IL-1β and ADAM17 are central regulators of β-defensin expression in *Candida* esophagitis

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**Am J Physiol Gastrointest Liver Physiol** 300: G547–G553, 2011. First published January 13, 2011; doi:10.1152/ajpgi.00251.2010.—**Candida albicans** resides on epithelial surfaces as part of the physiological microbiota. However, under certain conditions, it may cause life-threatening infections, including *Candida* sepsis. We have recently shown that human β-defensins (hBDs) hBD-2 and hBD-3 are upregulated in *Candida* esophagitis and that this antifungal host response is distinctly regulated by NF-κB and MAPK/activator protein-1 (AP-1) pathways. Here, we show that *C. albicans* induces hBD-2 through an autocrine IL-1β loop and that activation of the epidermal growth factor receptor (EGFR) by endogenous transforming growth factor-α (TGF-α) is a crucial event in the induction of hBD-3. To further dissect upstream signaling events, we investigated expression of the central sheddases for EGFR ligands ADAM10 and ADAM17 in the healthy and infected esophagus. Next, we used pharmaceutical inhibitors and small-interfering RNA-mediated knock down of ADAM10 and ADAM17 to reveal that ADAM17-induced shedding of TGF-α is a crucial step in the induction of hBD-3 expression in response to *Candida* infection. In conclusion, we describe for the first time an autocrine IL-1β loop responsible for the induction of hBD-2 expression and an ADAM17-TGF-α-EGFR-MAPK/AP-1 pathway leading to hBD-3 upregulation in the course of a *Candida* infection of the esophagus.

**THE GASTROINTESTINAL EPITHELIAL layer represents a barrier that is usually adequate to control commensal microbes but is often insufficient to protect against microbial pathogens. The polymorphic yeast *C. albicans* colonizes distinct epithelial regions of the body as a commensal, but the fact that it may cause severe and life-threatening diseases like *Candida* sepsis is of increasing clinical importance. *Candida* esophagitis is often the first manifestation before *Candida* sepsis develops and represents a severe threat to an immunocompromised individual. It is well established that epithelial cells of the esophagus are the central target of an oroesophageal invasive *Candida* infection and that the course of the infection is determined by both pathogen- and host-dependent factors (3, 27, 29, 37). Only very limited data exist on the host response counteracting bacterial and/or fungal infections in the esophagus (17, 42, 49). Protection is achieved by epithelial desquamation and formation of tight junctions or transmigration of macrophages. Once this physical barrier is penetrated, recognition of invading microbiota is the first step in the initiation of a fast immune response and involves the activation of pattern recognition receptors by microbial pathogens and their products (33, 41, 50), including antimicrobial peptides (AMPs). In the upper gastrointestinal tract, the subgroup of human β-defensins (hBDs) is supposed to be a critical component of both the innate and adaptive immune responses to *Candida* infections with distinct antifungal efficacies and mechanisms for hBD-2 and hBD-3 (12, 20, 43, 44). The expression of these two hBDs is under the control of proinflammatory cytokines like IL-1β, TNF-α, and epidermal growth factor (EGF) receptor ligands that in turn activate downstream effectors, including the transcription factors NF-κB or activator protein-1 (AP-1) (6, 40, 46, 47). Besides this direct antimicrobial effect, hBDs can protect the gastrointestinal mucosa by chemotactic properties recruiting polymorphonuclear leukocytes (PMN) to the site of infection (7, 46). There is some evidence that a toll-like receptor (TLR) 4-mediated interplay between PMNs and epithelial cells is crucial for the protective response against *Candida* infections (49), but the exact mechanisms remain elusive. We have recently shown that hBD-2 and hBD-3 are upregulated in *Candida* esophagitis and that the expression of α-defensins human neutrophil peptides 1–3 is also elevated (22), indicating an involvement of hBDs and PMNs in the immune response to the *C. albicans* infection (11). Our analysis of the signal transduction events in an in vitro model of the disease demonstrated a functional interaction of epithelial cells with PMNs and that NF-κB and MAPK/AP-1 are involved in distinct signaling pathways controlling hBD-2 and hBD-3 expression (40). We further demonstrated that hBD-3 expression is induced by transactivation of the EGF receptor (EGFR) independently of EGF.

