The Effect of *Candida albicans* on the Expression Levels of Toll-like Receptor 2 and Interleukin-8 in HaCaT Cells Under High- and Low-glucose Conditions

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**Abstract**

**Background:** The diabetics are prone to skin infections, especially with *Candida albicans*. It is important to elucidate the different antifungal abilities of patients with hyperglycemia and healthy controls for the treatment of this condition. The toll-like receptor 2 (TLR2) and interleukin (IL)-8 secreted by keratinocytes counteract *C. albicans*. **Aim:** This study aims to explore the differential expression of toll-like receptor 2 (TLR2) and interleukin (IL)-8 secretion by keratinocytes between controls and diabetic patients when challenged with *C. albicans*.

**Materials and Methods:** HaCaT cells were cultured in high-glucose (HG) Dulbecco’s modified Eagle’s medium (DMEM) and low-glucose (LG) DMEM. Then, they were exposed to *C. albicans* hyphae for 24 h. The expression levels of TLR2 and IL-8 were determined at different periods in both the HG and LG groups. Real-time polymerase chain reaction analysis, western blotting, and enzyme-linked immunosorbent assays were performed in this study. The morphological changes of HaCaT cells under two different glucose concentrations were also observed. **Results:** We found that the expression levels of both TLR2 and IL-8 increased and then decreased in the two groups. Notably, the IL-8 levels in the LG group were higher than those in the HG group at each time point (P<0.05), and the TLR2 levels in the LG group were higher than those in the HG group at the beginning of the experiment and after 24 h of treatment with *C. albicans* (P<0.05). In each group, the levels of IL-8 and TLR2 at the secretion peak were significantly different from those in the initial and the last period of observation (P<0.05). The cellular morphology of HaCaT cells treated with different concentrations of glucose was also similar. However, with prolonged coculture time, cell death increased. **Conclusion:** These observations showed that TLR2 and IL-8 act on the keratinocytes interacting with *C. albicans*, and HG status might affect the function of HaCaT cells by reducing the secretion of IL-8 and TLR2.

**Key Words:** *Candida albicans*, diabetes mellitus, HaCaT cell lines, interleukin-8, toll-like receptor 2

**Introduction**

The International Diabetes Federation has predicted that the worldwide epidemic of diabetes mellitus will reach 552 million by 2030, an increase of 7% compared to 366 million in 2011.[1] Although the major complications of diabetes are kidney disease, retinopathy, and peripheral neuropathy, skin infections are also common in diabetic patients and patients with hyperglycemia. Researchers have found that approximately 68% of diabetic patients had accompanying skin infections, which were the major cutaneous manifestations second only to pruritus.[6] *Candida albicans*, an opportunistic pathogen, can be found in the mouth, digestive tract, respiratory tract, vagina, and skin of healthy individuals as a commensal. Diabetic patients are susceptible to skin...
infections with *C. albicans*, which is one of the most common pathogens among diabetic patients in the clinic. However, the reasons for the susceptibility of diabetic patients to *C. albicans* are not well understood.

Keratinocyte resistance to *C. albicans* requires the combined action of the innate and adaptive immune systems. Toll-like receptors (TLRs) released from keratinocytes play a crucial role in the process of recognizing *C. albicans* and activating innate immunity.[3–5] TLRs are receptor proteins commonly observed on the surface of mammalian cells and are the major receptors for innate immune pattern recognition. Through the recognition of pathogen-associated molecular patterns (PAMPs), TLRs play a role in the anti-infection immunity that is primarily involved in the identification of pathogenic microorganisms and their products as well as inflammatory signal transduction.[6] When the skin and mucus membranes are infected by *C. albicans*, keratinocytes can identify the cell wall structure of mannans and transduce inflammatory signals through TLR2, resulting in the killing of *C. albicans* through production of nitric oxide (NO), β-defenseps, and other antimicrobial peptides.[7] However, past studies on this issue have resulted in conflicting findings.

