MYCOLOGY

Cytokine expression induced by *Candida albicans* in a model of cutaneous candidosis based on reconstituted human epidermis

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Skin equivalents based on reconstituted human epidermis have been used recently to establish models for allergic/irritant contact dermatitis and cutaneous candidosis. In the present study the cytokine expression pattern and the morphological alterations in experimental cutaneous candidosis were investigated by RT-PCR and histological analysis. In experimental cutaneous *C. albicans* infection the mRNA expression levels of interleukin (IL)-1 α , IL-1 β , IL-8, GM-CSF, Exodus-2, tumour necrosis factor- α and PSL (P-selectin ligand) were upregulated. Cytokine profile and histological features of infected skin (separation of keratinocytes, oedema, vacuolisation) were comparable to that seen in experimental contact dermatitis. These immunomodulatory and morphological similarities might reflect a common pathogenesis factor in both diseases.

Introduction

The local immune response of the host cells seems to be an important factor in mucocutaneous infection by *Candida albicans*. Studies with mice have demonstrated increased expression of chemokines and a Th1-type cell-mediated immunity in experimental oral [1] and vaginal candidosis [2–7]. Host defence mechanisms are poorly understood in cutaneous candidosis [8]. An earlier study established a three-dimensional model of cutaneous candidosis based on reconstituted human epidermis and demonstrated that secreted aspartyl proteinases are important virulence factors for infection [9]. In the present study the local production of cytokines during experimental cutaneous infection with *C. albicans* by RT-PCR was investigated.

Materials and methods

Candida strain and growth conditions

The clinical isolate *C. albicans* SC5314 was used for

the infection of the reconstituted epidermis [10]. Inocula were prepared as described previously [9].

Model of cutaneous candidosis

The reconstituted human epidermis for the in-vitro model of cutaneous candidosis was supplied by SkinethicTM Laboratory (Nice, France). It consists of a three-dimensional keratinocyte multilayer without any other cell type. All media for cell culture were prepared without antibiotics and antimycotic agents. The epidermal samples were infected with 2×10^6 *Candida* yeast cells in 100 μ l of phosphate-buffered saline (PBS) as described previously. Controls contained 100 μ l of PBS alone. In all experiments infected and uninfected cultures were incubated at 37°C with CO₂ 5% at 100% humidity for 24 and 48 h.

Light microscopy

Light microscopical studies were performed as described previously to evaluate histological changes during infection [9]. Semi-thin sections (1 μ m) were stained with toluidine blue 1% and pyronin G (Merck, Darmstadt, Germany) 1%. The histological changes in the epidermis were evaluated on the basis of 50 sections from 5 different sites for each infected epithelium.

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RNA analysis and reverse transcription

Reconstituted epithelia were lysed for 30 s in 1 ml of lysis buffer (RNeasy kit, QIAGEN, Hilden, Germany) with a mechanical blender (Ultraturrax, IKA, Karlsruhe, Germany). During RNA purification, genomic DNA was digested with RNAase-free DNAase (QIAGEN). Reverse transcription of total RNA was performed at 1 µg total RNA in 10 µl final volume for 55 min at 37°C with 100 U of Moloney murine leukaemia virus reverse transcriptase (Life Technologies GmbH, Eggenstein, Germany) in the presence of 1 mM each deoxynucleoside triphosphate (dNTP) (Amersham Biosciences, Freiburg, Germany), recombinant ribonuclease inhibitor (Promega, Madison, WI, USA) 10 U, 15 mM Tris-Cl (pH 8.4), 60 mM KCl, 3 mM MgCl₂, Tween 20 0.3%, oligo (dT)₁₅ 0.1 µg and 10 mM β-mercaptoethanol. After incubation for 5 min at 95°C the cDNA was finally diluted to 10 ng/µl with water and stored at -80°C.

