Over-expression of paneth cell-derived anti-microbial peptides in the gut of patients with ankylosing spondylitis and subclinical intestinal inflammation

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Abstract

Objectives. Subclinical gut inflammation has been demonstrated in patients with AS. Altered expression of paneth cell (PC) anti-microbial peptides have been reported in the inflamed ileum of patients with Crohn’s disease (CD). Here, we investigated the expression of PC-derived peptides in subclinical gut inflammation in AS.

Methods. Multiple adjacent mucosal biopsies from terminal ileum were obtained from 25 patients with AS, 30 CD and 15 healthy controls (HCs). Expression of human α-defensin 5 (HD-5), phospholipase A2 (PLA2), lysozyme and SOX-9 molecules was assessed by quantitative Taqman RT–PCR on mucosal samples. Immunohistochemistry with anti-human HD-5 antibody and genotyping of relevant NOD2 mutations was also performed.

Results. HD-5, PLA2 and lysozyme transcript levels were strongly increased in AS and CD with similar degrees of intestinal inflammation when compared with normal controls. Immunohistochemical evaluation showed a normal number of PCs in both AS patients with chronic gut inflammation and CD patients with less-inflamed ileal samples. Conversely, CD patients with higher degree of gut inflammation had a reduced number of PCs and low expression levels of HD-5.

Conclusion. In this study, we provide evidence that over-expression of PC-derived anti-microbial peptides occurs in the ileum of AS patients with subclinical gut inflammation, likely representing an important early alteration of the mucosal innate immune component and intestinal host defence in AS.

Key words: Ankylosing spondylitis, Intestinal inflammation, α-defensin 5, Paneth cells.

Introduction

Sub-clinical gut inflammation has long been demonstrated in patients with AS. The similarities in immunological abnormalities described in the inflamed gut of both AS and Crohn’s disease (CD) patients, and the observation that 7% of AS patients with more pronounced gut inflammation may progress towards the development of a clinically overt CD, has supported the hypothesis that gut inflammation of AS could represent a pre-clinical stage of CD [1–7].

The pathogenesis of intestinal inflammation in AS and CD is not yet clearly understood. However, evidence suggests that abnormal responses to a role of that loss of
tolerance to commensal microbiota may play a major role in the pathogenesis of intestinal inflammation in both CD (reviewed in [3]) and AS patients [4]. In the normal intestine, a broad range of anti-microbial peptides rapidly clear intestinal micro-organisms and exert a pivotal role in the maintenance of a homeostatic balance between factors regulating host immunity and host defences, thus maintaining the integrity of the mucosa.

Paneth cells (PCs), the intestinal secretory cells located at the bottom of the intestinal crypts, are well known to play an important role in innate host defence against intestinal micro-organisms via their ability to secrete lumen anti-microbial peptides and proteins such as lysozyme, secretory phospholipase A2 (sPLA2) and human α-defensins (HD)-5 and HD-6 [8, 9]. Importantly, altered expression of the α-defensins HD-5 and HD-6 has been demonstrated in the inflamed ileum of CD patients, whereas unchanged or even increased levels of the other PC products were found, suggesting that an imbalance of PC-derived anti-microbial factors might play a central pathogenic role in promoting intestinal inflammation [10-12]. In agreement with this possibility, genetic mutations in PC-related genes, such as NOD2 (which is strongly and specifically expressed by PCs in the terminal ileum [13-15] as well as ATG16L1), whose mutations induce abnormalities in the PC granule exocytosis pathway [16, 17], are regarded to play a central pathogenic role by contributing to increased susceptibility to gut inflammation and development of CD. Consistent with the fundamental function of PCs in regulating mucosal immunity, we have recently demonstrated that PCs are the major source of IL-23 in the intestinal mucosa in pathological (both in AS and CD) as well as physiological conditions [1]. This is likely to lead to impairment in the IL-23/IL-17 axis, which is a master regulator of innate and adaptive immune responses during intestinal inflammation [18].

