-12, HEPES, amphotericin B, gentamicin, and 0.25% trypsin/EDTA solution were from Invitrogen-GIBCO BRL (Grand Island, N.Y., USA). Fetal bovine serum was from Hyclone (Logan, Utah, USA). Enzyme-linked immunosorbent assay (ELISA) kits for human LL-37, hBD2, and hBD3 were from Hycult Biotech (Plymouth Meeting, Pa., USA), GenWay Biotech, Inc. (San Diego, Calif., USA), and Alpha Diagnostic International, Inc. (San Antonio, Tex., USA), respectively. Affinity-purified goat polyclonal antibody (Ab) against human LL-37 and rabbit polyclonal Abs against hBD2 and hBD3 were from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Fluorescein Alexa-Fluor 488-conjugated secondary Abs (donkey anti-goat IgG and donkey anti-rabbit IgG) were from Molecular Probes (Eugene, Oreg., USA). Ready-To-Go You-Prime First-Stand Beads were purchased from GE Health Care (Piscataway, N.J., USA). TaqMan Gene Expression Assay and real-time PCR Master Mix kits were from Applied Biosystems (Foster City, Calif., USA). RNeasy Mini kits were from Qiagen (Valencia, Calif., USA). Bovine insulin, human transferrin, hydrocortisone, human epidermal growth factor, cholera toxin A subunit, propidium iodide (PI), and all other reagents came from Sigma-Aldrich (St. Louis, Mo., USA).

Primary HCEC Cultures for AMP Induction by HKCA

Fresh human corneoscleral tissues from donors aged 23–64 years (within 72 h postmortem) which did not meet the criteria for clinical use were obtained from the Lions Eye Bank of Texas (Houston, Tex., USA). Human tissues were handled according to the tenets of the Declaration of Helsinki. Human limbal epithelial cells were cultured using explants from corneal limbal rims according to our previously published protocols [29, 30] . In brief, each limbal rim was trimmed and dissected into 2 × 2 mm explants. Each piece was placed in a supplemented hormonal epidermal medium containing 5% fetal bovine serum and incubated at 37° C under 5% CO 2 and 95% humidity. Corneal epithelial cell growth was carefully monitored, and the media were renewed every 2–3 days. Only the epithelial cultures without visible fibroblast con tamination were used for this study. Confluent corneal epithelial cultures were switched to serum-free supplemented hormonal epidermal medium for 24 h and then treated by HKCA for different time periods (2, 4, 8, 24, or 48 h) with multiple concentrations. Each experiment was repeated at least 3 times.

Total RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR

Total RNA was extracted from corneal tissues or HCECs using a Qiagen RNeasy Mini kit according to the manufacturer's protocol, then quantified by NanoDrop ND-1000 Spectrophotometer and stored at –80° C. The first-strand cDNA was synthesized by reverse transcription (RT) from 1 μg of total RNA using Ready-To-Go You-Prime First-Strand Beads as previously described [31, 32] . The real-time PCR was performed using the Mx3005P system (Stratagene) with 20 μl total reaction volume containing the following: 5 μl of cDNA that was generated from 50 ng of total RNA, 1 μl of TaqMan Gene Expression Assay primers and probe for human LL-37 (Hs00189038_ml), hBD1 (DEFB1, Hs00174765), hBD2 (DEFB4, Hs00175474_ml), hBD3 (DEFB103, Hs00218678_ ml), hBD4 (DEFB104, Hs00414476) or GAPDH (Hs99999905_ m1), and 10 μl TaqMan Gene Expression Master Mix. The ther-mocycler parameters were 50 ° C for 2 min, 95 ° C for 10 min, followed by 40 cycles at 95 ° C for 15 s and at 60 ° C for 1 min. A nontemplate control was included to evaluate DNA contamination. The results were analyzed by the comparative threshold cycle method and normalized by GAPDH, and then presented as relative fold changes in the expression levels in the treated groups versus the untreated controls [30, 32].

