The expression of innate immunity genes in Italian Crohn disease patients

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Crohn' disease (CD) is a chronic idiopathic inflammatory bowel disease characterized by the interaction of both hereditary and environmental factors. Intestinal flora and pathogens such as bacteria, viruses and fungi, are thought to be the first step leading to an inflammatory status, which is subsequently amplified in genetically susceptible patients thus triggering the disease. Since the innate immune system is believed to be very important in regulating the flora of the gastrointestinal tract, we decided to study the influence of two important molecules of the innate immune system in CD. Frozen intestinal biopsies from 49 Crohn patients and 10 healthy individuals were collected at the gastroenterology unit of Children's Hospital Burlo Garofolo in Trieste and innate immunity gene expression was evaluated by using both *in situ* RT-PCR and quantitative PCR.
We have analyzed the expression and localization of both *MBL2* and *DEFB1* genes in intestinal biopsies of Italian Crohn patients by *in situ* RT-PCR and quantitative PCR. *DEFB1* is expressed equally in all subjects. Importantly, *MBL2* transcripts were upregulated in CD patients compared to healthy controls. *MBL2* expression in controls is normally extremely low, detectable only by quantitative PCR with a Taqman probe.
We demonstrated the *MBL2* and *DEFB1* expression in intestinal biopsies of patients suffering from CD. Our results showed that the *MBL2* gene is expressed by cells in the basal lamina, whilst *DEFB1* is expressed by epithelial cells.

Key words: *MBL2*, *DEFB1*, innate immunity, Crohn disease, gene expression.

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Crohn disease (CD) is a chronic idiopathic inflammatory bowel disease characterized by the interaction of both hereditary and environmental factors. One etiologic hypothesis is that the normal intestinal flora or perhaps specific pathogens such as bacteria, viruses and fungi, are thought to initiate the first step leading to an inflammatory state, which is subsequently amplified in genetically susceptible patients thus triggering the disease (Yamamoto-Furusho, 2006; Wehkamp, 2006; Sands, 2007). Since the innate immune system is believed to be very important in regulating the flora of the gastrointestinal tract, we decided to study the influence of two important molecules of the innate immune system in CD.

Mannose-binding lectin (MBL) is a serum lectin that plays a pivotal role in natural immunity. It has a role of *ante-antibody* in the first line of host defense and activates the classical pathway of the complement and mediates the phagocytosis (Turner, 1998). MBL is a C-type reactive protein synthesized by liver hepatocytes. The importance of MBL in first line host defense and immune system regulation is supported by recent findings which demonstrate that individuals with low MBL levels are more prone to severe and repeated infections or autoimmune diseases (Eisen, 2003).

The human beta defensin 1 (hBD-1) is constitutively expressed by the epithelial cells of the skin, in the upper-airways and in the genito-urinary tract. It plays a direct antimicrobial activity and also shows a chemotactic activity on immature dendritic cells linking CCR6. The peptide is encoded by the *DEFB1* gene. Dahariwall *et al.* (2003) demonstrated, by RT-PCR, the expression of the *DEFB1* gene in the small intestine of healthy people. Furthermore, they showed that the *DEFB1* expression seems to vary between individuals: in fact mRNA wasn't detected in every sample.

Moreover, it has been shown (Fellerman, 2006) that a lower *DEFB4* gene copy-number in the

defensin locus could be associated with increased risk of developing colonic Crohn disease, further suggesting a role for defensins in Crohn disease.

In our study, we have analyzed the expression and localization of *MBL2* and *DEFB1* genes in intestinal biopsies from Italian CD patients by *in situ* RT-PCR (IS RT-PCR) and Quantitative PCR (Q-PCR).