In the present study, we investigated the expression of PC-derived anti-microbial peptides in the subclinical gut inflammation of AS patients, and provided evidence for the first time that ileal biopsies from patients with AS and recent-onset CD display a marked up-regulation of PC anti-microbial peptides. This suggests that early stages of intestinal inflammation in AS and CD are characterized by abnormal PC function in response to local environmental stimuli leading to an altered control of mucosal immune homeostasis.

Patients and methods

Twenty-five consecutive patients with active AS without clinical symptoms of bowel inflammation were systematically referred for an oncoscopy with biopsies. Thirty CD patients with ileal involvement, with a disease duration no longer than 6 months and who were never treated with steroids or immunosuppressants at the time of sample collections, were also enrolled. Patients with isolated colonic CD were excluded from this study. Fifteen healthy controls with a routine ileocolonoscopy lacking significant pathology were randomly selected as controls [healthy subjects (HSS)]. Ileocolonoscopy was performed by a single gastroenterologist (S.P.) who, where possible, from within a 2-cm² area, retrieved multiple pinch biopsies from the terminal ileum of patients and controls. Samples were processed as previously described [1]. Briefly, samples for RNA and DNA extraction were immediately snap frozen in dry ice following withdrawal of the endoscope and then stored at −80°C in RNA until later use. One or more biopsies were also embedded in paraffin for histological examination and immunohistochemistry. All patients gave written informed consent according to the Declaration of Helsinki before ileocolonoscopy was performed. The study was approved by the ethics committee and the institutional review board of the University of Palermo.

Demographic and clinical characteristic of patients and controls are summarized in Table 1. The AS group consisted of 18 men and 7 women ranging from 28 to 55 years of age. The diagnosis for each patient was made according to the modified New York criteria [19]. Disease duration since diagnosis was 5 (4.2) months. Disease activity was evaluated by the use of the BASDAI [20], with a BASDAI ≥4 defining an active disease. All the patients were HLA-B27 positive. At the time of mucosal biopsies were obtained, the mean (s.d.) of BASDAI score was 6.5 (3.2) and none of the patients had ever received cytotoxic therapy. The CD group consisted of 19 men and 11 women, ranging from 32 to 48 years of age. The diagnosis of CD was based on standard criteria using clinical parameters, radiological, endoscopic and histopathological findings in every case. CD disease activity was defined using the CD activity index [21] together with endoscopic and histopathological data. At the time of sample collection, the mean (s.d.) of CD activity index score was 330 (62.5). The control group consisted of ileal specimens from 15 subjects (8 males and 7 females) with ages ranging from 41 to 58 years.

Quantitative Taqman RT-PCR for HD-5, sPLA2 and lysozyme mRNA

Total RNA was extracted as previously described [1] using the Qiagen RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA), with or without DNase I digestion to avoid genomic DNA contamination. First-strand cDNA was synthesized from 1 μg of total RNA with a Thermoscript RT–PCR System for First-Strand cDNA Synthesis (Invitrogen, Carlsbad, CA, USA). For quantitative Taqman real-time evaluation of the target genes and the two endogenous controls, β-actin and 18S, primers and probe were obtained from Applied Biosystems (Foster City, CA, USA) (complete list is reported in Table 2). Samples were run in triplicate with an equal loading of 20 ng of cDNA/well and detected using the ABI PRISM 7900HT Instrument; ABI PRISM 7900HT Sequence Detection System Version 2.1 (Foster City, CA, USA). Results were then analysed using the ABI PRISM 7900HT Sequence Detection System Version 2.1. Relative quantification was assessed using the Comparative threshold cycle (Ct) Method with cDNA from normal human lymph nodes used as calibrator.
Histology and immunostaining of PCs in the intestinal mucosa

Tissue samples were fixed in 10% buffered formalin, dehydrated in ethanol and paraffin embedded. Sections (4 μm thick) were deparaffinized and rehydrated. Haematoxylin and eosin-stained slides of AS specimens were divided as previously described [1] in two subgroups: those with normal histology or acute inflammation and those with chronic inflammation. Haematoxylin and eosin-stained paraffin sections from CD patients were blindly scored for inflammation (Grades 0–2) by a gastrointestinal pathologist (A.R.).