Immunofluorescent Staining

The HCECs on 8-chamber slides were fixed with 100% methanol at 4 ° C for 10 min and permeabilized with 0.2% Triton X-100 in PBS at room temperature for 10 min. Primary goat polyclonal Ab against human LL-37 and rabbit polyclonal Abs against hBD2 or hBD3 (1: 50, 4 mg/ml) were used for indirect immunofluorescent staining as described previously [33, 34]. Alexa-Fluor 488-conjugated secondary Abs were applied, and PI was used for nuclear counterstaining. Secondary Abs alone without primary Abs or isotype IgG were used as negative controls. The results were photographed with epifluorescence microscopy (Eclipes 400; Nikon, Garden City, N.Y., USA) using a digital camera (DMX 1200; Nikon).

Enzyme-Linked Immunosorbent Assay

The supernatants of culture media were collected from HCECs with or without exposure to HKCA for 48 h and stored at –80 ° C before use. Double-sandwich ELISA for human LL-37, hBD2 and hBD3 was performed according to the manufacturer's protocols. The microplates were read by Tecan Infinite M200 with Magellan V6.55 software. The replicate samples were averaged. Results are presented as mean ± SD from 5 separated experiments.

Statistical Analysis

Student's t test was used to compare the differences between the 2 groups. One-way ANOVA test was used to make comparisons between the 3 groups, and the Dunnett test was further used to compare each treated group with the control group. Statistical significance was set at $p < 0.05$.

Results

LL-37 Expression Was Stimulated in Primary HCECs Exposed to HKCA

The only human cathelicidin is LL-37, which plays important roles in mucosal defense. LL-37 is a member of a large family of cationic AMPs expressed in many species; it has a broad spectrum of antimicrobial activity and many immunmodulatory effects. LL-37 is mainly produced by neutrophils, macrophages, and various epithelial cells.

In this study, RT-quantitative real-time PCR (qPCR) with GAPDH as an internal control was used to determine the mRNA expression of LL-37 in primary HCECs challenged with HKCA at different time periods (2–24 h) and concentrations (10^3–10^6 cells/ml); untreated cultures were maintained as controls. LL-37 mRNA was expressed at low levels in primary cultured HCECs. Its expression increased significantly with stimulation by HKCA at 10^6 cells/ml, peaking at 4 h (fig. 1a). The stimulation of LL-37 by HKCA was in a concentration-dependent manner. HKCA at 10^3 –10^4 cells/ml did not increase the LL-37 expression, while 10^5 –10^6 cells/ml of HKCA significantly stimulated LL-37 mRNA levels up to 4.33 ± 1.81 fold when compared with controls (fig. 1 b).

The stimulated LL-37 production was further quantified by ELISA in the medium supernatants of HCECs. As shown in figure 1 c, the secreted LL-37 in the media was 27.9 ± 3.4 pg/ml in normal controls, and increased to 39.4 ± 5.1, 63.2 ± 7.9 ($p < 0.05$), and 109.1 ± 18.2 pg/ml ($p < 0.01$) in HCECs exposed for 48 h to HKCA at 10^4, 10^5, and 10^6 cells/ml, respectively. Immunofluorescent staining was performed to further confirm LL-37 protein production in HCECs. As shown in figure 1 d, LL-37 protein was detected in the cytoplasm of some cells with weak positive staining of LL-37 Ab in the untreated HCEC controls. The LL-37 immunoreactivity was markedly increased in the HCEC cultures exposed to HKCA at 10^6 cells/ml for 48 h.

Differential Induction of β-Defensins in HCECs Exposed to HKCA

The hBDs are expressed widely in skin and mucosal epithelia where they contribute to initial host defense. In general, hBDs are secreted from epithelial cells in mature form either onto to the cell surface or into the fluid surrounding these cells. The hBD1–4 are the most important defensins produced by human epithelial cells in response to infectious or inflammatory stimuli. However, it is not clear how hBDs are used by HCECs to respond to fungus.

