Modulation of VIPergic phenotype of enteric neurons by colonic biopsy supernatants from patients with inflammatory bowel diseases: Involvement of IL-6 in Crohn's disease

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First published: 31 August 2017

https://doi-org.proxy.insermbiblio.inist.fr/10.1111/nmo.13198

Citations: 6

Funding information

This work was supported by an unrestricted grant obtained from the “Direction de la Recherche Clinique” (University hospital Nantes, France) and the “Institut National de la Santé et de la Recherche Médicale” (INSERM, Paris, France) and by an unrestricted grant obtained from the “Region pays de La Loire” (SYNEMIC project).

FS has received a financial support from the SanTdige foundation (IMAD, Nantes, France).

Arnaud Bourreille and Michel Neunlist are equally contributed to the study.

Abstract

Background

Neuroplastic changes in the enteric nervous system (ENS) observed during IBD might participate in physiopathological processes. Vasoactive intestinal polypeptide has been shown to be involved in intestinal inflammation and barrier functions. We aimed to investigate the modulation of VIP expression in colonic biopsies of IBD patient, the ability of soluble factors from biopsies to reproduce in vitro these modulations and identify soluble factors responsible.

Methods

VIP and cytokines mRNA expressions were assessed in colonic biopsies of healthy subjects (HS) and IBD patients from inflamed (I) and non-inflamed areas (NI). Supernatants (SUP) of biopsies were applied to primary culture of ENS and VIP and cytokines mRNA expressions were assessed. The role of cytokines in SUP induced changes in VIP expression was evaluated.

Key Results

VIP mRNA expression was lower in biopsies of patients with Crohn's disease (CD) than Ulcerative Colitis (UC) but unchanged as compared to HS. VIP mRNA and protein expression were lower in primary culture of ENS incubated with SUP-CD than with SUP-UC. Furthermore, in CD but not UC, SUP-I reduced VIP expression in the ENS as compared to SUP-NI. Next, IL-6 but not IL-5, IL-10, IL-17, IFN-γ or TNF-α reduced VIP expression in the ENS. Finally, in CD, SUP-I incubated with anti-IL-6 antibody increased VIP expression as compared to SUP-I alone.

Conclusions & Inferences

Mucosal soluble factors from IBD induce VIP neuroplastic changes in the ENS. IL-6 was identified as a putative soluble factor responsible in part for changes in VIP expression in CD.

Key Points

- Neuroplastic changes and in particular VIP expression in the enteric nervous system observed during inflammatory bowel disease might participate in physiopathological processes.
- We demonstrated that mucosal soluble factors differentially modulate VIP neuroplastic changes observed in IBD according to the disease.
- Among different putative soluble factors, IL-6 was in part responsible for reduced VIP expression in CD.

1 INTRODUCTION

Inflammatory bowel diseases (IBD), which include Crohn's disease (CD) and Ulcerative Colitis (UC) are characterized by a chronic inflammation affecting the gastrointestinal (GI) tract (CD) and the colon (UC).[1] The etiology of IBD is unknown but results from inappropriate immune responses in genetically susceptible host.[2] Current medical treatment has focused mainly on the modulation of the immune response during the last decade.[3] However, IBD are multifactorial and increasing evidences suggest that, besides dysregulated immune response, abnormalities occur at different levels such as the intestinal epithelial cells (IEC), fibroblasts and also the enteric nervous system (ENS).

The ENS plays a major role in regulating GI functions such as paracellular permeability, proliferation of IEC, motility and the immune response.[4] Changes in the ENS have been observed in IBD, encompassing modifications in neuronal/glial morphology, in neuromediator production, and in electrophysiological properties.[5, 6] These alterations could be responsible for functional changes observed in IBD such as modifications of motility, intestinal permeability and secretory responses, and even, visceral sensitivity.[7-9]