Immunohistochemistry for HD-5 [1] was performed by means of the biotin-free NovoLink polymer Detection System (Novocastra, Newcastle upon Tyne, UK). Before staining, slides were boiled in citrate buffer, pH 6.1, for 1 min with high power and 9 min with medium power and then cooled to room temperature for 20 min, to retrieve antigenicity. Endogenous peroxidase activity was blocked by incubation for 10 min with 3% hydrogen peroxide before the primary antibody, a mouse mAb to human α-defensin NP5 (Abcam, Cambridge, UK) was added to the sections at proper concentrations (1:50 dilution in PBS/0.1% BSA) and incubated for 1 h at room temperature in a humidified chamber. An isotype-matched irrelevant antibody was used as a negative control. Following three washes with PBS, slides were incubated for 30 min with the NovoLink Polymer (Novocastra) conjugated with peroxidase. After three further washings, peroxidase activity was visualized using diaminobenzidine chromogen (Novocastra), slides rinsed and counterstained with haematoxylin before dehydrating and mounting in Depex (VWR International, Oslo, Norway). The number of HD-5-expressing cells was determined by evaluating reactive cells on microphotographs taken from three randomly selected high-power microscopic fields (magnification ×40) under a Leica DM2000 optical microscope using a Leica DFC320 digital camera (Leica, Rijswijk, The Netherlands). More than 30 crypts were analysed for each patient and the number of HD-5-expressing cells is reported as mean (S.D.). Scoring was performed by two experienced pathologists who were blinded with regard to subject group.

Mutation analysis

Genotyping of genomic and/or cDNA for the functionally relevant NOD2 mutations (SNP8, SNP12 and SNP13) was performed using TaqMan technology (Applied Biosystems). In brief, amplification reactions (25 ml) were carried out with 20 ng of template DNA, 16 TaqMan Universal Master Mix buffer (Applied Biosystems),

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**Table 1** Baseline characteristics of the patients and controls

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>CD patients (n = 30)</th>
<th>AS patients (n = 25)</th>
<th>Controls (n = 15)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (s.d.), years</td>
<td>40 (6.5)</td>
<td>37 (8)</td>
<td>50 (5.4)</td>
<td>0.001</td>
</tr>
<tr>
<td>Sex: male, n (%)</td>
<td>19 (63)</td>
<td>18 (72)</td>
<td>8 (53)</td>
<td>0.001</td>
</tr>
<tr>
<td>Disease duration, mean (s.d.), months</td>
<td>4 (2.5)</td>
<td>5 (4.2)</td>
<td>NA</td>
<td>0.001</td>
</tr>
<tr>
<td>Intestinal area involved, n (%) of patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ileum</td>
<td>30</td>
<td>NA</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>Ileum and colon</td>
<td>9</td>
<td>NA</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>CRP, mean (s.d.) mg/l^a</td>
<td>32 (12)</td>
<td>7.3 (2.7)</td>
<td>NA</td>
<td>0.001</td>
</tr>
<tr>
<td>Concomitant medical treatment, n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>–</td>
<td>–</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>–</td>
<td>11</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>Immunosuppressants</td>
<td>–</td>
<td>–</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>5-Aminosalicylates</td>
<td>–</td>
<td>–</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>CD Activity Index score, mean (s.d.)^b</td>
<td>330 (62.5)</td>
<td>NA</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>BASDAI score, mean (s.d.)^c</td>
<td>NA</td>
<td>6.5 (3.2)</td>
<td>NA</td>
<td>–</td>
</tr>
</tbody>
</table>

^aP-value vs patients with CD. ^bScores for the CD Activity Index range from 0 to 600, with higher scores indicating more severe disease. ^cScores for the BASDAI range from 0 to 10, with higher scores indicating more severe disease. NA: not applicable.