Using RT-qPCR, we detected the expression of hBD1– 4 in the untreated primary HCECs. The expression of hBD1 appeared to be most abundant, while hBD4 was expressed at very low levels in HCECs. Interestingly, the expression of both hBD1 and hBD4 were constitutive since their mRNA levels did not significantly increase or decrease in HCECs exposed to HKCA in different concentrations (10^3 –10^6 cells/ml) for the different time periods (4–24 h). In contrast, hBD2 and hBD3 were expressed at levels between hBD1 and hBD4 in normal HCECs as compared with their threshold cycle values in real-time PCR. These two hBDs appeared to be largely inducible as their mRNA levels were markedly increased in the HCECs challenged by HKCA (fig. 2). The mRNA levels of both hBD2 and hBD3 reached peak levels at 4 h after stimulation by HKCA at 10^6 cells/ml (fig. 2a), and

the stimulated mRNA expression by HKCA was concentration-dependent (fig. 2 b). The mRNA expression of hBD2 and hBD3 increased 3.75 ± 1.31 and 4.91 ± 1.09 fold, respectively, in HCECs exposed to HKCA at 10^6 cells/ml (fig. 2 b).

Similar to their mRNA expression, HCECs produced significantly higher levels of hBD2 and hBD3 proteins in the culture media by HKCA in a dose-dependent manner (fig. 2 c). The production of hBD2 in normal HCECs was 1.28 ± 0.33 ng/ml, and significantly increased to 1.75 ± 0.37 ng/ml, 3.31 ± 1.21 ng/ml ($p < 0.05$), and 4.33 ± 1.67 ng/ml ($p < 0.01$) in HCECs exposed to HKCA at 10^4, 10^5, and 10^6 cells/ml, respectively. The production of hBD3 was much lower than that of hBD2 by HCECs. It was 60.2 ± 10.4 pg/ml in normal controls, and significantly increased to 102.1 ± 27.1 pg/ml ($p < 0.05$), 167.2 ± 28.1 pg/ ml ($p < 0.01$), and 296.9 ± 81.8 pg/ml ($p < 0.001$) in HCECs challenged by HKCA at 10^4, 10^5, and 10^6 cells/ml, respectively. The induction of these two hBDs by HKCA was further confirmed by immunofluorescent staining. As shown in figure 2 d, the protein production of hBD2 and hBD3 was observed in the cytoplasm of most cells of untreated primary HCECs, and their immunoreactivity was markedly increased in the cells exposed to HKCA at 10^6 cells/ml for 48 h.

Discussion

Mucosal surfaces are under constant threat of pathogenic attack [35] . The production of AMPs is one of the critical protective mechanisms in innate immune responses to various infections and injuries. AMPs are known to have diverse functions, including direct antimicrobial activity, phagocyte chemotaxis, and contribution to wound healing [6, 36–38] . The eye, in particular the cornea, is a special case. The avascular cornea is poorly served by blood vessels, and corneal damage can lead to visual loss due to serious infection or the subsequent inflammatory response [39] . The production of AMPs by corneal epithelium is of particular significance because many AMPs are inducible by inflammatory stimuli, and production is at the actual site of infection. AMPs including LL-37 and β-defensins have been documented to play important roles in protecting the ocular surface from infections by pathogens [10, 38, 40, 41]. However, AMP production by the ocular surface in response to fungus has not been documented. We hypothesized that AMPs may be an ocular defense mechanism for keratomycosis. The present study was to explore whether HCECs produce AMPs, LL-37, and hBDs in response to fungal challenge.

The wide expression among a broad range of organisms indicates the evolutionary conservation of the cathelicidin gene, part of the innate immune system. The cathelicidins contain a cathelinlike domain and an antimicrobial domain termed LL-37. Humans and mice express only one cathelicidin protein, named LL-37 and cathelicidin-related antimicrobial protein (CRAMP), respectively, while other species are able to express multiple cathelicidin peptides. LL-37 is derived from the cleavage of human cationic antimicrobial protein-18, and has been shown to mediate various host responses, including bactericidal action, chemotaxis, angiogenesis, epithelial wound repair, and activation of chemokine secretion [42] . Human LL-37 has been well known to have antimicrobial and immunomodulatory multifunctions against bacterial and viral infections [12, 42–46]. A few studies have revealed

that cathelicidin may have antifungal activity against *C. albicans* in skin infection [47]. However, the antifungal role of LL-37 in ocular surface disease has not been elucidated.

