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ABSTRACT

Cytokine mRNA quantification in gastro-intestinal biopsies of dogs with idiopathic chronic enteropathies by Real Time RT-PCR: preliminary results

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Keywords Cytokine · Dog · Inflammatory bowel disease · Intestine

Introduction

Cytokines are small peptide proteins produced by immune cells that facilitate communication between cells, have important effector functions via activation of directly cytotoxic compounds and stimulation of activity of immuno-mediatory T-cells during bacterial and viral infections. Recent studies have underlined the important role played by CD4+T helper cells and cytokines in the immunopathogenesis of human inflammatory bowel disease (IBD) (Ridyard et al., 2002). Studies showed increased synthesis and action of pro-inflammatory cytokines and a defective secretion of immunoregulatory cytokines in intestinal tissue and peripheral blood of patients with Crohn's disease (CD) or ulcerative colitis (UC) (Bustin, 2002). The aim of this study was to quantify the expression of m-RNA by real-time PCR encoding pro-inflammatory (IFN-γ; TNF-α) and immunoregolatory (IL-4) cytokines in intestinal biopsies of dogs affected with inflammatory bowel disease (IBD).

Materials and methods

Dogs were separated into two groups: group 1 was made up of 11 dogs (6 males and 5 females), the median age was six years (range, 1–12 years), suffering from chronic

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diarrhoea at the time of presentation; group 2 was made up of 4 dogs without chronic diarrhoea. In group 1, haematochezia, fecal mucus, tenesmus, increased frequency of defecation, vomiting, weight loss, dependent the site of the disease, had been reported for at least 3–4 weeks. Eight dogs showed predominantly small-bowel clinical signs upon clinical examination, two dogs showed signs of large bowel disease, while in one dog mixed signs were noted. Haematological tests, serum biochemical profile (with folate and cobalamin measurements, trypsinlike immunoreactivity) and urinalysis was performed for all dogs. A repeated faecal flotation examination was negative for parasitic ova and *Giardia* cystis. All the dogs had been fed with a new protein commercial diet and they had been given intermittent antibiotic treatment with either transient or no clinical improvement. Upper gastrointestinal endoscopy was performed in eight dogs, colonoscopy in two dogs and gastroduodeno-colonoscopy in one dog; the four dogs in group 2 had gastroduodenoscopy for other reasons (gastric foreign bodies and megaesophagus). Multiple endoscopic biopsies were taken from the gastric body, fundus and pyloric antrum; biopsies were also obtained from the mucosa of the proximal and distal duodenum and from all parts of the colon and rectum. For histopathological examination biopsies were fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned at 3–4 μm and stained with hematoxylin and eosin and Periodic-acid-Shiff (PAS). For real-time RT-PCR, two endoscopic biopsies, taken from adjacent areas of the pyloric antrum and duodenum for the upper tract, and from the colon and rectum for the lower tract, were placed immediately in RNA-later (Qiagen). The histopathological evaluation, performed out blind by a single veterinary pathologist, was based on: mucosal architecture and lamina propria or epithelium cellularity; severity of changes was scored on a scale from 0 (normal), 1 (mild), 2 (moderate) or 3 (severe) on the basis of previously published guidelines (Peters et al., 2005).

Total RNA was extracted from the endoscopic biopsy homogenates (total tissue mass 10–20 mg) using the RNeasy total RNA isolation kit according to the manufacturers' recommendations (Qiagen, Milan, Italy). All RNA samples were treated with RNase-Free DNase Set 50 (Qiagen) at room temperature for 45 min to remove any traces of genomic DNA. The final RNA was eluted in 40 μl of RNase-free water and stored at −80°C before use. cDNA was synthesized with the M-MLV-reverse transcriptase Kit (Invitrogen, Milan, Italy), using the manufacturer's protocol. Primers and TaqMan MGB probes for TNF-a, IFN-γ and IL-4 were designed to bind specifically to canine cytokine cDNA using Primer Express Sofware (http://www.appliedbiosystems.com/support/apptech/) according to published canine cytokine mRNA sequences (GenBank). G3PDH (Glyceraldehyde-3-phosphate dehydrogenase) was used a housekeeping gene.

Real-time PCR data were normalized in each individual sample using the level of G3PDH expression. Gene expression was measured by the comparative cycle threshold method ($\Delta\Delta C_T$) and was reported as the n-fold (n-fold=$2^{-\Delta\Delta CT}$) difference relative to the normalized expression of reference samples (cDNA preparations made from biopsy specimens belonging to heathy dogs). Comparisons between the n-fold values of the two groups were performed using the *t*-Student test. Significance was taken at $P<0.05$.

Results

In group 1, histopathological examination revealed, in eight biopsies (2 colonic mucosa and 6 duodenal mucosa), a predominantly lymphocytic-plasmacytic cell infiltrate within the lamina propria; in one case duodenal lymphangectasia and moderate lymph-plasmacytic infiltrates were associated; one case had severe duodenal lymphangectasia without

inflammatory cells. Biopsies from two dogs revealed a predominantly eosinophilic infiltration of the lamina propria of antrum and duodenal samples. In one dog, histological features were suggestive of histiocytic ulcerative colitis (HUC). The severity scores ranged from 1 to 3 (mean 2.1). The four clinically normal dogs of group 2 had histologically normal biopsies. Cytokine mRNA of TNF-α was quantified in all the samples of duodenal mucosa with inflammatory processes and in only one sample of the pyloric antrum, in all three colonic biopsies, with very high expression in the samples from the dog affected by HUC. No cytokine mRNA of TNF-α was quantified in rectal biopsies. Cytokine mRNA expression of IFN-γ was measured in seven duodenal biopsies and in the same biopsy with mRNA expression of TNF-α; expression was also quantified in two colon biopsies. mRNA of IL-4 was quantified in only one duodenal biopsy. m-RNA expression of TNF-α and IFN-γ was found in all group 2 duodenal biopsies. No significant difference in the level of cytokine m-RNA expression was found between the biopsies of the two groups: TNF-α ($P=0.208$) and IFN-γ ($P=0.472$).

Discussion

TNF-α and IFN-γ are considered to be pro-inflammatory cytokines, related to type I immunity regulated by the Th1 subset of CD4+T lymphocytes. M-RNA transcripts of these cytokines in duodenal mucosa of dogs affected by inflammatory bowel disease was not statistically different compared to dogs without signs of gastro-intestinal disease and normal mucosa. This is in agreement with one of two previously published investigations into the role of cytokines in canine IBD (Fujiwara et al., 2002; Peters et al., 2005) The expression of mRNA TNF-α and IFN-γ in biopsies of normal dogs suggested that pro-inflammatory cytokines are present in normal mucosa and that there does not appear to be a relationship between the simple expression of cytokines, clinical signs and inflammatory infiltration.

Higher levels of pro-inflammatory cytokines were identified in one sample with a severe inflammatory infiltrate formed by periodic acid-Shiff positive (PAS) histiocytes. This observation could be correlated to the severity of mucosal damage or to the high presence of cells involved (macrophages). In conclusion, we believe that the information from the quantification of gene expression of other cytokines and in a greater number patients, trough a continuation of the current work could provide new information about canine inflammatory bowel disease.

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