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TNF-α Induced Expression of Enteric Neuronal Neuropeptide Y (NPY) Influences Intestinal Epithelial Barrier Functions

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Introduction: The integrity of the intestinal epithelium is crucial to maintain boundaries between luminal contents and mucosal immune system. Increased epithelial permeability has been noted in 10-20% of pre-symptomatic Crohn's disease patients. We investigated if enteric neurons producing neuropeptide Y (NPY) could influence epithelial permeability and modulate TNF-α signaling. **Methods:** Caco2- BBE epithelial cells were grown on transwell plates and NPY was added to the basolateral medium (0.1 μM). The epithelial permeability was measured by transepithelial resistance (TER) and flux of FITC-Dextran (4 Kd) at 2 and 24 h. The changes in the expression of tight junction proteins were compared in control and NPY-treated cells by real-time polymerase chain reaction (qRT-PCR) and Western blotting. The enteric neurons transfected with NPY promoter constructs (-1078 (full length), -952, -836, -769, -728, -597, -448, -278) were treated with TNF-α, and NPY promoter activity was assessed by luciferase assay. NPY expression in enteric neurons treated with TNF-α was also determined by qRT-PCR. **Results:** The addition of NPY increased the TER and FITC-Dextran flux across the CaCo2-BBE monolayer ($p<0.05$), thus increasing colonic epithelial permeability. NPY also induced a two-fold increase in the claudin-2 expression at mRNA ($p< 0.01$) and protein ($p< 0.05$) levels. NPY mRNA was up regulated by two-fold in TNF-α treated enteric neurons ($p< 0.05$) as seen by qRT-PCR. Luciferase assay demonstrated a significant increase in the NPY promoter activity, specifically in the regions between -728 and -836 of the NPY promoter ($p< 0.05$. Transcription factor (TRANSFAC) analysis showed Activator Protein-1 (AP-1) binding sites in this region of the NPY promoter, supporting the responsiveness to TNF-α. **Conclusions:** TNF-α upregulates NPY expression in enteric neurons; NPY in turn alters barrier functions of the colonic epithelium via modulation of leaky tight junction proteins like Claudin-2. Taken together, enteric neurons producing NPY aggravate pro-inflammatory signaling and increases epithelial permeability. Our studies demonstrate a role of NPY regulation of epithelial permeability and participation of enteric neuronal signaling in propagating inflammation during inflammatory bowel disease.

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Epigenetic Regulation of ICAM-1 Gene Expression by IL-1β and NO in Colonic Inflammation

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Background and Aim: Numerous reports show that endogenous nitric oxide (NO) can be a protective or a deleterious agent in inflammatory responses. At high concentrations, NO is deleterious by inducing excessive oxidative stress via the formation of peroxynitrites. However, the cellular mechanisms of the protective role of NO in inflammation are poorly understood. We investigated the epigenetic mechanisms by which NO suppress the expression of ICAM-1 gene, a prominent proinflammatory mediator. Methods: We used rat colonic muscularis externa and rat colonic circular smooth muscle cells (RCCSMCs). Results: 20 ng/ml IL-1β time-dependently increased ICAM-1 expression in both colonic muscularis externa and RCCSMCs. S-Nitrosoglutathione (GSNO: 25 μM to 250 μM), an NO donor, concentration-dependently attenuated this increase. The incubation of muscularis externa with 20 ng/ml IL-1β for 0, 0.5, 1 and 3 hours significantly increased Acetyl histone H3K18. Chromatin immunoprecipitation (ChIP) assays with anti-Acetyl histone H3K18 and H4K12 antibodies showed that IL-1β treatment time-dependently increased the acetylation of both histone H3 and H4 at the rat ICAM-1 core promoter region containing two NF-κB binding sites. In support of this, incubation of RCCSMCs with 1 μM histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), significantly increased ICAM-1 protein expression, suggesting that histone acetylation plays a critical role in ICAM-1 gene expression and IL-1β induces ICAM-1 by increasing histone acetylation at ICAM-1 core promoter. Therefore, we further hypothesized that NO attenuates ICAM-1 up-regulation by IL-1β by decreasing histone acetylation. To test this hypothesis, RCCSMCs were incubated with 20 ng/ml IL-1β in the presence or absence of 25 μM GSNO for 24 hours. We found that both H3K18Ac and H4K12Ac were markedly enhanced by IL-1β, and GSNO significantly abrogated IL-1β-induced H3K18 and H4K12 acetylation. Our *In Vivo* data support the cellular findings. TNBS-induced inflammation (68 mg/kg) significantly enhanced the mRNA and protein expression of ICAM-1 on day-1. It also suppressed HDAC3 activity and enhanced the acetylation of H3K18 and H4K12 in muscularis externa. The treatment of these tissues with GSNO time-dependently reversed these inflammatory responses. Conclusions: Both IL-1β and NO modulate ICAM-1 gene expression through epigenetic modification of histones around the NF-κB binding domains of ICAM-1 promoter, which might alter NF-kB transcriptional activity. Inhibition of histone acetylation with histone acetytransferase (HAT) inhibitor is a potential therapeutic target in abrogating inflammatory response and smooth muscle dysfunction.

