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Dipeptidyl peptidase-4 expression is reduced in Crohn's disease ☆,☆☆

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ABSTRACT

Background: Dipeptidyl peptidase 4 (DP4) is a serine protease that preferentially cleaves N-terminal dipeptides from polypeptides containing proline or alanine as the penultimate amino acid. DP4 inactivates glucagon like peptide-2 (GLP-2), a trophic peptide with cytoprotective and reparative properties in the injured gut; therefore DP4 potentially inhibits repair processes. DP4 also modulates the activity of GLP-1 and polypeptide YY (PYY) which regulate appetite and motility. No data are yet available on the tissue and plasma expression of DP4 in inflammatory bowel disease (IBD).

Methods: Tissue and plasma were studied from active CD and healthy controls for DP4 quantification. Experiments were also carried out in a reductionist Caco-2 cell line model of intestinal inflammation with TNFα incubation. DP4 expression was studied by tissue Western blotting and plasma enzymelinked immunosorbent assay (ELISA), in addition to quantitative polymerase chain reaction (qPCR).

Results: There was a ~2.7-fold decrease in DP4 protein in CD tissue ($p = 0.05$). Plasma DP4 in CD was also significantly lower than the control group. A negative correlation between plasma DP4 levels and inflammatory activity as measured by C-reactive protein was observed. In Caco-2 cells an ~18-fold increase ($p < 0.0001$) in DP4 protein expression was seen after incubation with TNFα at a concentration of 25 ng/μl for 48 hours paralleled by a 2-fold increase in DP4 mRNA.

Discussion: DP4 is reduced in tissue and plasma in active Crohn's disease. This is unlikely to represent simple downregulation induced by inflammation since the key proinflammatory cytokine strongly upregulated DP4 expression in Caco-2 cells. Clearly a more complex situation exists in vivo. We propose that reduced DP4 activity limits the cleavage of regulatory peptides, for example potentiating the trophic signal from GLP-2. Pharmacological DP4 inhibition may present an additional therapeutic target in IBD.

1. Introduction

Dipeptidyl peptidase 4 (DP4) is a serine type protease that cleaves N-terminal dipeptides from polypeptides containing proline or alanine as their penultimate amino-acid [1]. This membrane-bound glycoprotein is a unique multifunctional protein, acting as a receptor and proteolytic molecule. It is expressed on the cell surface of various cell types including enterocytes [2] and the related model cell line Caco-2 [3]. Proteolytic cleavage of the membrane bound DP4 results in a soluble homodimer with a molecular weight of 210–220 kDa [4].

Due to this wide biochemical repertoire, DP4-mediated effects span multiple physiological systems. For example, due to its regulatory role on enteroendocrine hormone activity, DP4 has effects on gut mucosal permeability and appetite regulation. It decreases the bioavailability of the anorectic hormone glucagon-like peptide-1 (GLP-1) [5,6], the intestinotrophic enteroendocrine hormone, glucagon-like peptide-2 (GLP-2) [7,8], and the incretin gastric inhibitory peptide (GIP) [6,9]. However, the dipeptidase cleavage step activates the anorectic polypeptide YY (PYY) [10,11].

DP4 activity has the potential role to intensify intestinal inflammation. In keeping with this, dextran sodium sulphate (DSS) mouse models of intestinal inflammation have shown disease severity and

Abbreviations: ANOVA, analysis of variance; CD, Crohn's disease; CDAI, Crohn's disease activity index; CRP, C-reactive protein; DP4, dipeptidyl peptidase 4; DSS, dextran sodium sulphate; EEC, enteroendocrine cell; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; GIP, glucose-dependent insulinotropic polypeptide; GLP-1/2, glucagon-like peptide ½; HRP, horseradish peroxidase; HSK, house-keeping gene; IBD, inflammatory bowel disease; LNA, locked nucleic acid analogues; PVDF, polyvinylidene fluoride; PYY, polypeptide YY; RA, rheumatoid arthritis; rhTNFα, recombinant human tumour necrosis factor alpha; RT qPCR, real time quantitative polymerase chain reaction; SDHA, succinate dehydrogenase complex, subunit A; SLE, systemic lupus erythematosus; TBS, tris buffered saline; TMB, 3,3′,5,5′-tetramethylbenzidine; UC, ulcerative colitis; WT, wild type.

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☆☆ Moran G.W., O'Neill C. and McLaughlin J.T. conceived the study and planned the experimental work. Moran G.W. carried out the experimental work, analysed the data and drafted the manuscript. O'Neill C. and McLaughlin J.T. led in analysing the data and critically reviewing the manuscript. Padfield P. participated strongly in optimising the experimental work.

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histological improvement upon treatment with DP4 inhibitors [12,13]. No data are yet available on the tissue and plasma expression of DP4 in human inflammatory bowel disease (IBD). In this study, DP4 expression in Crohn's disease (CD) was studied and related to the degree of underlying inflammation assessed by C-reactive protein (CRP) and the Crohn's disease activity index (CDAI) [14].

DP4 is expressed by epithelial enterocytes as previously described [15]. To assess whether the reduction in DP4 observed was due to direct inhibition of its epithelial biosynthesis, the cellular monolayer system Caco2 was exposed to varying concentrations of recombinant human recombinant TNFα (rhTNFα) for different time periods.

2. Methods

2.1. Basic tissue protocol and patient recruitment

All tissue was obtained from patients with active CD and controls undergoing endoscopic procedures for clinically indicated reasons. Tissue from the terminal ileum was used in this study from both patients and controls. For protein studies, tissue was immediately placed on dry ice and stored at −80 °C within 2 hours of sampling. Twenty-six patients with active ileocolonic CD and twenty healthy controls were recruited for this study (Table 1). Disease activity and localisation were clinically defined by independent endoscopic, histological, radiological and biochemical investigations. The CDAI was used as a validated measure of disease activity [14].