Among key neuromediators involved in the control of GI functions is vasoactive intestinal polypeptide (VIP). VIP reduces intestinal paracellular permeability by increasing tight junction protein expression.[7] In addition, VIP reduces proinflammatory cytokines production (IL-1β, IL-6, IL-12) and increases the expression of the regulatory cytokine IL-10.[10, 11] Plasticity affecting different neurochemically identified subpopulations of enteric neurons has been reported in UC and CD.[12] However, changes in VIP expression observed in IBD are contradictory.[13-16] The mechanisms and mediators responsible for these alterations remain also currently unknown. However, as has been demonstrated in irritable bowel syndrome (IBS), alterations of the ENS could be due to the release of soluble factors of the mucosal cellular microenvironment.[17, 18]

Therefore, after characterizing VIP expression in colonic biopsies of IBD patients, we evaluated the ability of supernatants (SUP) from biopsies to induce changes in VIP expression in primary culture of ENS. Finally, we aimed to identify mediators responsible in part for the neuroplastic effects observed.

2 PATIENTS AND METHODS

2.1 Patients

Twenty-seven patients with IBD, 16 with an established CD, 11 with UC were enrolled in the study. The demographic and clinical characteristics of the patients are detailed in Table 1. There were no statistical differences between groups concerning most of the items evaluated. Twenty-two asymptomatic healthy subjects (HS) who underwent ileocolonoscopy for the detection of colonic polyps were also included and were matched for age and sex. None of the controls were receiving immunosuppressors or corticosteroids. Colonic biopsies were obtained and stored in the bio-collection of the “Institut des Maladies de l'Appareil Digestif (IMAD)”. All patients included in the bio-collection gave written and informed consent before the endoscopic work-up. The clinical and demographic data were collected at the time

of colonoscopy and recorded in a computerized database securely coupled to the biological collection. This bio-collection was started in 2008 and was approved and registered by the French Ministry of Science and Research “DC-2008-402”.

Table 1. Demographic and clinical patient characteristics

	Crohn's disease (n = 16)	**Ulcerative colitis (n = 11)**	**Healthy controls (n = 22)**
Sex ratio (M/F)	7/9	8/3	10/12
Age (y) median (min-max)	34 (17-49)	38 (22-58)	49 (34-67)
Duration of the disease (y) med (min-max)	9 (1-22)	7 (1-17)	NA
Treatment (%)			NA
IS	8 (50)	6 (55)	
Anti-TNF	9 (56)	4 (36)	
Prednisone	2 (16)	4 (36)	
5 ASA	3 (19)	7 (63)	

- IS, Immunosuppressor; 5 ASA, 5-amino-salycilate; NA, not applicable.

2.2 Biopsies and supernatants

Twelve biopsies were taken from the colon near endoscopic inflamed (I) and non-inflamed (NI) areas of the disease. Six biopsies were taken from the right colon, macroscopically undamaged in HS. In all cases, biopsies were taken using standard biopsy forceps without needles (FB210K, Olympus co., Japan) and were immediately immersed in 4°C saline solution.

Two biopsies per area, ie, inflamed or not, were crushed in Lysing Matrix D tubes (MPBio, Thüringer, Germany) containing 600 µL buffer RA1 (Macherey-Nagel, Düren, Germany) and 6 µL β-mercaptoethanol (Sigma–Aldrich, Saint-Louis, USA) and stored at −80°C for further mRNA amplification. The four remaining biopsies were cultured in Krebs/Hepes (1 mL/30 mg) at 37°C. After 25 minutes incubation, the supernatants (SUP) were removed and filtered (0.22 µm, Spin-X Corning Incorporated, Amsterdam, Holland), centrifuged (1 minute, 10 000 g) and stored at −80°C for further experiments.

2.3 VIP and cytokine mRNA expression in biopsies by real-time quantitative polymerase chain reaction

The total RNAs were extracted from colonic biopsies using NucleoSpin®TriPrep (Macherey-Nagel EURL, Hoerdt, France) according to the manufacturer's instructions. For reverse transcription, 1 µg purified total RNA was denatured and subsequently processed using SuperScript III Reverse Transcriptase (Invitrogen, Saint-Aubin, France) according to the manufacturer's instructions.

cDNAs were amplified by real-time PCR using Rotorgene2000 (Qiagen, Courtaboeuf, France). Reactions were performed in a total volume of 15 µL composed of 1.25 µL forward and reverse primer, 7.5 µL Light cycler 480 SYBR Green I Master (Roche, Mannheim, Germany) and 5 µL cDNA at a concentration of 0.8 ng μL^{-1} RNA equivalent. All primers

were designed using the software Oligo 4 (Molecular Biology Insights, Cascade, USA) and were produced by Sigma. Before repeated experiments, the validity of each amplification product for each primer pair was tested by sequencing. The full sequence of each primer set, and their accession numbers, are detailed in Table 2.