**Table 2** List of primers and probes for Taqman real-time PCR

<table>
<thead>
<tr>
<th>Gene product</th>
<th>mRNA accession number</th>
<th>Assay Id</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human DEFA5</td>
<td>NM_021010.1</td>
<td>Hs00360716_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Human PLA2G2A</td>
<td>NM_000300.2</td>
<td>Hs00179898_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Human Lyz</td>
<td>NM_002392.2</td>
<td>Hs01548808_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Human SOX-9</td>
<td>NM_000346.3</td>
<td>Hs00165814_m1</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Human β-actin</td>
<td>NM_001101</td>
<td>Cat N 4326315E</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>Eukaryotic 18S</td>
<td>NM_001101</td>
<td>Cat N 4319413E</td>
<td>Applied Biosystems</td>
</tr>
</tbody>
</table>
900 nM of each primer and 200 nM of each fluorogenic probe. Detection of fluorescence signals was performed using the ABI PRISM 7700 detection system and the results were analysed as described previously by use of the Sequence Detection System Software Version 1.7 (Applied Biosystems).

Statistical analysis
Statistical analysis for quantitative variables was performed using the non-parametric Kruskal–Wallis test with Dunns’ post-test, when analysing multiple groups and the Mann–Whitney U-test when confronting two groups. Pearson’s correlation analysis was utilized to quantify the expression associations between the genes of interest. Differences were considered significant when P < 0.05.

Results
Histology
Of the 25 patients with active AS who underwent ileocolonoscopy (mean BASDAI score 6.5), subclinical ileal inflammation was observed in 17 (68%). As previously described [1, 5], patients with AS were divided into two main groups: those (12 patients) with normal histological features or minor inflammation (acute lesions) and those (13 patients) with chronic inflammation. CD patients were also divided in two main groups: those with moderately active or inactive disease (Grades 0–1; 13 patients) and those with active disease (Grade 2; 17 patients).

HD-5 mRNA expression is increased in inflamed ileum of AS patients and correlates with inflammatory state but not NOD2 mutation status
We investigated whether subclinical gut inflammation in AS patients was characterized by a modulation of PC-derived anti-microbial peptides. As shown in Fig. 1A, a marked and significant increase in expression of HD-5 mRNA transcripts was observed in the ileum of AS patients. The increase in HD-5 mRNA was significantly higher in patients with chronic rather than in those with acute inflammation, without any association between NOD2 status and the mRNA levels of HD-5.

In CD, ileal HD-5 mRNA expression was related to the degree of gut inflammation but not to the NOD2 status (Fig. 1A). Patients with active disease generally had a reduced HD-5 expression, when compared with either controls or patients with lower degree of inflammation. Patients with recent-onset CD and the lowest degree of gut inflammation showed the highest levels of HD-5 similar to those observed in the chronic inflamed AS patients. All together these findings suggest that in CD, HD-5 deficit may be the result of the inflammatory changes, rather than the initiating event before inflammation occurs.

sPLA2, Lysozyme (Lyz) and SOX-9 mRNA expression is increased in the ileum of AS patients
Lyz and sPLA2 mRNA transcripts were significantly increased in the intestine of AS patients, when compared with control intestinal biopsy specimens (P < 0.001 and 0.001, respectively). As seen for HD-5 expression, the increase in Lyz and sPLA2 mRNA, was particularly evident in mucosal biopsy specimens obtained from AS patients with chronic as compared with acute inflammation (Fig. 1B and C). CD patients with lower degree of gut inflammation also had a significant increase in Lyz and sPLA2 expression levels, when compared with those patients with highly inflamed ileum or controls (Fig. 1B and C).

A positive relationship between HD-5 and sPLA2 mRNA was found in AS (r = 0.89, P < 0.001) as well as in CD patients (r = 0.78, P < 0.001). These results confirm the link between PC density and HD-5 expression previously described [1, 10, 22].