Some chemokines released from keratinocytes are also involved in chronic inflammation and impaired wound healing in diabetic patients.[8] Several researchers have suggested that the production of pro-inflammatory cytokines could be directly induced by dermatophytes at the transcriptional level in human keratinocytes and that there are differences in the ability of keratinocytes to induce cytokine production with different dermatophytes.[9,10] The secretion of interleukin (IL)-8 was increased after stimulation with *C. albicans* in keratinocytes.[10,11] Phospholipomannan (PLM), a type of glycolipid expressed on the surface of the *C. albicans* cell wall, acts as a member of the PAMP family. Simultaneously, *C. albicans*-native PLM initiates inflammatory responses characterized by the production of cytokines (IL-6 and IL-8) and activation of the TLR2, nuclear factor-κB (NF-κB), and p38MAPK signaling pathways in the infected skin.[6,12]

Although there are many controversies about the function of TLR2 in keratinocytes infected with *C. albicans*, few researchers have investigated the differences in TLR2 between diabetic and normal skin infected with *C. albicans*. We hypothesized that TLR2 and IL-8 levels were significantly increased in keratinocytes under high-glucose (HG) conditions compared with those under normal-glucose conditions; thus, diabetic patients or patients with hyperglycemia are more easily infected with *C. albicans* due to the immune deficiencies. To verify our hypotheses, we cultivated HaCaT cell lines with *C. albicans* in HG and low-glucose (LG) media and measured the expression of TLR2 and IL-8 in HaCaT cells in both these conditions.

**Subjects and Methods**

**Cell culture and corresponding reagents**

The immortalized keratinocyte cell line HaCaT provided by our laboratory was recovered from liquid nitrogen and then cultured in 25 cm² cell culture flasks using HG Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, USA) in which the glucose concentration was 4.5 g/l and LG DMEM in which the glucose concentration was 1 g/l, both supplemented with 10% fetal bovine serum (FBS) (Life Technologies, USA), at 37°C in a humidified atmosphere containing 5% CO₂. The HG and LG concentrations used in our experiments were consistent with those previously used by other researchers. The day after cell recovery, the medium was replaced and then replaced every 2–3 days after the cells were washed with phosphate buffered saline (PBS) each time. When the cells reached 70–80% confluence, they were digested with 0.25% trypsin accompanied by 0.02% ethylene diamine tetraacetic acid (EDTA) in HEPES [N-(2-Hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)] and then subcultured. The subsequent experimental procedures were carried out using the second to fourth passage cells.

**Candida albicans hyphae induction**

*C. albicans* was kindly provided by the Mycology Laboratory of our hospital. Yeast cells were activated by being transferred two times in Sabouraud dextrose agar containing 5% chloramphenicol. In this study, the yeast was adjusted to 1 × 10⁷/ml in a blood cell counting plate in liquid Sabouraud medium and cultured at 150 r/min shaking speed under 37°C for 20 h to reach logarithmic growth phase. The yeast suspension was transferred to a centrifuge tube and centrifuged at 3000 r/min for 15 min with two PBS washes. Then, the yeast was adjusted to 2 × 10⁶/ml in RPMI1640 culture medium with 10% FBS and cultured with 150 r/min shaking speed under 37°C for 3 h. Staining by trypan blue showed that approximately 90% of *C. albicans* transformed into the hyphal phase with a viable count of more than 95% by microscopy.

**Coculture of HaCaT keratinocytes with Candida albicans**

When HaCaT cells with a good state of growth were passaged to the second or fourth generation and reached attachment to the plate of 80–90%, the cells in the HG and LG groups were inoculated on several 6 well plates with a density of 10⁴/ml in 2 ml for each well. Then, 1 ml RPMI1640 medium was added to the blank control wells, and 1 ml *C. albicans* suspension was added to the other wells. Overall, the ratio of *C. albicans* to HaCaT cells was 1:1. All the 6 well plates were incubated at 37°C with 5% CO₂.
Real-time polymerase chain reaction of toll-like receptor 2