RT-PCR

In a final volume of 20 µl (with oil overlay) PCR samples contained dimethyl sulphoxide 5%, 60 mM KCl, 10 mM Tris-Cl (pH 8.4), 1.6 mM MgCl₂, Tween 20 0.3%, 0.2 mM of each dNTP, 10 pmol of each primer, Taq DNA polymerase (Promega) 0.6 U and 20 ng of cDNA. Thirty cycles of 1 min at 95°C, 1 min at 65°C, 1 min at 72°C (5 min final) were performed. PCR-amplified products were visualised by agarose gel electrophoresis of 10-µl samples and ethidium bromide staining. A 100-bp ladder (Amersham Pharmacia Biotech, Freiburg, Germany) was used as a DNA mol. wt marker.

The following human primers were used (the sequences are given in the 5' to 3' direction):

interleukin (IL)-1α

5' primer CAC TCC ATG AAG GCT GCA TGG
3' primer ACC CAG TAG TCT TGC TTT GTG G
631-bp product

IL-1β

5' CGA TCA CTG AAC TGC ACG CTC CG
3' GGT GAA GTC AGT TAT ATC CTG GCC G
431-bp product

IL-8

5' GCA GCT CTG TGT GAA GGT GCA G
3' GCA TCT GGC AAC CCT ACA ACA G
365-bp product

GM-CSF

5' GTG GCC TGC AGC ATC TCT GCA C
3' CCT GGA CTG GCT CCC AGC AGT C
396-bp product

GAPDH

5' GCA CCA CCA ACT GCT TAG CAC C
3' GTC TGA GTG TGG CAG GGA CTC
637-bp product

IFN-γ

5' GCA GAG CCA AAT TGT CTC CT

3' ATG CTC TTC GAC CTC GAA AC

290-bp product

TNF-α

5' GGG ACC TCT CTC TAA TCA GCC CTC
TGG

3' GACGGCGATGCGGCTGATGG

287-bp product

Exodus-2

5' CAA GCG CTC TCA GGC AGA GC

3' TCT GGA CCT GGC CTG CTG

420-bp product

TGF-α

5' CAG ATC CTG TCC AAG CTG CGG C

3' GAA TGG TGG CCA GGT CAC CTC GGC

600-bp product

PSL

5' CTT CTT CGT GTG CAC TGT GGT G

3' GAT GGC AGA GTG AGC TAA GGG

255-bp product

GAPDH primers were used as a positive internal mRNA control. To verify the absence of genomic DNA, the designed primers amplified across an intron. The expected size of the amplicons proved that only cDNA was amplified.

Results

Epidermal morphology after infection with *C. albicans*

C. albicans infection of the reconstituted human epidermis confirmed the previously described morphological alterations, i.e., oedema, vacuolisation and detachment of cells (Fig. 1).

Basal cytokine expression of uninfected epidermis

Expression of cytokines was monitored 0, 24 and 48 h after incubation with PBS. The housekeeping gene GAPDH served as a positive control for RT-PCR reaction and demonstrated equal amounts of cDNA in the different samples. RT-PCR analysis demonstrated

Fig. 1. Model of cutaneous candidosis based on reconstituted human epidermis. *C. albicans*-induced morphological alterations 24 h after infection include separation and detachment of cell layers, oedema and vacuolisation.

constant mRNA expression levels for GAPDH, IL-1 α , IL-1 β , IFN- γ , Exodus-2, TGF- β , TNF- α and PSL in all uninfected epidermal samples at the indicated time points (shown for 48 h PBS in Fig. 2). No signals were seen for G-CSF, GM-CSF, M-CSF, hsp-70, the cytokines IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-13, IL-14, IL-15, IL-16, IL-17 and the chemokines BCA-1, HCC-1, I-309, I-TAC, IP-10, MCP-1, MIG, MIP-1 α , MIP-1 β , MIP-3 β , RANTES, SDF-1 β and TARC (Fig. 2 and data not shown).