In the small intestine, transcriptional up-regulation of PC-specific markers has been demonstrated to be dependent by SOX-9 [23], a central determinant in PC differentiation [23, 24]. SOX-9 mRNA appeared to be over-expressed in both AS chronic inflamed and CD patients with low degree of intestinal inflammation, but not in controls (Fig. 1D), apparently confirming the central position of SOX-9 in the regulation of intestinal epithelium homeostasis. In this regard, a positive correlation was found in both AS patients with chronic inflammation and CD patients with low inflammation, between SOX-9 and both HD-5 (AS: r = 0.86, P < 0.01; CD: r = 0.93, P < 0.001) and sPLA2 (AS: r = 0.81, P < 0.01; CD: r = 0.88, P < 0.01).

Normal number of PCs in early ileal inflammation
Given the strong up-regulation of HD-5 mRNA in mucosal specimens from patients with AS, we next investigated its protein expression. Representative anti-HD-5, immunostainings are shown in Fig. 2. In ileal specimens of AS and CD patients with similar degrees of ileal inflammation, HD-5 expression was comparable (Fig. 2A and B). We then compared the number of HD-5+ cells in samples from AS patients, CD patients and normal controls. As shown in Fig. 2C, terminal ileal biopsy specimens from AS patients, independently by the degree of inflammation, showed a number of HD-5+ expressing cells [mean (s.d.) of normal histology/acute inflamed 49 (9) and chronic inflamed 40 (5)], similar to patients with CD and low inflamed ileum [mean (s.d.) 46 (8)] and controls [mean (s.d.) 42 (11)]. Patients with CD and severe intestinal inflammation showed significantly reduced PC [mean (s.d.) 19 (8)].

NOD2 mutation analysis
Four (16%) of the AS patients were positive for CARD15 polymorphisms, three were heterozygous for at least one mutation and one was homozygous for the 1007fs allelic variant. Three patients had chronic gut inflammation and high levels of HD-5 mRNA and one displayed normal histology. In the CD population, a carrier frequency of 26% (8 of 30 patients) was observed. Four CD patients were positive for SNP13 (three heterozygotes and one homozygote). Two were positive for SNP8 and two patients displayed a mutation for SNP12 (heterozygotes only).
One of the four SNP13 heterozygotes was also positive for SNP12. Five out of eight patients carrying NOD2 mutation had normal to increased levels of HD-5 mRNA. In the control group, two individuals (16%) displayed NOD2 polymorphisms, one was heterozygous for SNP12 and one for SNP13. No relationship was observed between NOD2 mutations and HD-5 mRNA levels in either patients or controls.

**Discussion**

In the present work, we provide evidence that PC-derived anti-microbial HD-5, sPLA2 and lysozyme transcripts are overexpressed in the ileum of patients with AS. In our study, HD-5 mRNA levels, in both AS and CD patients, were influenced by degree of inflammation rather than the NOD2 status, apparently supporting the hypothesis of an acquired defensin deficiency, likely due to the loss of epithelium occurring in the course of active inflammation. In fact, AS patients with chronic inflammatory lesions and CD patients with recent-onset disease and low degree of intestinal inflammation, similarly displayed a normal number of PC with significant over-expression of PC-derived anti-microbial peptide mRNA levels.

PCs are specialized secretory intestinal epithelial cells located at the bottom of the crypts of Lieberkühn in the small intestine displaying an essential role in the modulation of gut immune homeostasis, through the production of anti-microbial peptides and immunoregulatory cytokines such as IL-23 [1]. PC secretes anti-microbial peptides such as lysozyme, sPLA2 and HD-5 and HD-6 luminally [8, 9]. Defensins are small cationic arginine-rich anti-microbial peptides that contain six cysteine residues in conserved spacing pattern [25]. In humans, six α-defensins have been described, with HD-5 and HD-6 mainly expressed in PCs [26, 27]. Although defensins were first identified as anti-microbial peptides, evidence supports the concept that defensins may also play an important function in both innate and adaptive response of the small intestine against bacteria [27]. Chemoattraction of macrophages, T lymphocytes and mast cells represent...
an immunomodulatory function that is evolutionarily conserved within the HD family [28]. Defensins may also modulate adaptive immune response regulating the production of pro-inflammatory cytokines (such as IL-1β) [29] and chemokines (such as IL-8) [30].