Ligands of the EGFR, including transforming growth factor-α (TGF-α), heparin-binding EGF (HB-EGF), and amphiregulin, are expressed as transmembrane precursors. These are released from the cell surface following proteolytic cleavage of the extracellular domain (ectodomain shedding) by zinc-dependent metalloproteases. Of particular importance in mediating EGFR ligand shedding is the ADAM (a disintegrin and metalloprotease) family of membrane glycoproteins. Using mouse embryonic fibroblasts lacking candidate sheddases, ADAM10 and ADAM17 have been identified as the central sheddases of the EGFR ligands in response to various stimuli (34). How-

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ever, the nature of individual ADAMs required for EGFR ligand shedding, the mechanisms leading to the activation of ADAMs, their role in EGFR activation, and their interaction with other signaling pathways remain poorly defined. Borrelli-Pages et al. (5) establish relevance of ADAM17 to TGF-α shedding and EGFR signaling in breast cancer, while others reported ADAM17-independent shedding of TGF-α and amphiregulin (16) after calcium influx. ADAM10 has been shown to be critical for EGF cleavage under some conditions (34).

In the present study, we explored the molecular mechanisms underlying the induction of hBD expression in an in vitro model of *Candida* esophagitis and clinical specimens from patients with *Candida* esophagitis.

**METHODS**

**Materials.** Cell culture medium was purchased from PAA-Labs (Linz, Austria), FBS was from Seromed (Berlin, Germany), and recombinant TNF-α was from Sigma. The hydroxamate-based inhibitors GL-254023X (10-fold selective for ADAM10 over ADAM17) and GW-280264X (preferential ADAM17 inhibitor) were kindly provided from Andreas Ludwig (RWTH, Aachen, Germany).

**Organism and cell culture.** OE21 cells (European Collection of Cell Cultures), a moderately differentiated esophageal squamous carcinoma cell line, were cultured in RPMI 1670 supplemented with 10% FCS and divided every 3 days. Cells for experimental purposes were cultured in six-well cell culture plates with the same medium and used at subconfluence. *C. albicans* [a clinical isolate from esophagitis (22)] was taken from a frozen strain suspension (BHI, glycerine). Ten microliters were transferred to 10 ml TSB-Bouillon and incubated overnight at 37°C. The cells were then separated from the medium by centrifugation at 2,800 g for 15 min, washed twice with PBS, and finally resuspended in RPMI for each experiment. The final concentration of *C. albicans* was 1 × 10^4/ml.

**Human tissues.** Patients underwent endoscopy for the diagnostic work-up of dysphagia. Biopsies from macroscopically healthy and diseased areas of the esophagus were taken. The study (AZ158/01) was approved by the Ethics Committee of the University of Kiel, Germany, and all patients gave their written informed consent before the investigation. For detailed description, refer to our prior work (22).

**Immunohistochemistry.** Normal esophagus samples were obtained from cadavers donated to the Institute of Anatomy at the Christian-Albrechts-University of Kiel. After fixation in 4% paraformaldehyde, the tissue was embedded in paraffin, sectioned, dewaxed, and rehydrated followed by heat-induced antigen retrieval in citrate acid buffer (10 mM, pH 6.0). Slides were incubated with 3% aqueous H2O2 for 5 min at room temperature to block endogenous peroxidase activity and subsequently blocked with serum (1:5 in Tris-buffered saline)
from the species in which the secondary antibody was raised. The primary antibodies mouse anti-ADAM17 antibody (R&D Systems, Minneapolis, MN) and rabbit anti-ADAM10 antibody (Millipore, Schwalbach, Germany) were diluted in Tris-buffered saline (0.15 M NaCl and 0.05 M Tris, pH 7.6) and incubated for 1 h at room temperature. Sections were incubated for 30 min with secondary biotinylated antibodies (Dianova, Hamburg, Germany) followed by incubation for 30 min with StreptABComplex/HRP (Dako Diagnostics), developed with Liquid DAB (Biogenex, San Ramon, CA), and counterstained with Mayer’s hemaluna (Merck, Darmstadt, Germany).