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Human Derived Enteroglial Cells Toll-Like Receptors mRNA Expression and Modulation by Pathogen and Probiotic Bacteria

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Background and Aim: Enteric glial cells (EGC) are involved in intestinal inflammation and can be activated by exogenous stimuli, and foreign antigens (i.e. gliadin). Toll-Like Receptors (TLRs) are immune receptors able to recognize pathogen conserved molecular patterns. Whether EGC express TLRs or respond to bacterial stimuli is unknown. We aimed to investigate if TLRs are expressed by human EGC and if their expression is differentially modulated by pathogens or probiotics bacteria. Materials and Methods: Pure EGC were obtained according to method previously described by our group. Briefly, myenteric plexus preparations were isolated from ileum of patients undergoing surgery and enzimatically dissociated. Ganglia were plated and cell cultures were grown to subconfluence. After 21 days, EGC were purified using Dynal Magnet® and characterized by immunofluorescence to confirm the absence of contaminating cells (i.e. fibroblasts). TLRs 1, 2, 4, 7 and 9 mRNA expression were evaluated at baseline and after 24 hours (h) incubation of EGC with the probiotic strain Lactobacillus Paracasei F19 (LP F19) and the pathogen Enteroinvasive *Escherichia coli* (EIEC). The bacteria/cells ratio was 0.1/1 and 100/1. Quantitative Real Time PCR was performed to evaluate specific mRNAs fold increases respect to untreated controls. Data were obtained with 3 experiments and expressed as mean±SD. Results: Human purified EGC physiologically expressed TLR 1, 2, 4, 7 and 9 mRNAs, with TLR 1 and 4 transcripts being the most expressed. With a bacteria/cells ratio of 0.1/1, EIEC induced a higher significant increase than LP F19 of TLR 2 (4.1±0.1 and 2.7±0.4; $p<0.05$), TLR 7 (8.0±0.2 and 0.6±0.2; $p<0.05$) and TLR 9 (8.8±1.7 and 1.0±0.2; $p<0.05$). Conversely, only a trend to an increased expression of TLR 4 was observed with LP F19 respect to EIEC (2.4±0.1 and 1.3±0.4; p=NS). At 100/1 bacteria/cell ratio EIEC determined an 80% of cellular mortality, while LP F19 induced an higher expression of TLR 7 (17.8±4.1) and 9 (21.2±4.9) respect to lowest bacteria/cell ratio, while the other TLRs remained unmodified. Conclusion: We showed, for the first time, that pure cultures of human EGC express TLRs' mRNA and are able to discriminate between pathogen and probiotic bacteria. This data support the hypothesis that enteric glia is involved in the host-pathogen interaction.