Table 2. Real-time PCR information

Gene	Species	Accession number	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product length (bp)
S6	Human Mouse Rat	NM_001010	CCAAGCTTATTCAGCGTCTTGTTACTCC	CCCTCGAGTCCTTCATTCTCTTGGC	130
NSE	Human Mouse Rat	NM_001077651	TCAGACTCCAGACCAAAACCATAAAGG	GCGGATGAATCCCACCCCTC	202
VIP	Human	NM_003381	CGGCATGGCCTCTTTACAGGGC	ACTCCATCAGCATGCCTGGCA	184
VIP	Rat	NM_053991.1	TTGGCAAACGAATCAGCAGTAG	ATTTGCTTTCTAAGGCGGGTGT	100
IL-6	Human	NM_000600	CAATGAGGAGACTTGCCTGGTGAA	TGTGGTTGGGTCAGGGGTGGTT	195
IL-6	Rat	NM_012589.1	TTGTTGACAGCCACTGCCTTCCC	TCTGACAGTGCATCATCGCTGTTCA	200
IL-17	Human	NM_002190	AAACAACGATGACTCCTGGG	GAGGACCTTTTGGGATTGGT	199
IFN-γ	Human	NM_000619	CCAGAGCATCCAAAAGAGTGTGGAG	GCTGGCGACAGTTCAGCCATCA	179
TNF-α	Human	NM_000594	CGCTCTTCTGCCTGCTGCACT	ACTGGAGCTGCCCCTCAGCTT	173
IL-10	Human	NM_000572	GAGGCTACGGCGCTGTCATCG	CGCCACCCTGATGTCTCAGTTTCG	189
IL-5	Human	NM-000879	GAACTCTGCTGATAGCCAATGAGAC	AGTTTGACTCTCCAGTGTGCCTATT	113

The same standard reaction condition was used for each primer pair, consisting of a denaturation at 95°C for 10 second, hybridization at 60°C for 15 second, and elongation at 72° for 15 second. This cycle was repeated 45 times. Samples were analyzed in duplicate and

a standard curve for each gene was prepared using serial dilutions from the cDNA extracted from colonic biopsies of each pool of patients. All the results obtained were expressed as a ratio of cytokine or VIP/S6 (in biopsies) mRNA expression levels.

2.4 Primary cultures of ENS

Primary cultures of ENS were performed as previously described.[19] In brief, the small intestines of rat embryos E15 (35-45 per isolation) from three pregnant Sprague–Dawley rats (CERJ, Le Genest St Isle, France) were removed and finely diced in Hank's buffered salt solution (Sigma). Tissue fragments were collected in 10 mL medium (Dulbecco's modified Eagle's medium [DMEM]-F12 [1:1] medium) and digested at 37°C for 15 minute in 0.1% trypsin (Sigma). The trypsin reaction was stopped by adding 20 mL medium containing 10% fetal calf serum and then treating with 0.01% DNase I (Sigma) for 10 minute at 37°C. After triturating with a 10 mL pipette, cells were centrifuged at 500 g for 10 minute. Cells were counted and then seeded at a density of 2.4×10^5 cells/cm^2 on 24-well plates previously coated for 6 hour with a solution of gelatin (0.5%; Sigma) in sterile phosphate-buffered saline (PBS). After 24 hour, the medium was replaced with a serum-free medium (DMEM-F12 [1:1] containing 1% N-2 supplement [Life Technologies, CergyPontoise, France]). Cells were maintained in culture for 14 days. Half of the medium was replaced at D6 and D11.[19]

2.5 Effect of colonic supernatants on primary culture of ENS

Primary ENS cultures were incubated in duplicate in 24-well plates, in the absence or presence of SUP obtained from HS (n = 12), patients with CD (n = 12) and patients with UC (n = 6). After 12 days of culture, 50 μL SUP were added for 48 hour in 950 μL serum-free DMEM-F12 (1:1) containing 1% N-2 supplement. After 48 hour treatment, the supernatants of the ENS cultures were collected and stored at −80°C. ENS primary culture was used either for the quantification of the VIP protein or for the quantification of its mRNA expression.