Inhibitor studies. OE21 cells were pretreated with specific inhibitors or neutralizing antibodies for 30–60 min before and during Candida or cytokine stimulation. The neutralizing antibodies against the EGFR (Upstate), TGF-α (R&D Systems), and IL-1 receptor antagonist (IL-1RA; R&D Systems) were used at the indicated concentrations. The metalloproteinase (MP) inhibitors for ADAM10 (GI-254023X) or ADAM17 (GW-280264X) were used as described (18).

Isolation of PMN. PMN were purified using LeucoSep tubes according to the instructions of the manufacturer (Greiner Bio-One). In brief, 3 ml of Ficoll-Paque were preloaded in a 14-ml LeucoSep tube by centrifugation for 30 s at 1,000 g. The heparinized whole blood samples of healthy volunteers were diluted with equal volumes of PBS, and 6 ml of the diluted blood were added to a LeucoSep tube. The cell separation tubes were centrifuged for 15 min at 800 g without braking at room temperature. The cell suspension was collected, and the cells were washed two times in PBS (for 10 min at 640 g and 470 g, respectively, for the two successive wash steps) and resuspended in complete RPMI medium before counting.

Stimulation in the Transwell coculture model. In the Transwell setting, 2 × 10⁵ OE21 cells were cultured in the bottom compartment of a six-well culture plate. After 24 h, PMNs were added to the bottom compartment, and C. albicans was seeded in the top Transwell compartment (Costar, Bodenheim, Germany) and cultured for an additional 24 h.

RNA isolation and cDNA synthesis. After separation of the OE21 cells from the PMNs, total RNA from OE21 cells was isolated using the RNeasy Kit from Qiagen (Hilden, Germany) and reverse-transcribed into single-stranded cDNA, as described previously (23).

Primer. The oligonucleotide sequence and product size for each primer pair used have been described previously (2, 22).

Quantification of gene expression by real-time PCR. Real-time PCR analyses were performed as previously described (23). Standard curves for each mRNA were constructed by cloning the purified PCR products containing the target sequence into pCR-Blunt II-TOPO vector (Invitrogen). Concentration of the reference plasmid was measured spectrophotometrically and transformed into number of copies per microliter by calculation. The absolute mRNA transcript number in each sample was calculated by use of calibration curves.

Small-interfering RNA transfection. For knock down of RelA/p65, ADAM10, or ADAM17, cells were seeded into six-well plates (2 × 10⁵ cells/well) and grown overnight and then transfection with 12 μl/well RNAiFect reagent (Qiagen) and 2 μg/well of either Stealth negative control small-interfering RNA (siRNA; Invitrogen) or Stealth RelA/p65, Stealth ADAM10, or Stealth ADAM17 siRNA (Invitrogen) was performed for 48 h.

Electrophoretic mobility shift mobility assay. Nuclear extracts were prepared as described previously (39) and incubated with a γ-32P-labeled oligonucleotide containing a consensus NF-κB or consensus AP-1 site (Promega, Mannheim, Germany). After 30 min incubation at room temperature, samples were separated by gel electrophoresis at 100 volts and 4°C. Gels were dried and exposed to X-ray Hyperfilm (Amersham, Freiburg, Germany).

Statistics. Data are presented as means ± SD and analyzed by Student’s t-test. A P value <0.05 was considered as statistically significant.