SOX-9 belongs to the SOX family of transcription factors related to a gene involved in sex determination called (sex determining region Y) SRY. SOX-9 expression requires an active beta-catenin-(T-cell factor), Tcf complex. SOX-9 has been demonstrated to regulate the differentiation of PC through the transcriptional regulation of several marker mRNAs of these cells [23]. In this regard, our demonstration, in both AS and CD patients, of a strong positive correlation between the levels of sPLA2 (a marker of PC mass) [22], with either HD-5 (a PC-specific gene) or SOX-9 genes strongly confirms the central position of SOX-9 in the regulation of intestinal epithelium homeostasis in the inflamed ileum of AS and CD patients.

Altered production of PC-derived HD-5 has been demonstrated in patients with CD. Two studies by Wehkamp et al. [11, 12] suggest that HD-5 deficiency, linked primarily to the NOD2 SNP13 (1007fs) genotype, is likely to be an initiating event in the pathogenesis of the disease. Mutations in the NOD2 gene (SNP8, SNP12 and SNP13) are strongly associated with ileal location of CD, contributing towards 50% of pure ileal disease [13]. NOD2 is a cytosolic receptor for muramyl dipeptide, which is derived from the bacterial cell wall component peptidoglycan [31], and is expressed mainly by monocytes and PCs [15, 32]. Although NOD2 variants seem not to significantly affect the risk of developing primary AS [33], variants of NOD2 have been demonstrated to increase the risk to developing ileal CD in AS. NOD2 mutations seem not sufficient, however, to explain the observed defensin deficiency in CD patients, since not all NOD2 mutations have effects on α-defensin production. In particular, the NOD2 frame-shift mutation does not affect other α-defensins, and it has been recently demonstrated that the reduced levels of HD-5 mRNA are primarily linked to the loss of surface epithelium as a consequence of intestinal inflammation [10].

**Fig. 2** HD-5 expression by PCs in the terminal ileum of CD and AS patients (A and B). Representative photomicrographs showing 3-μm-thick paraffin-embedded sections of distal ileal biopsy specimens obtained from patients with CD (A) and AS (B), stained for HD-5. In both CD and AS patients with similar degrees of ileal inflammation (A and B), numbers of PCs were similar. (C) Number of HD-5+ cells in the mucosa. Results are expressed as the number of positive cells per field. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. NS: normal subjects.
In summary, this is the first study to demonstrate that HD-5 mRNA over-expression occurs in the terminal ileum of AS patients at levels comparable with those found in early CD patients, suggesting that dysfunction of PCs occurs already in a relatively early stage of gut inflammation and certainly before the onset of clinical symptoms. In addition, our results are consistent with the hypothesis that HD-5 deficiency in CD occurs independently from NOD2 status, being the consequence of the loss of surface epithelial cells with concomitant reduction in PC number. In this regard, over-expression of PC-derived anti-microbial peptides as well as pro-inflammatory cytokines, such as IL-23, could be the primary moment in the pathogenesis of chronic inflammatory gut disorders. Once the disease is manifested, a deficiency in defensin could be the result of the loss of epithelial integrity, resulting in bacterial invasion and further inflammatory response. The underlying pathogenetic mechanism related to the aberrant expression of HD-5 in the gut of AS patients need further investigation.

Rheumatology key messages

- Up-regulation of PC-derived anti-microbial peptides occurs in the ileum of patients with AS.
- Dysfunction of PCs characterizes early stages of gut inflammation probably in response to unknown bacterial triggers.
- Immunomodulatory function of defensin could play a pivotal role in driving intestinal inflammation.

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