In our previous study [48], we have shown that the mouse cathelicidin gene CRAMP was upregulated in response to *C. albicans* in a murine model of experimental fungal keratitis. The CRAMP expression increased up to 40-fold during the onset of posttraumatic *C. albicans* keratomycosis. The CRAMP expression levels dose-dependently increased with increased fungal load. In the present study, we found that human LL-37 production was significantly increased in primary HCECs exposed to HKCA. This is demonstrated at both mRNA and protein levels as evaluated by RT-qPCR, ELISA, and immunofluorescent staining, respectively (fig. 1), suggesting a novel innate response of corneal epithelial cells in response to fungal infection.

Defensins, the small cysteine-rich cationic proteins, serve a central role in innate immunity in all species of plants and animals. In humans, defensins are widely expressed and found in neutrophils, in the skin, and in mucosal epithelia. Most defensins are potent antibiotics, and some have chemotactic and toxin-neutralizing activities [11, 12, 37, 49] . β-Defensins 1–4 are well-characterized members of the defensin family and display strong antimicrobial activity. For example, murine β-defensin (mBD)-3 was found to promote disease resolution in *Pseudomonas aeruginosa* infections. BALB/c mice were more susceptible to *P. aeruginosa* keratitis when expression of mBD2 or mBD3, but not mBD1 or mBD4, was knocked down by siRNA [49–51] . However, the hBD expression induced by fungus in primary human corneal epithelial cell has not been reported.

In this study, all four of these hBDs were detected in primary HCECs. hBD1 and hBD4 were constitutively expressed by HCECs, whereas hBD2 and hBD3 were inducible by HKCA at both mRNA and protein levels as evaluated by RT-qPCR, ELISA, and immunofluorescent staining (fig. 2). This pattern of hBDs expression in response to fungus was supported by previous reports that showed hBD2 expression is inducible by conditions in response to bacterial challenge [37, 52].

In conclusion, we have demonstrated that the human corneal epithelium expressed at least 2 groups of AMPs, LL-37 and hBDs, among which LL-37, hBD2, and hBD3 were largely inducible in response to HKCA. These findings suggest that the human corneal epithelium may participate in antifungus defense through producing LL-37 and β-defensins, a novel mechanism of innate immune response. Since the current therapies are often ineffective, several new treatment strategies for fungal keratitis have been studied, such as the new drug that targets fungal iron acquisition [53] and riboflavin/ultraviolet light-mediated crosslinking against fungal infection in ocular surface [54]. Based on our study, modifying the level of these multifunctional peptides through pharmacotherapy or gene therapy may provide new strategies for managing keratomycosis. Further studies are necessary to investigate the functional role of these stimulated AMPs in vitro using HCECs and in vivo using a mouse model of fungal keratitis.