RESULTS

Expression of hBD-2 and hBD-3 is induced by C. albicans through distinct pathways via autocrine IL-1β and TGF-α. We have recently shown that a functional interaction of epithelial cells with PMNs in response to Candida infection of the esophagus leads to NF-κB and AP-1 activation differentially contributing to regulation of hBD-2 and hBD-3 expression (40). To further dissect the upstream signaling events leading to distinct regulation of hBD expression, we used our established coculture system of epithelial cells (OE21 cells) with PMNs for the in vitro analysis. There is growing evidence that innate epithelial defense induced by microbial stimuli is significantly modulated by autocrine action of several cytokines.
and growth factors, including IL-1β (26) and ligands of the EGFR system (4, 6). Because we have shown that IL-1β is strongly upregulated in *Candida esophagitis* (22) and that the induction of hBD-3 depends on EGFR transactivation (40), we first analyzed a putative functional interaction of IL-1β and EGFR signaling in *C. albicans*-mediated regulation of hBD expression.

Real-time PCR experiments revealed a strong upregulation of IL-1β expression (Fig. 1A) by *C. albicans* in combination with PMNs in OE21 cells. The proposed functional role of IL-1β in the induction of hBD-2 and hBD-3 by *C. albicans* was next analyzed by incubating OE21 cells in the presence or absence of the IL-1RA. As shown in Fig. 1B, inhibition of IL-1β signaling by IL-1RA strongly reduced hBD-2 induction but had only a little effect on hBD-3 expression (Fig. 1C).

In contrast, preincubation of OE21 cells with blocking antibodies against the EGFR or its ligand TGF-α reduced *C. albicans*/PMN-induced hBD-3 expression (Fig. 1C), whereas hBD-2 expression was only slightly affected by EGFR or TGF-α blockade (Fig. 1B). Because we have shown that NF-κB is involved in the regulation of the hBD-2 expression (40), we next analyzed whether the observed induction of IL-1β expression (Fig. 1A) by *C. albicans* in combination with PMNs in OE21 cells is NF-κB dependent. After transfection of the OE21 cells with siRNA directed against the RelA/p65 subunit of NF-κB (40), the induction of IL-1β expression was strongly reduced (Fig. 1D). Interestingly, the inhibition of IL-1β signaling by IL-1RA reduced the *C. albicans*-induced expression of IL-1β mRNA after 24 h (Fig. 1E).

**ADAM10 and ADAM17 are expressed in the healthy esophagus and further upregulated in *Candida esophagitis*.** Ligands of the EGFR are expressed as transmembrane precursors that are released from the cell surface by shedding of the extracellular domain. ADAM10 and ADAM17 are the main sheddases of EGFR ligands in response to various stimuli, but their expression and role in the gastrointestinal tract remains elusive. We first analyzed ADAM10 and ADAM17 expression in biopsies and sections of the upper gastrointestinal tract of healthy volunteers. As shown in Fig. 2A, ADAM10 and ADAM17 are highly expressed in the healthy esophagus while their expression in the stomach is considerably lower. ADAM10 expression in the duodenum is nearly as high as in the esophagus while ADAM17 expression remained on a low level in the complete gastrointestinal tract (Fig. 2A). Immunohistochemistry confirmed the predominant epithelial expression of ADAM10 and ADAM17 (Fig. 2B). Biopsies from *C. albicans*-infected esophagus showed a marked upregulation of
ADAM10 and ADAM17 transcript levels (Fig. 2C). In the same biopsies, we have already described a strong upregulation of hBD-2 and hBD-3 (22).