Total RNA of the cocultured cells was extracted with TRIzol reagent (Gibco BRL, USA) according to the manufacturer’s instructions after 1, 6, and 24 h, and the purity and concentration of the sample were determined on a spectrophotometer. cDNA was then synthesized using 2 μg total RNA with a PrimeScript RT Reagent Kit (Taigen, Hilden, Germany) following the manufacturer’s specifications. Primer sequences were synthesized by Invitrogen (Carlsbad, CA, USA) (Table 1). Real-time polymerase chain reaction (RT-PCR) was then carried out with SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa Bio, Dalian, China) and ROX plus (TaKaRa Bio) on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). Multiple changes of mRNA expression were calculated using the 2^ΔΔCT method, where ΔCT is the difference between the genes TLR2 and β-Actin, and ΔΔCT for the sample = ΔCT of the actual sample – ΔCT of the lowest expression sample.12,13

Western blot analysis of toll-like receptor 2

The total protein of each well was obtained by the radioimmunoprecipitation assay lysis buffer after 1, 6, and 24 h of cocultivation. The protein concentration was measured using bicinchoninic acid assays (Solarbio, China). Different samples were assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and polyvinylidene difluoride (PVDF) membranes (Millipore Immobilon-P, Darmstadt, Germany). PVDF membranes were then blocked with PBS containing 5% nonfat dry milk and 0.05% Tween 20 for approximately 2 h.14 The PVDF membranes were probed with primary antibodies (TLR2: rabbit-derived mouse antibody, SAB1300199, Sigma, USA; β-Actin: mouse-derived antibody, TA-09, Zsbio, China) both at a dilution of 1:1000 overnight in 4°C and then incubated with the corresponding secondary antibodies conjugated to horseradish peroxidase (TLR2: rabbit antibody IgG, zb-2301, Zsbio, China; β-Actin: mouse antibody IgG, A4416, Sigma, USA) at a dilution of 1:5000 and 1:1000, respectively, for 2 h at room temperature. Protein bands were exposed and then photographed, and the gray value was measured with Gel-Pro Analyzer Software (Media Cybernetics, Rockville, MD, USA). The TLR2 level was standardized by the gray value ratio of TLR2/β-Actin.

Table 1: The primer sequences used for polymerase chain reaction

| Gene      | Primer sequences (5’ to 3’) |
|-----------|-----------------------------|
| TLR2 F    | TTTTCACTGCTTTCAACTGGTA      |
| TLR2 R    | TGGAGAGGCTGTAGATGAC         |
| β-actin F | GCATGGGTCAGAAGGATTCCCT     |
| β-actin R | TGTCGCCAGTGGGTTGACGAT      |

TLR: Toll-like receptor

Analysis of interleukin-8 secretion

After 1, 4, 6, and 12 h of cocultivation of cells and C. albicans, the supernatants were harvested by centrifugation at 2500 r/min for 20 min at 4°C. The IL-8 level in each well of the 6-well plates was determined by an IL-8 enzyme-linked immunosorbent assay kit (Nanjing SenBeiJia, China) according to the manufacturer’s specifications.

Statistical analysis

All the above experiments were carried out more than three times, and the data were shown as the mean with standard deviation. Student’s t-test was used between two groups, and analysis of variance was used in each group to evaluate the difference. P<0.05 was defined as statistically significant.

Results

The morphology of HaCaT cells cultured in HG and LG media with or without C. albicans at different periods

We selected 0, 1, 4, 6, 12, and 24 h as the six time periods to observe the morphology and to detect the expression of cytokines and proteins of cells. The first time point at 0 h [Figure 1a] represented the 2–4 generations of stably growing HaCaT cells, while 1, 4, 6, 12, and 24 h time points represented the times after C. albicans treatment of HaCaT cells. The concentration of C. albicans used in Figure 1b-d was 2×10^4/ml. We observed the general morphological changes of the cells by an electron microscope; images are shown in Figure 1.

The expression of toll-like receptor 2 mRNA in the high- and low-glucose groups

The culture medium of HaCaT cells was exposed to different concentrations (1 and 4.5 g/l) of glucose. RT-PCR was performed to determine whether C. albicans could influence the expression of TLR2 mRNA in HaCaT cells. The results revealed that mRNA levels of TLR2 in both the HG and LG groups had similar changes, resulting in a time-dependent increase. The mRNA levels began to increase at 1 h, reached the peak value at 6 h, and remained high at 24 h after stimulation. Compared with the HG group, the LG group had higher levels of TLR2 mRNA at each time point. These results are shown in Figure 2a and b.