Regulation of cytokine mRNA expression during epidermal infections with *C. albicans*

Regulation of cytokine mRNA expression during infection of reconstituted epidermal samples (over 24 and 48 h) with *C. albicans* induced new expression of IL-8 and GM-CSF. During the course of infection, the amounts of mRNA coding for IL-1 α , IL-1 β , Exodus-2, TNF- α and PSL were upregulated while the levels of

GAPDH and IFN- γ mRNA remained constant. Expression signals for TGF- β were upregulated after 24 h but not detectable 48 h after infection (Fig. 2).

Discussion

Models of superficial candidosis based on reconstituted epidermis have been used successfully to investigate virulence factors of *C. albicans*, such as the gene family of secreted aspartyl proteinases [9]. The present investigation demonstrated that uninfected reconstituted human epidermis is able to express a cytokine profile which is very similar to that observed *in vivo* or in other in-vitro studies [11–13]. Moreover, it demonstrated a basal expression of PSL and Exodus-2 by keratinocytes which has not been reported before. Expression of Exodus-2 was demonstrated in lymph node tissue and in monocytes and is important for the adhesion and migration of human T cells [14]. PSL- (P-selectin ligand) expression by endothelial cells and

Fig. 2. Kinetics of cytokine expressions in uninfected reconstituted human epidermis (48 h PBS) and 24 and 48 h after stimulation with *C. albicans*. GAPDH was used as an internal mRNA control. M, 100-bp mol. wt marker.

platelets is important for leucocyte migration [15]. To investigate if the expression of Exodus-2 and PSL by epidermal keratinocytes may also play a significant role in host inflammatory response, the regulation of these genes after infection with *C. albicans* was studied. Infection of reconstituted human epidermis with *C. albicans* increased the expression levels of IL1- α , IL-1 β , Exodus-2, TNF- α and PSL, while the amount of TGF- β transcripts was decreased. As expected, relatively constant mRNA expression levels during infection were seen for the housekeeping gene GAPDH, proving equivalence of the skin culture samples used. IFN- γ mRNA levels were also equal. Expression of the pro-inflammatory cytokines IL-8 and GM-CSF, which are chemotactic for monocytes, lymphocytes and neutrophils [11], was induced *de novo* by infection with *C. albicans*. The great influence of these cells in the in-vivo defence against invading fungal pathogens confirms the relevance of this candidosis model to pathogenesis. Both chemotactic mediators IL-8 and GM-CSF are especially important for the recruitment of neutrophils [11], which are frequently seen in cutaneous candidosis *in vivo*. Increased transcription levels of IL1- α , IL-1 β , TNF- α , Exodus-2 and PSL stimulated by experimental *C. albicans* infection are linked *in vivo* with a protective Th1-type cytokine response or with chemotaxis and activation of macrophages, neutrophils and lymphocytes [11, 12, 16]. Recently, it was demonstrated that local production of TGF- β contributes to disease progression in *C. albicans* infection [6]. Down-regulation of the Th2-type cytokine TGF- β in the model used in the present study supports the induction of a protective Th1 response. Induction of a protective Th1-type cytokine response by *C. albicans* was demonstrated previously in several murine models for systemic or mucosal infections [1, 2, 16]. This study presents evidence that chemotaxis and the Th1-type of cytokine response are induced in the earliest stage of contact with the pathogen and without the influence of other immunocompetent cells. Recently, skin equivalents were also used to investigate the cytokine profiles triggered by allergic and sensitising agents [17–21]. A critical review of the results of studies dealing with this topic showed that a clear discrimination between an allergic or a toxic immune response by analysis of the cytokine mRNA expression is difficult. Cytokine expression patterns observed in reconstituted human epidermis after treatment with allergic or toxic agents, or both, are similar to those seen after infection with *C. albicans*. In previous studies, the skin reactions seen in experimental *C. albicans* infection have been explained as a sort of biological contact dermatitis of the primary irritant or allergic type [22–25]. The present study found morphological alterations and a cytokine profile of reconstituted human epidermis induced by *C. albicans* infection, which are similar to that found in irritant/allergic contact dermatitis [18]. This might suggest an irritant/allergic effect of *C. albicans* in the pathogenesis of cutaneous candidosis.

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