**ADAM17 mediates AP-1 but not NF-κB activation by C. albicans.** As shown recently, hBD-2 and hBD-3 expression in *Candida* esophagitis (40) is mainly controlled by NF-κB- and AP-1-dependent pathways. Electro mobility shift assays (EMSA) revealed that these two transcription factors are activated by *C. albicans* together with PMNs in our cellular model system (Fig. 3A). To elucidate a functional involvement of ADAM10 and ADAM17 in the immune response to *C. albicans* infection, we first analyzed effects of ADAM inhibition on NF-κB and AP-1 activation. Two strategies for ADAM inhibition were applied. We preincubated OE21 cells with pharmacological metalloproteinase inhibitors with preferential selectivity for ADAM10 (GI-254023X) or ADAM17 (GW-280264X). To get higher specificity, OE21 cells were transfected with siRNA directed against ADAM10 or ADAM17. Efficacy of knock down of ADAM10 or ADAM17 expression was assessed by real-time RT-PCR (Fig. 3). The ADAM10 inhibitor (GI-254023X) or siRNAs directed against ADAM10 had no effect on NF-κB activation (Fig. 3), whereas interference with ADAM17 shedding had no effect on NF-κB activation (Fig. 3E). The ADAM10 inhibitor (GI-254023X) or siRNAs directed against ADAM10 neither affected NF-κB nor AP-1 activation (Fig. 3, D and E).

**Effect of ADAM17 inhibition on hBD induction by C. albicans.** We next analyzed the effects of ADAM inhibition on *C. albicans*-mediated hBD-2 and hBD-3 induction. In line with the central role of NF-κB for the regulation of hBD-2 expression in *Candida* esophagitis (40), we observed no significant effects on hBD-2 expression by ADAM10 or ADAM17 inhibition using chemical inhibitors or specific siRNAs (Fig. 4, A and B). In contrast, interference with ADAM17-mediated shedding of EGFR ligands resulted in a significant inhibition of hBD-3 induction by *C. albicans*/PMNs in our cellular model. As shown in Fig. 4C, pharmaceutical inhibition of ADAM17 activity by the ADAM17 inhibitor (GW-280264X) strongly reduced the *C. albicans*-induced increase in hBD-3 expression. Furthermore, specific interference with ADAM17 by siRNA-mediated knock down confirmed the crucial role of this shedding enzyme in hBD-3 regulation in *C. albicans* infection of the esophagus (Fig. 4D).

**DISCUSSION**

We have recently shown that the β-defensins hBD-2 and hBD-3 are two important AMPs upregulated in the esophageal epithelium during the course of a *Candida* esophagitis (40). We established an in vitro model for *Candida* esophagitis that was used to demonstrate the close functional interaction of epithelial cells with PMNs in response to *Candida* infection (40). In the present work, we further dissected upstream events leading to the distinct signaling pathways governing hBD-2 and hBD-3 induction in *Candida* infection.

By using IL-1RA, we demonstrated that IL-1β is a central mediator of *C. albicans*-mediated NF-κB-dependent hBD-2 induction. In contrast, hBD-3 expression in the course of a *Candida* infection of the esophagus was independent of IL-1β but relied on an ADAM17-TGF-α-MAPK/AP-1 signaling cascade.

*C. albicans* is an opportunistic fungal pathogen causing life-threatening mucosal and systemic infections in immunocompromized humans. Several reports indicated that auto- and paracrine action of IL-1β is a critical component in the host defense against *Candida* infection (14, 15). In the cellular defense and expression of hBD-2 and hBD-3, we have shown that IL-1β is a critical component in the host defense against *Candida* infection (14, 15). In the cellular defense and expression of hBD-2 and hBD-3, we have shown that IL-1β is a critical component in the host defense against *Candida* infection (14, 15).
In this study, *C. albicans* together with PMNs led to a significant upregulation of IL-1β expression. Furthermore, blockade of IL-1 receptor-dependent signaling strongly reduced hBD-2 induction, confirming a critical role of secreted IL-1β in the antifungal response. This is in line with the recent observation that autocrine and paracrine signaling of IL-1β in the course of *Candida* infection is tightly controlled and first requires transcriptional induction and subsequent production of pre-IL-1β by the NLRP3 inflammasome (14, 15). The transcriptional part of IL-1β-dependent antifungal responses is regulated by activation of the known fungal pattern recognition receptors TLR2 and Dectin-1 (13–15, 30). Nevertheless, for IL-1β secretion, a second signal is required, leading to caspase-1-mediated cleavage of pro-IL-1β controlled by the NLRP3 inflammasome (14, 15, 19, 25). Several reports indicated that the activation and formation of the inflammasomes are mediated by intermediate intracellular signals, including the production of reactive oxygen species (ROS) (1, 14, 38). It is well established that inflammation of the esophagus leads to mucosal production of ROS (31) and that *C. albicans* mediates IL-1β secretion through induction of ROS in other models (14).