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Decreased Activity of DNase-I Predisposes to Immune-Mediated Complications in IBD Patients During Anti-TNFA Treatment

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Aim TNFalpha antagonism with monoclonal antibodies is an effective therapy for severe IBD. An induction of apoptosis of inflammatory cells contributes to this therapeutic effect. Desoxyribonuklease (DNase) I is an enzyme involved in chromatin metabolism and has been implicated in degrading DNA during apoptosis. A deficiency in DNase I enzyme, and thereby a failure to remove DNA from nuclear antigens, promotes disease susceptibility to autoimmune disorders. We have hypothesized that a deficiency in DNase I activity in IBD patients treated by anti-TNFalpha agents could lead to autoimmune phenomena. Materials and methods The study included 92 IBD patients treated with infliximab or adalimumab (57 women and 35 men, mean age 34±11 years): 49 patients with uncomplicated biological treatment and 43 patients with different skin complications, including psoriasiform and atopic-like exanthema, with supposed immunopathological origin. Two age-matched control groups were examined: 25 SLE patients (19 female, 6 male) and 20 healthy blood donors (10 women and 10 men). Serum DNase I activity was determined by the enzyme-linked immunosorbent assay (Orgentec, Germany). A cut-off value of 75% of DNase I activity was assessed.The statistical analysis was performed using the Statistica program (StatSoft, USA). Mann-Whitney U test was performed to compare DNase I activity between examined cohorts. A p value less than 0.05 was considered significant. Results DNase I activity in IBD patients with uncomplicated biological treatment was significantly higher compared to IBD patients with skin involvement after the anti-TNFalpha therapy (75.5±11.7% and 47.8±14.2, respectively, $p<0.001$). DNase I activity in IBD patients (both uncomplicated and with post-treatment skin complications) was significantly lower compared to healthy blood donors (75.5±11.7% and 47.8±14.2, respectively, versus 91.9±10.8%, $p < 0.001$). However, DNase I activity in all IBD patients was significantly higher compared to SLE patients ((75.5±11.7% and 47.8±14.2, respectively, versus 31.3±19.8%, $p<0.001$). DNase I activity in patients with complete treatment response did not differ compared to partial- and non-responders. DNase I activity in female IBD patients was significantly lower compared to male patients, regardless of the response/non-response or uncomplicated/complicated treatment (59.9±19.7% and 66.8±16.4%, $p = 0.0294$). Conclusions Activity of DNase I was significantly decreased in IBD patients with skin immunopathological complications during anti-TNFalpha therapy. Thus, measurement of DNase I activity might be used to identify subjects at increased risk for skin immunopathological adverse events.

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Biomarkers Associated With Progressive Behaviour in Crohn's Disease

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Aims:Crohn's disease (CD) is variable in severity among patients.Progressive CD is not well defined and is currently assessed using crude clinical characteristics.Biomarkers may be useful in identifying patients at risk of progressive CD.The aims were to identify phenotypic, genotypic and serologic variables associated with progression of CD from B1 (inflammatory) to B2 (fibrostenotic) or B3 (penetrating) behaviour as defined by the Montreal Classification.Methods:A retrospective chart review was conducted on 364 CD patients recruited between 2002-04 who had at least five years of follow-up and serum/DNA samples taken at enrolment.Age of diagnosis,gender,ethnicity,smoking status,extraintestinal manifestations,disease location and behavior,requirement for steroids or surgery and time to progression from B1 to B2 or B3 were determined using clinical records.Patients were censored at the date of disease behaviour change or date of most recent assessment,whichever was earliest.The anti-glycan antibodies gASCA, ACCA, ALCA, AMCA, anti-L and anti-C were assayed.Genotyping was performed on a panel of IBD-relevant SNPs and genetic additive models were applied.Survival analysis was performed and probability estimates based on Kaplan-Meier methods were calculated.Differences between survival curves were analyzed by the Log-rank test.Cox-proportional hazard regression models were used to evaluate continuous factors.Results:The proportions for age categories A1,A2 and A3 were 24%,67% and 9% respectively.The proportions for disease location L1,L2 and L3 were 40%, 23% and 35% respectively.The median time to latest follow up from diagnosis was 12.4(5.0-50.0) years.Out of 288 patients who were B1 at diagnosis,168 (58%) patients progressed to either B2 or B3.Age at diagnosis <40 years (p=0.008),small bowel disease location (either L1 or L3, p=0.0005),gASCA IgG,gASCA IgA, and AMCA (p=0.005, 0.02, 0.04 respectively) were statistically significant factors associated with disease progression.SNPs significantly associated with evolution to more severe disease behavior are shown in Table 1.Conclusions:Serum and genetic markers are able to prospectively identify individuals at risk of progression to more severe disease behaviour in CD.