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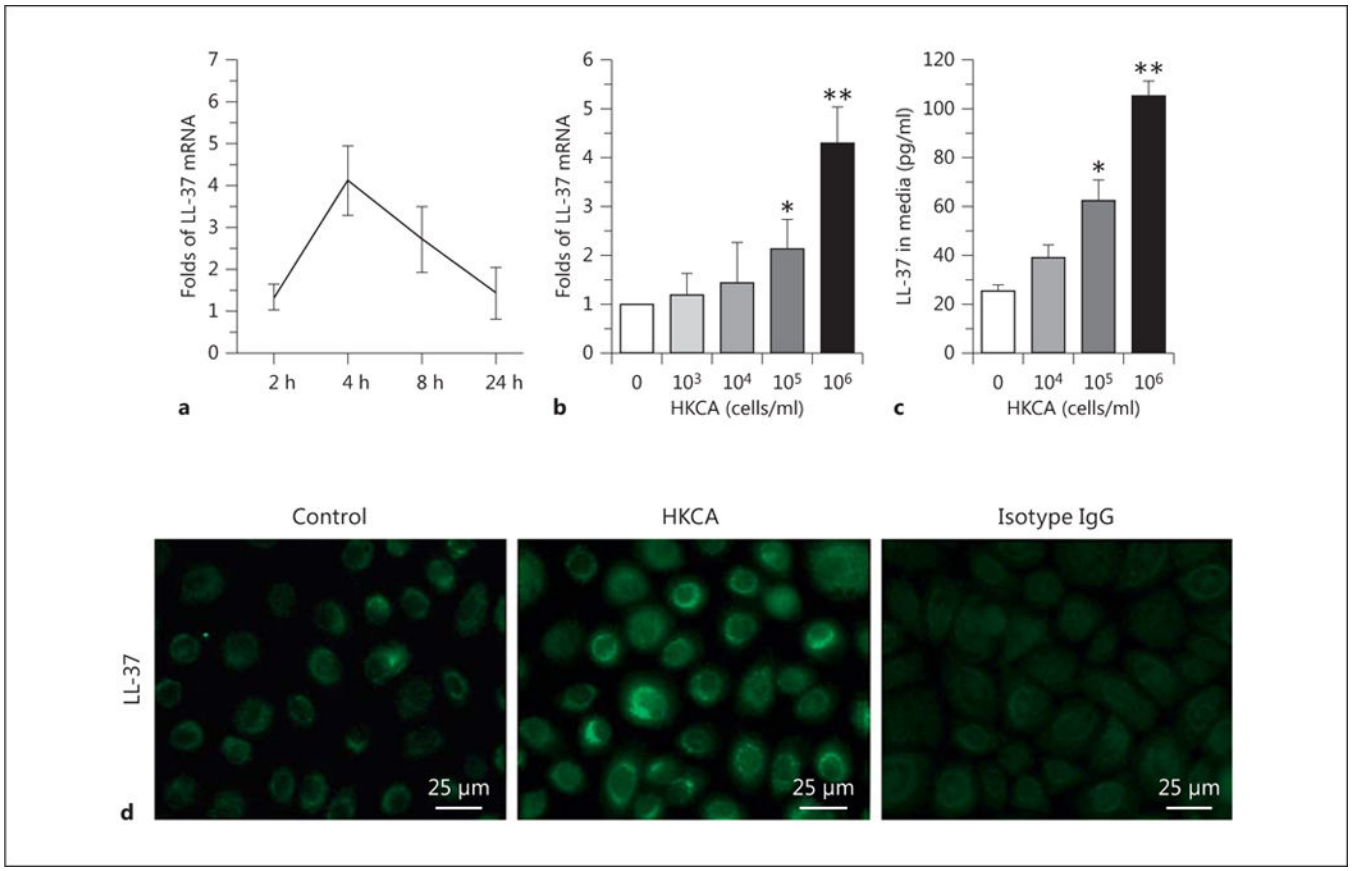

Fig. 1.

HCECs produce LL-37 in response to stimulation with HKCA. Primary HCECs were exposed to HKCA at different doses (10^3 –10^6 cells/ml) for different time periods (2–48 h), using untreated cultures as normal controls. **a** The time course of LL-37 mRNA induction in HCECs exposed to 10^6 cells/ml of HKCA evaluated by RT-qPCR with GAPDH as an internal control. **b** Dose-dependent stimulation of LL-37 mRNA in HCECs by HKCA for 4 h. **c** LL-37 protein levels were quantified by ELISA in the media supernatants of HCECs with or without exposure to HKCA for 48 h. Data were presented as mean ± SD (n = 5). *$p < 0.05$, $p < 0.01$ vs. controls. **d** Representative images showing immunofluorescent staining of LL-37 (green; colors refer to the online version ** only) with isotype IgG as negative control in HCECs exposed to HKCA (10^6 cells/ml) for 48 h. PI was used as nuclear counterstaining (red; data not shown). ×400.