Material and Methods

Frozen colonic intestinal biopsies from 49 Crohn patients and 10 healthy individuals were collected at the gastroenterology unit of Children's Hospital Burlo Garofolo in Trieste (Italy). The study was approved by the Ethical Committee. Direct IS RT-PCR was performed as described by Boniotto et al. (Boniotto, 2003) using Cy3 fluorescent nucleotides (GE healthcare, Piscataway, NJ), and specific primers (for *MBL2*: forward 5'-ACCAGGC-CAAGGGCTCAG-3', reverse 5'-AAGGTGAGC-CACTTTTTGATACGT-3'; for *DEFB1*: forward 5'-CCAGTTCCTGAAATCCTGAGTGT-3' reverse 5'-CTGTGAGAAAGTTACCACCTGAGG-3'). Negative controls were used for retrotranscription and amplification, without Reverse Transcriptase (RT) or primers. After the amplification, slides were counterstained with DAPI (Vectashield, Burlingame, CA) and directly observed under a fluorescent microscope. Total RNA was prepared from colonic biopsies of CD patients and controls following standard procedures. RNAs were quantified by spectrophotometer and reverse transcribed. First-strand cDNA was synthesized using random hexamers and SuperScript II Reverse Transcriptase (Gibco BRL). Q-PCR was performed using primers and Taqman probes of two ABI Assay on Demand following manufacturer's instructions, Hs00175093_m1 for *MBL2* and Hs00174765_m1 for *DEFB1* genes, on ABI Prism™ 7900*HT* Sequence Detector System (Applied Biosystems, Foster City CA). Human *HPRT1 (HGPRT)* was used as endogenous control.

The differentially expressed genes were quantified using the comparative Ct method. Normal cDNA was used as calibrator sample; the gene expression values from each gene were normalized toward the *HPRT1* housekeeping gene; expression data were obtained with the ΔCt method (Livak 2001). Data generated by the SDS 2.1 software are expressed as the mean fold differences between normal patient(s) and diseased sample(s) RQ value with the interquantile range (IRQ). Data were subsequently analyzed by a t-Student test with a significance *p- value* of 0.001.

Results

Figure 1 shows the localization and expression of *MBL2* and *DEFB1* genes on intestinal biopsies from CD patients. *DEFB1* is expressed by epithelial cells, while *MBL2* expression is localized at the level of the basal lamina (Figure 1). The cell type expressing the *MBL2* gene has not been identified yet. Controls, both without primers or RT, were characterized by the absence of amplification signal (*data not shown*).

MBL2 was not expressed in the intestinal biopsy of healthy controls, while *DEFB1* mRNA was present both in CD patients and healthy individuals (*data not shown*).

Q-PCR performed on RNA extracted from the biopsies confirmed the results obtained *in situ*. *DEFB1* was equally variable in both CD patients and controls but no difference was evidenced between the two groups. *MBL2* was detected in CD patients and resulted up-regulated if compared to healthy controls *MBL2* RNA transcripts were upregulated in CD patients compared to healthy controls (36.7 times more; IRQ 2.01) where *MBL2* expression was extremely low, detectable only by Q-PCR with a Taqman probe and not with an *in situ* RT PCR experiment.

Discussion

CD is a complex disease mainly caused by an over response of the immune system of the host against the physiological intestinal flora or against specific pathogens isolated from Crohn disease patients. Recently, polymorphisms in genes which encode proteins involved also in immune response have been associated with the disease.

In this study, we demonstrated *MBL2* and *DEFB1* expression in intestinal biopsies of patients suffering from CD. Our results show that the *MBL2* gene is expressed at the level of the basal lamina, whilst *DEFB1* is clearly expressed by epithelial cells. It is known that MBL is produced by liver hepatocytes and its expression varies among individuals. However, recently Boniotto *et al.* (2003) demonstrated that the gene is expressed in intestinal biopsies from celiac patients. Our Q-PCR results clearly indicate the presence of *MBL2* expression in cells in the basal lamina in CD biopsies. Healthy normal have very low *MBL2* expression that is undetectable by IS RT-PCR techniques and only identified by a Taqman probe.

