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Aligned alteration of enteric neurons, smooth muscle cells and inflammatory markers involved in stricture formation in a rat model of Crohn's disease

Petra Talapka

Department of Physiology, Anatomy and Neuroscience, University of Szeged, Szeged, Hungary

Crohn's disease (CD) is a multifactorial, relapsing disorder with chronic inflammation involving all layers of the gut wall. The development of obstructive strictures associated with CD causes major complications in patients. Because no effective therapies are available to prevent stricturing we wanted to gain a better understanding of its pathogenesis by developing a rat model suitable to investigate the involvement of the enteric neurons, the intestinal smooth muscle cells (SMCs) and different inflammatory markers in the intestinal stricturing.

Colitis was induced by an enema of 2,4,6-trinitrobenzenesulfonic acid (TNBS, 10 mg) in 25% ethanol. Tissue samples were taken from control, as well as once, twice and three times treated rats from the inflamed segment, and also proximal and distal to the inflamed segment of the colon in different time points between 2 and 120 days. Quantitative features of myenteric neurons were investigated after HuC/D immunohistochemistry. The expression of multiple inflammatory markers was determined by RT-PCR. The strictures were studied by transmission electron microscopy.

The number of myenteric neurons decreased significantly in all three colonic segments in the acute phase of inflammation. However, 8 days after the TNBS treatments no further changes in the neuronal number was detected until the end of the investigation. Strictures developed at 60th day after the first TNBS treatment and the frequency of strictures increased until day 120th. Thickened muscle layers, expanded intercellular spaces and matrix deposition characterized the strictures. Loosed SMCs with the morphological sign of apoptosis was frequently seen, while enteric ganglia were morphologically intact. HO-1 mRNA was upregulated in all samples from the TNBS-treated rats in the acute phase of the inflammation, and the HO-1 level remained high until day 120th. The increased activity of MMP9 after repeated treatments referred to severe local tissue injury. TGF-β2, but not TGF-β3 was expressed in each tissue samples from the rats with colitis. This expression profile of TGF-β isoforms is characteristic to CD.

The repeated induction of TNBS colitis enhanced intestinal stricturing making this rat model suitable to investigate its pathogenesis. Our preliminary findings indicate that aligned alteration of enteric neurons, smooth muscle cells (SMCs) and different inflammatory markers have a critical role in the development of intestinal strictures. After repeating TNBS treatments, decreased extension of mucosal inflammation was observed when compared to rats treated with TNBS only once. Therefore, a preconditioning effect of repeated TNBS treatment was suggested. Based on the evaluation of quantitative properties of the enteric neurons seemed that this preconditioning did not evolve in the enteric neurons. Ultrastructural morphometry revealed an increased amount of extracellular matrix deposition and increased number of SMCs with proapoptotic markers. Consequently, the distance between SMCs and myenteric ganglia increased, which might be responsible for the default innervation of SCMs and the formation of intestinal strictures.

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Supervisors: Éva Fekete; Mária Bagyánszki
E-mail: talapka.petra@gmail.com

Characterization of the innate and adaptive immune responses induced by the opportunistic human pathogen *Candida parapsilosis*

Adél Tóth

EMBO Candida Group, Department of Microbiology, University of Szeged, Szeged, Hungary

The genus *Candida* comprises more than one hundred species, of which less than 20 have been associated with human infections. Depending on the age group and geographical region, *C. parapsilosis* is the second or third most common species after *C. albicans* and *C. glabrata* causing invasive candidiasis. Although in recent years there has been a great progress in the understanding of immune responses induced by *C. albicans*, little is known about the immunity against *C. parapsilosis*.

During our study, we examined the innate and adaptive immune responses induced by *C. albicans* and *C. parapsilosis*. Firstly, we compared the cytokine responses evoked by *C. albicans* and *C. parapsilosis* using an *in vitro* model of human peripheral blood mononuclear cells (PBMCs). PBMCs were stimulated with heat killed *C. albicans* or *C. parapsilosis*, and the cytokine production was measured by enzyme-linked immunosorbent assay (ELISA). *C. parapsilosis* induced similar quantities of TNFα and IL-6, and slightly lower amounts of IL-1β in human PBMCs compared to *C. albicans*. However, stimulation of PBMCs with *C. parapsilosis* resulted in higher IL-10 and lower IFNγ production compared to *C. albicans*, indicating a skewed T helper cell response. Furthermore, *C. parapsilosis* induced much lower IL-17 and IL-22 production compared to *C. albicans*. Following intracellular cytokine staining, flow cytometric analysis confirmed that the decreased production of IL-17 and IL-22 was in line with a lower number of IL-17 producing cells. Blocking of the three classical