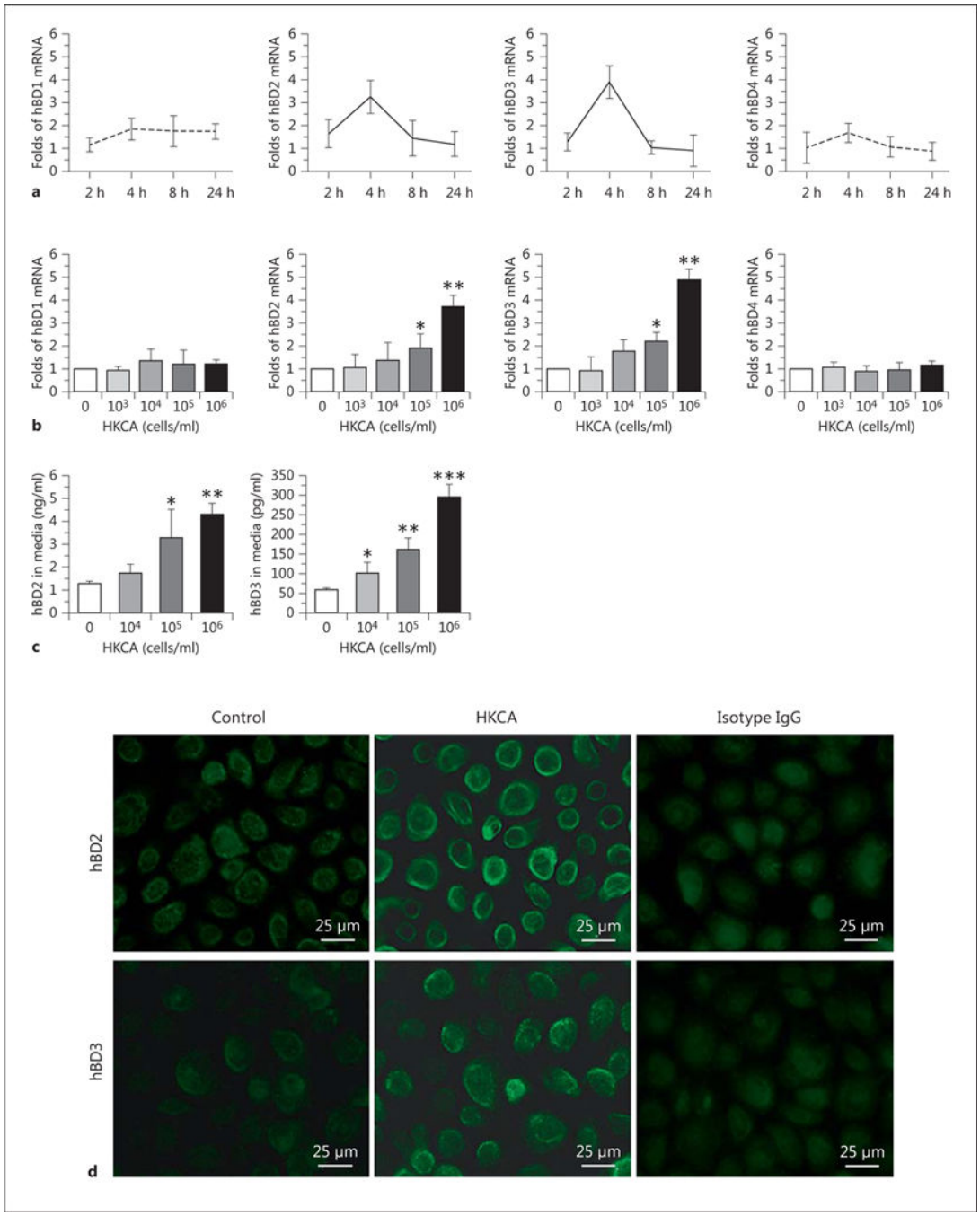

Fig. 2.

Differential responses of hBD1–4 in HCECs challenged by HKCA. Primary HCECs were exposed to HKCA at different doses (10^3 –10^6 cells/ml) for different time periods (2–48 h), using untreated cultures as normal controls. **a** The time course of mRNA induction of hBD1–4 in HCECs exposed to 10^6 cells/ml of HKCA evaluated by RT-qPCR. **b** Dose-dependent stimulation of mRNA expression of hBD1–4 in HCECs by HKCA for 4 h. **c** The protein levels of hBD2 and hBD3 were quantified by ELISA in the media supernatants of HCECs with or without exposure to HKCA for $p < 0.05$, $p < 48$ h. Data were presented as

mean ± SD (n = 5). * $p < 0.05$, **$p < 0.01$, *$p < 0.001$ vs. controls. **d** Representative images showing immunofluorescent staining of hBD2 and hBD3 (green; colors refer to the online version only) with isotype IgG as negative control in HCECs exposed to HKCA (10^6 cells/ml) for 48 h. PI was used as nuclear counterstaining (red; data not shown). ×400.