Interestingly, hBD-3 has an ~10-fold higher fungicidal activity compared with hBD-2 (20, 43, 44), indicating that it might be clinically more relevant than hBD-2 in the antifungal response. Therefore, we wanted to further investigate the upstream events leading to the observed *C. albicans*-mediated transactivation of the EGFR independent of EGF and followed by an AP-1-regulated strong induction of hBD-3 expression (40). Recent evidence suggests that EGFR-mediated response in epithelial cells is a central pathway producing innate immune responses to a variety of infectious stimuli (4, 6, 9, 24). With the use of blocking antibodies for the EGFR ligands EGF, HB-EGF, amphiregulin, and TGF-α, we were able to show that, most likely, TGF-α is involved in the EGFR-mediated upregulation of hBD-3 expression (Fig. 1C and Ref. 40). The EGFR mostly targets a pathway independent of NF-κB to regulate hBD-3 but not hBD-2 as indicated by the data presented in this paper. This is in line with observations in *Helicobacter pylori* infection (6) and oral dysplasia (21). Currently, we are not able to explain why the inhibition of the EGFR has no effect on hBD-2 expression while inhibiting AP-1 activation. There is some controversy about the relevance of the NF-κB and AP-1-binding sites in the promoter of hBD-2 for full induction of hBD-2 expression after treatment with IL-1β or infection with microbiota. Wehkamp and colleagues (45, 48) showed that the parallel activation of NF-κB and AP-1 is needed for full transcriptional activation of the hBD-2 promoter after IL-1β stimulation or *Escherichia coli* infection. We were able to show recently that both NF-κB and AP-1 activation are required for full upregulation of the hBD-2 mRNA after treatment with either supernatants of *C. albicans* or the coculture of *C. albicans* with PMNs (40). Inhibition of the NF-κB or of the MAPK/AP-1 pathway significantly reduced the induction of hBD-2 expression, confirming the central role of both transcription factors (40). Nevertheless, EGFR inhibition does not affect hBD-2 expression in *Candida* or *H. pylori* infection (6), indicating that EGFR activation by TGF-α induced a MAPK pathway that is not capable to induce hBD-2 expression.

TGF-α is produced as a transmembrane precursor that is released after shedding of its ectodomain by the ADAM family of membrane glycoproteins. ADAM10, -12, and -17 are the main sheddases involved, but their role in EGFR activation in disease and its interaction with other signaling pathways remain poorly defined (16, 34). By pharmaceutical and siRNA-mediated inhibition of ADAM10 and ADAM17, we were able to establish ADAM17 as the central sheddase in the immune response to a *Candida* infection of the esophagus (Figs. 3 and 4). This critical role of ADAM17 as a mediator of innate immune signaling is in line with recent reports for airway epithelial cells (4, 24, 32) and in a dextran sodium sulfate colitis model (10).

The present analysis of signal transduction events describes the divergent signaling in esophageal epithelial cells that may govern hBD-2 and hBD-3 expression in esophageal *Candida* infection. Our data are the first report of an ADAM17-TGF-α-EGFR-MAPK/AP-1 pathway leading to antifungal hBD-3 expression in the course of a *Candida* infection of the esophagus. Furthermore, we were able to show that hBD-2 expression depends on an autocrine IL-1β loop.

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**DISCLOSURES**

No conflict of interest to declare.

**REFERENCES**