2.2. Protein extraction from human tissue

Tissue was thawed on ice for a few minutes and then washed twice in ice cold phosphate buffered saline (PBS). Approximately 1 ml of ice-cold tissue extraction buffer (sodium chloride 120 mM, HEPES 25 mM, Triton-X-100 1%, ethylenediaminetetraacetic acid 2 mM, sodium fluoride 25 mM, sodium orthovanadate 1 mM), 0.2% sodium dodecyl sulphate (Fisher Scientific, Loughborough, UK) and protease inhibitor solution (Sigma) 3% was prepared in a separate Eppendorf. This was then vortexed and 100 μl transferred to a Dounce homogeniser (VWR international, Leicestershire, UK) along with the tissue. The whole procedure was undertaken on ice. The tissue sample was homogenised with 20 vertical strokes of the loose pestle and 20 vertical strokes of the tight pestle. Two to three rotational strokes were applied at the end. The sample was incubated on ice for 30 minutes with gentle agitation. The sample was then transferred to a fresh Eppendorph tube and spun in a microcentrifuge at 13,000 rpm for 15 minutes at 4 °C. The supernatant was used for further studies.

2.3. Cell culture

Caco-2 cells (passages 45–55) were routinely grown at 37 °C and 5% CO_2 in a constant humidity environment in MEM medium (Gibco, Invitrogen), containing non-essential amino acids (1%), 50 U/ml penicillin and 50 μg/ml streptomycin, glutamine (2 mM) and 10% foetal calf serum. Cells were split at confluence at a split ration of 1:10 using 0.05% trypsin/0.02% EDTA (Invitrogen) and then grown in six well plates (Greiner Bio-one, Gloucestershire, UK). When Caco-2 cells reached 80% sub-confluency, the medium was replaced with serum-free medium for at least 1 hour prior to addition of rhTNFα at the concentrations specified. The cells were then harvested and the expression of DP4 protein and RNA studied as indicated.

Table 1
Description of the two groups studied. Disease activity described through C-reactive protein (CRP) and the CDAI.

	Crohn's disease	Healthy controls
Number	26	20
Sex	9 males; 17 females	10 males; 10 females
Age (years)	42.3 ± 2.9	42.6 ± 2.8
CDAI	174.5 ± 14.26	n/a
CRP (mg/L)	20.4 ± 5.4	n/a

Table 2
Sense and antisense primer sequences used in the quantitative PCR experiments.

Primer name	Primer sequence
DP4 sense	CCAAAGACTGTACGGGTTCC
DP4 antisense	ACAAAGAACTTTACAGTTGGATTCAC
GAPDH sense	AGCCACATCGCTCAGACAC
GAPDH antisense	GCCCAATACGACCAAATCC
SDHA sense	AGAAGCCCTTTGAGGAGCA
SDHA antisense	CGATTACGGGTCTATATTCCAGA

2.4. Effects of rhTNFα on DP4 expression

Caco-2 cells were placed in serum free medium for 1 hour prior to the addition of rhTNFα (R&D systems Ltd) at 25, 50 and 100 ng/ml [16]. Cells were incubated for 6, 12, 24 and 48 hours and then harvested for further analysis.

2.5. Protein extraction, SDS–PAGE and immunoblotting

This was performed as described [17], using goat anti-rabbit DP4 (R&D systems, Abingdon, UK) as the primary antibody and an horseradish peroxidase (HRP) conjugated secondary antibody (BioRad, Hemel Hempstead, UK). In case of protein derived from gut tissue the sample and Laemmli buffer were warmed together at 37 °C for 5 minutes in a water bath leading to a DP4 product with a molecular weight of 220 kDa [18]. For all protein derived from Caco-2 cells, the protein and Laemmli buffer mix were heated at 100 °C for 2 minutes leading to a product of 110 kDa as previously shown [2,3,19].

2.6. RNA expression studies

RNA from Caco-2 cells was prepared using Trizol (Invitrogen) according to manufacturer's instructions. RNA samples were DNase treated using the DNA Free Turbo Kit (Ambion, Paisley, UK) as per manufacturer's instructions. RNA was bioanalysed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA). Absorbance was read at 220–350 nm and the 260:280 nm ratio was used as an indicator of RNA purity. The Nanodrop software algorithm produced an estimate of RNA concentration from absorbance spectra. RNA integrity was assessed using the Agilent 2100 Bioanalyser [Agilent Technologies, Edinburgh, UK].

First-strand cDNA synthesis was performed using up to 1 μg of RNA from individual samples and the ImProm-II™ Reverse Transcription System (Promega Corporation, Madison, USA) in a final reaction volume of 20 μl according to the manufacturer's instructions.

The Human Universal Probe Library system [20] (Roche Diagnostics, Switzerland), employing proprietary locked nucleic acid analogues (LNA) of fluorescence resonance energy transfer hydrolysis probes, was used for real time quantitative polymerase chain reaction (RT qPCR) to measure expression levels in genes of interest. Using the Roche Online Assay Design Centre, specific primers and an associated probe were selected for the gene of interest transcript. Where primers showed homology to other regions within the human transcriptome, the assay was redesigned. The primers were submitted to Basic Local Alignment Search Tool (BLAST®) (http://www.ncbi.nlm.nih.gov/BLAST/). Primer sets and dual labelled LNA probes were purchased (Metabion, Planegg-Martinsried, Germany; Roche Diagnostics respectively).