The expression of toll-like receptor 2 in the high- and low-glucose groups

After examining the expression of TLR2 at the RNA level, we further measured the protein levels of TLR2 in HaCaT cells extracted from the two mixed culture groups. We observed that compared to the expression at the beginning (0 h) of infection with C. albicans, the level of TLR2 was elevated from the 1st h until the 6th h, which was consistent with the mRNA expression data [Figure 3a-c].
The expression of interleukin-8 in the high- and low-glucose groups

To determine the amount of IL-8 produced by the HaCaT cells that were not exposed to C. albicans, we set 0 h to represent the amount of IL-8 produced only by HaCaT cells. The results are shown in Figure 4a and b. When the HaCaT cells in HG or LG conditions were infected by C. albicans, the IL-8 levels showed the same trend: they began to rise at the 1st h, were maintained steadily for 6 h, and decreased significantly at 12 h. Although the trends of the two groups were similar, the expression level of IL-8 in the LG group was significantly higher than that in the HG group. The results demonstrated that C. albicans could stimulate the HaCaT cells to secrete IL-8 against pathogens more strongly in the LG group.

Discussion

C. albicans infection in diabetic patients has become increasingly prevalent and is a global health concern; it influences the quality of life of the patients. However, C. albicans infectious dermatosis easily recrudesces and is difficult to cure. The mechanisms of C. albicans infection in diabetes are closely related to defects in immune function. Diabetic patients have deficiencies in systemic immune function. First, combined with a high level of glucose in the skin tissue, long-term metabolic disorders in terms of sugar and protein can lead to a poor nutritional status and a defective tissue repair in diabetic patients. Secondly, sensory impairment due to peripheral neuropathy and microvascular damage or atherosclerosis can lead to an insufficient blood supply in the skin. Third, excess urination caused by diabetes can lead to chronic dehydration of the skin, resulting in cracks and microinjury.

As a result, the function of keratinocytes in the skin immune system is impaired. Keratinocytes are the major component of the epidermis and one of the most important immune cells. Under normal conditions, keratinocytes can express major histocompatibility complex-II antigen, which plays an auxiliary role in the immune response mediated by T-cells. Moreover, it can produce many cytokines involved in the local immune response. In addition, keratinocytes can perform phagocytosis and process antigens, which is beneficial for the acquisition and presentation of antigens to Langerhans cells. Therefore, impaired keratinocyte function has an important role in C. albicans infection of diabetic patients. All these changes will be especially conducive to the reproduction of fungus and deficiencies in skin functions.

IL-8 and TLR2 participate in C. albicans infection of the skin. We conducted the present study to look into the status of these molecules in skin infection with C. albicans in diabetes. We found that IL-8 and TLR2 secreted from HaCaT cells infected with C. albicans in a HG environment were different from those in a LG environment though they showed a similar trend of change.

First, C. albicans can stimulate the production of TLR2, which has a vital function in anti-inflammatory
actions. Tessarolli V et al. [14] found that C. albicans PLM was important in the activation of TLR2. Studies have shown that TLR2 played an essential role in protecting the host against C. albicans infection by MyD88 [13, 15]. It was also reported that TLR2 ablation caused a strong inhibition of cytokine and chemokine (tumor necrosis factor-alpha and macrophage inflammatory protein-2) productions in vitro after infection with C. albicans to enhance the inflammatory response [16, 17]. In vivo experiments [14, 18, 19] demonstrated that TLR2-/- mice were more easily infected with or injured by C. albicans due to the decreased phagocytic activity of neutrophils and macrophages, NO production, and myeloperoxidase activity. However, Villamón et al. [20] indicated that TLR2-/- mice were capable of mounting vaccine-induced resistance to C. albicans and acquiring specific humoral responses, similar to wild-type mice. Netea et al. [5] concluded that the absence of TLR2 led to incremental resistance to C. albicans in TLR2-/- mice.