MAP kinases (p38, ERK, JNK) resulted in decreased cytokine production after stimulating PBMCs with *C. albicans* or *C. parapsilosis*, indicating that these kinases are all involved in the signal-transduction following the recognition of the two *Candida* species. However, cytokine levels indicated that there are certain differences in the signal-transduction following the immune sensing of *C. albicans* and *C. parapsilosis* by PBMCs. Additionally, decreased cytokine production following the inhibition of Dectin-1 revealed that this receptor plays a role in the recognition of both *C. albicans* and *C. parapsilosis*. To further elucidate the immune responses evoked by *C. parapsilosis*, we examined the interactions of this pathogen with human primary monocytes-derived macrophages. As secreted fungal lipases have been shown to play in important role an pathogenesis, we compared the response of human macrophages to a wild type (wt) as well as a lipase deficient ($lip^{-/-}$) *C. parapsilosis* strain that has been previously established in our lab. When co-cultured with macrophages, both strains induced a significant increase in the expression of TNFα, IL-1β, IL-6, IL-8 and PTGS-2 (prostaglandin-endoperoxide synthase 2) genes in host cells after 12 hours, as determined by quantitative real-time PCR. Notably, macrophages stimulated with lipase mutant *C. parapsilosis* showed at least two-fold higher expression of these pro-inflammatory mediators compared to those infected with lipase-producing (wt) *C. parapsilosis*. Additionally, we examined the phagocytosis of wt and $lip^{-/-}$ *C. parapsilosis* strains by human PBMC-derived macrophages using quantitative imaging flow cytometry. We found that although after 2 hours both strains were phagocytosed to the same extent by host cells, the rate of internalization and phagolysosome fusion was higher in case of $lip^{-/-}$ *C. parapsilosis*. These findings confirm the role of fungal lipases as important virulence factors during *C. parapsilosis* infection and support the hypothesis that these microbial compounds have anti-inflammatory potential. Taken together, our results contribute to the better understanding of the immune response induced by *C. parapsilosis*, and highlight the role of fungal lipases during host-pathogen interactions.

Supervisor: Attila Gácser
E-mail: toth.adel.86@gmail.com

Investigating the structure and the mechanism of action of *Neosartorya fischeri* antifungal protein

Máté Virágh

Department of Microbiology, University of Szeged, Szeged, Hungary

Today there is a substantial demand for new antimicrobial compounds because of the increasing number of fungal infections. The defensin-like antimicrobial peptides produced by filamentous fungi are interesting in this respect, because they can inhibit the growth of several filamentous fungi. Different defensin-like antimicrobial miniproteins have been isolated from seven ascomycetous filamentous fungal species (*Aspergillus clavatus*, *Aspergillus giganteus*, *Aspergillus niger*, *Fusarium polyphialidicum*, *Neosartorya fischeri*, *Penicillium chrysogenum*, *Penicillium nalgiovense*). Until now the *Penicillium chrysogenum* antifungal protein (PAF) and the *Aspergillus giganteus* antifungal protein (AFP) have been intensively studied. Although AFP and PAF generate similar symptoms in the susceptible organisms, they have different mode of actions. AFP disturbs the cell wall biosynthesis by specific inhibition of chitin synthases, and PAF evokes programmed cell death via G-protein signal transduction pathway. The *Neosartorya fischeri* antifungal protein (NFAP) consists of 57 amino acid residues and has a calculated molecular mass of 6625.5 Da and a pI of 8.93. Our *in silico* structure modelling revealed that NFAP contains five antiparallel β-strands connected by three loops, and showing a β-barrel topology in general, which is stabilized by three intramolecular disulfide bridges. Previously we demonstrated that NFAP effectively inhibits the growth of numerous filamentous fungi including human and plant pathogens and the model organism, *Aspergillus nidulans*. As in the case of similar proteins, the high yield production of NFAP is not resolved despite the available knowledge of the nature of its 5′-upstream transcriptional regulation elements in response to environmental signals and stress. For the future investigation it would be important that NFAP could be producible in a non-sensitive, easily fermentable, "generally recognized as safe" fungus. On the other hand the understanding the exact antimicrobial effect and the mode of action of NFAP are essential for its future practical applications.

For these reasons, we carried out the heterologous expression of the *nfap* gene in *Pichia pastoris* KM71H by using the pPICZαA vector. After purification, the final yield of the hNFAP from 1000 ml ferment broth was 2.4±0.2 mg, which is twofold amount compared to the native producer, *Neosartoria fischeri* NRRL 1881. N-terminal sequencing experiments revealed that the first 5 amino acid residues of the purified heterologous protein is LEYKG (which corresponds well to the determined amino acid sequence of the native NFAP) and the different ion chromatograms from the mass spectrometry correspond six out of all peptides found by analysis of mass lists. Based on the signal dispersion of the amide region (6-10 ppm), it is proven that the protein exists in folded state. Tertiary structure determination needs further NMR investigations using isotope-labelled NFAP. The antifungal activity of the hNFAP was the same as described in the case of NFAP. Based on our previous studies, it seems that the antifungal mechanism of NFAP differs from what was described in the case of AFP and PAF. For revealing the exact mechanism of action of NFAP fluorescent microscopic investigations and antifungal susceptibility assays on *Aspergillus nidulans* mutants in protein kinase C/mitogen-activated protein kinase and cAMP/protein kinase A signalling pathway are in progress.