At the moment, the cell type that expresses

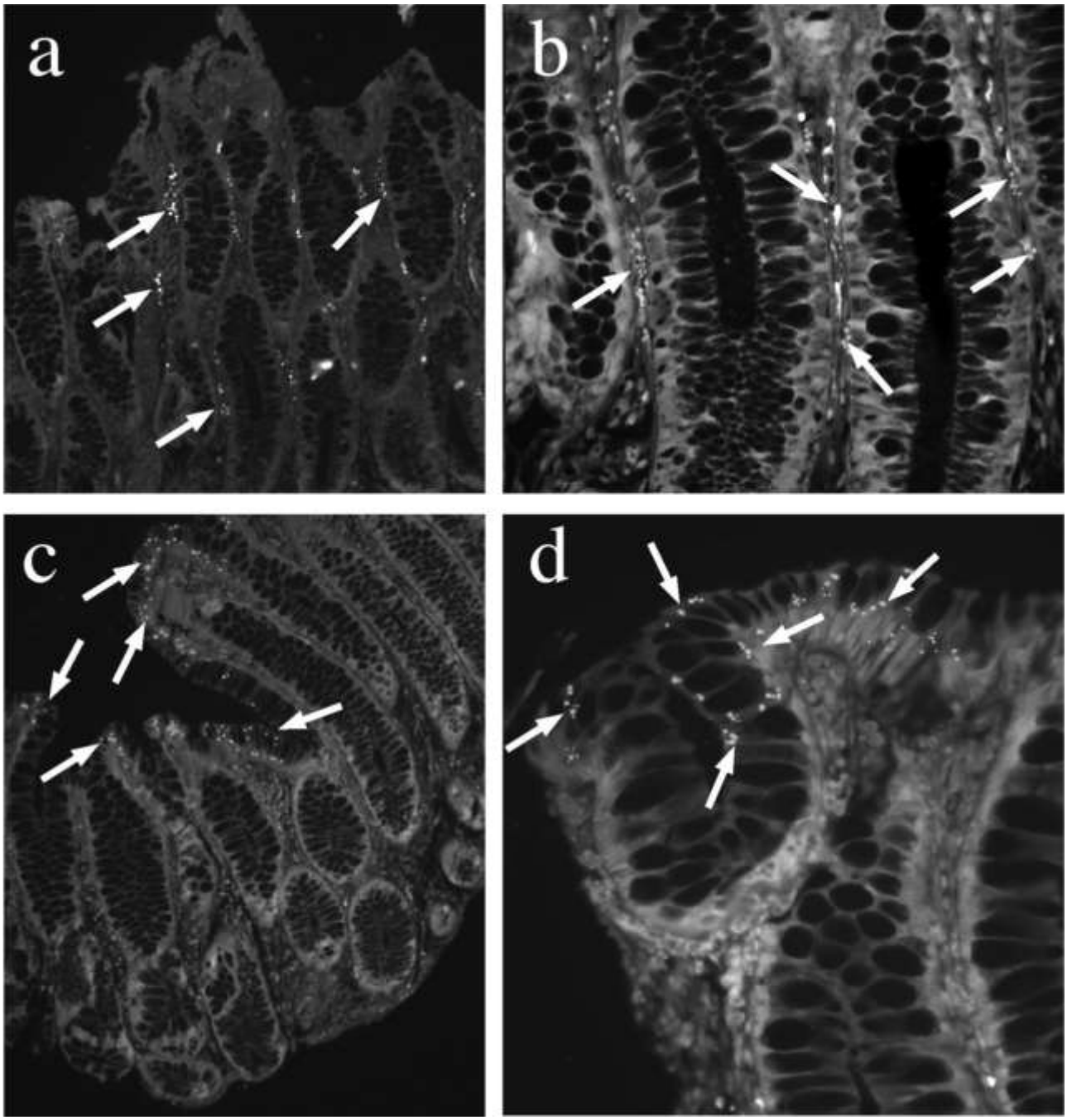

Figure 1. CMBL2 and DEFB1 expression in Crohn patients' biopsies as revealed by in situ RT-PCR. a) Crohn patient intestinal biopsy: MBL2 expression visible at the level of the basal lamina (arrows). Magnification is 100X. b) Crohn patient intestinal biopsy: MBL2 amplification signal localization in the area of the basal lamina (arrows). Magnification is 400X. c) DEFB1 expression localized in the epithelial cells (arrows). Magnification is 100X. d) DEFB1 gene expression in a Crohn patient: the amplification signal is localized in the epithelium of the intestinal biopsy (arrows). Magnification is 400X.

MBL2 in the basal lamina is unknown. The role of the protein in the disease process is also unknown. However, as already suggested by Boniotto *et al.* (2003), we can hypothesize that *MBL2* is expressed by cells of the immune response infiltrated in Crohn inflamed intestines, as its expression is absent in the biopsies of healthy controls.

As regards *DEFB1*, we confirm the results obtained by O'Neil *et al.* (1999) who detected gene expression in epithelial cells of the small and large bowel but in our study no difference in *DEFB1* expression was found between CD patients and

healthy controls. It should be interesting to further investigate the differential expression of inducible defensins, such as human beta defensin 2 and 3 whose impaired production has been suggested to be linked to the onset of Crohn' s disease (Wehkamp J., 2005).

In conclusion we confirmed the expression of *DEFB1* gene in colonic lamina propria; furthermore we show a differential expression of *MBL2* expression between patients and healthy controls, suggesting a role for MBL in the etiopathogenesis of Crohn disease.

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