For the effect of glucose concentration on the level of TLR2, Lan et al. [21] reported that there was no obvious difference in the expression of TLR2 between normal and HG-cultivated keratinocytes, but the expression of BD3 (human β-defensins), which can inactivate a spectrum of bacteria, fungi, and enveloped viruses in vitro, induced by TLR2 in epidermal keratinocytes.
was inhibited in HG conditions compared to normal conditions. Researchers concluded that the HG condition was responsible for the reduction of BD3, which in turn resulted in frequent infection in diabetic patients. These varying results may be due to the methods used for experiments such as in vitro or in vivo analyses. In addition, in our study, glucose concentration combined with fungal infections affected the production of TLR2, which was different from previous studies.

IL-8 has a vital function in inflammatory reaction to *C. albicans*. Diabetic patients are prone to skin infections. Similarly, patients with diabetes or hyperglycemia have a slower healing process than individuals with normal plasma glucose. Following *C. albicans* infection, activation of TLR2 through signal transduction pathways, such as, NF-κB and MAPKs38 can cause the delivery of cytokines such as IL-8, thereby regulating the inflammatory response.\(^{[22]}\)

As for the effect of glucose concentration on IL-8 level, Lan *et al.*\(^{[21]}\) demonstrated that keratinocytes in HG environments enhanced IL-8 production through an epidermal growth factor receptor-regulated kinase pathway in a reactive oxygen species-dependent manner. Interestingly, this is different from what we observed after HaCaT cells were infected with *C. albicans*. In addition to the explanations for the TLR2 results presented above, which were similar to those for IL-8, these differences may also be because the injured skin in HG environments has a more serious inflammatory reaction with high levels of IL-8, resulting in difficulties in wound healing. However, this reaction is not sufficient to fight against inflammation in the skin of patients with high plasma glucose infected with *C. albicans*. Therefore, without a high level of IL-8, the infection will spread or persist. These two situations are different but overlapping.

Some studies have examined the effects of HG or LG on keratinocytes and they found that keratinocytes showed reduced migration and decreased proliferation under hyperglycemic conditions. These impaired physiological events provided a logical explanation for the poor wound healing frequently observed in patients with diabetes.\(^{[23]}\) Moreover, microscopic analysis revealed that the cellular morphology among the keratinocytes treated with different concentrations of glucose was not significantly different.\(^{[21]}\) We observed similar results in our experiments.

In this study, we found that the differences in TLR2 at the 1\(^{\text{st}}\) h and 24\(^{\text{th}}\) h between the HG and LG groups were significant. These results may be due to the interaction of *C. albicans* with the HaCaT cells, which can result in complex changes in cellular function. Over time, we observed that the cell death increased and *C. albicans* proliferation was enhanced, resulting in decreased production of TLR2 and IL-8 to resist the fungus. As a result, we found that TLR2 and IL-8 increased slightly from the 1\(^{\text{st}}\) h to the 6\(^{\text{th}}\) h, and at the 24\(^{\text{th}}\) h, the levels in the two groups were substantially reduced, especially in the HG group.

We are also aware of some limitations in this study. First, although the HaCaT keratinocytes possess full epidermal differentiation capacity and can express differentiation-specific keratins as well as other markers, such as involucrin and filaggrin, they have the potential of unlimited growth, which is different from normal human epidermal keratinocytes. Thus, HaCaT cells cannot completely represent the keratinocytes. Second, the nutrient conditions and other factors *in vitro* are different from those *in vivo*, indicating that *in vivo* experiments should be performed in the future to confirm our findings. Third, in addition to TLR2 and IL-8, other proteins or cytokines such as hBD and IL-6...
may also have anti-inflammatory as well as antifungal effects in these processes.

**Conclusion**

Our study showed that HaCaT cells under HG conditions secrete less TLR2 and IL-8 compared with those under LG conditions when infected with C. albicans, which can further contribute to a more comprehensive understanding of the infection caused by C. albicans and to improve the clinical control of C. albicans-related dermatosis in the at-risk population of diabetes mellitus patients.

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Nil.

**Conflicts of interest**

There are no conflicts of interest.

**What is new?**

- The secretion of TLR2 and IL-8 by HaCaT cells is different under low and high glucose conditions and also when infected with Candida albicans.

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