2.6 Analysis of VIP protein expression

Primary culture of ENS was treated with 125 μL RIPA (Millipore, Temecula, CA, USA) with protease inhibitors for 30 minute on ice and centrifuged at 10 000 g for 15 minute at 4°C. The VIP protein was measured in the cell culture supernatants and in the cells by ELISA (Peninsula laboratories, Bachem, California, USA), according to the manufacturer's instructions. The neuron-specific enolase (NSE) was measured by RIA (Diasorin, Antony, France) in the same conditions and the total amount of VIP protein was normalized to the NSE concentration.

2.7 Analysis of VIP mRNA expression

For quantification of the mRNA expression, primary culture of ENS treated with 300 μL buffer RA1 and stored at −80°C. Quantification of VIP mRNA expression by quantitative polymerase chain reaction was performed as described previously and results were normalized to HU mRNA expression.

2.8 Effects of cytokines on primary cultures of ENS

After 12 days of culture, cells of ENS primary cultures were incubated with various cytokines at different concentrations for 48 hour. Cytokines and their concentrations were used as follow: IL-5: 0.1, 1, and 10 ng mL^{-1}; IL-6: 10, 50, and 100 ng mL^{-1}; IL-10: 0.1, 10, and 100 ng mL^{-1}; IL-13: 0.5, 5, and 50 ng mL^{-1}; IL-17: 1, 25, and 50 pg mL^{-1}; IFN-γ: 10, 100, and 1000 ng mL^{-1}; TNF-α: 1, 10, and 100 ng mL^{-1}. All cytokines were purchased from EurobioAbcys (Courtaboeuf, France).

After 48 hour, the supernatants were collected and stored at −80°C. Adherent cells were used for quantification of the VIP protein by ELISA. Adherent cells were treated with 125 μL RIPA with protease inhibitors for 30 minute on ice and then centrifuged at 10 000 g for 15 minute at 4°C. The results were normalized to controls.

In a second set of experiments, the effect of SUP on primary ENS cells was tested in the absence or presence of an anti-IL6 polyclonal antibody (ab6672, Abcam, Cambridge, UK) at 200 μg mL^{-1}. The polyclonal antibody was first incubated in the SUP for 4 hour at 37°C, before being added to primary ENS cells for 48 hour at the same concentration (2%). A nonspecific rabbit IgG (ab2410, Abcam, Cambridge, UK) at 200 μg mL^{-1} was also used in the same conditions. Cells were treated as previously described, VIP was measured by ELISA and PCR and, the results were normalized to controls.

2.9 Statistics

Quantitative variables were described by the number of values for which information was provided, mean, standard deviation (SD), median, first and third quartiles. Data were compared by paired or unpaired non-parametric test: Mann–Whitney or Wilcoxon rank sum test and Kruskal–Wallis test followed by a Dunn's test. Correlations between variables were assessed by the Spearman test and simple linear regression. Differences were considered significant for a $P < 5\%$. Analyses were performed using Graphpad prism software version 5.0 (Graphpad Software Inc, La Jolla, CA, USA).

3 RESULTS

3.1 VIP mRNA expression in colonic biopsies

We first measured VIP mRNA expression in colonic biopsies of patients with UC or CD, and in HS. VIP mRNA expression (mean (±SD)) in patients with CD was significantly lower as compared to UC (0.97 ± 1.77 and 3.12 ± 3.49, respectively; Kruskal–Wallis followed by Dunns comparison; $P = .0008$) (Figure 1A). There was no other difference in VIP mRNA expression between HS and UC or between HS and CD.

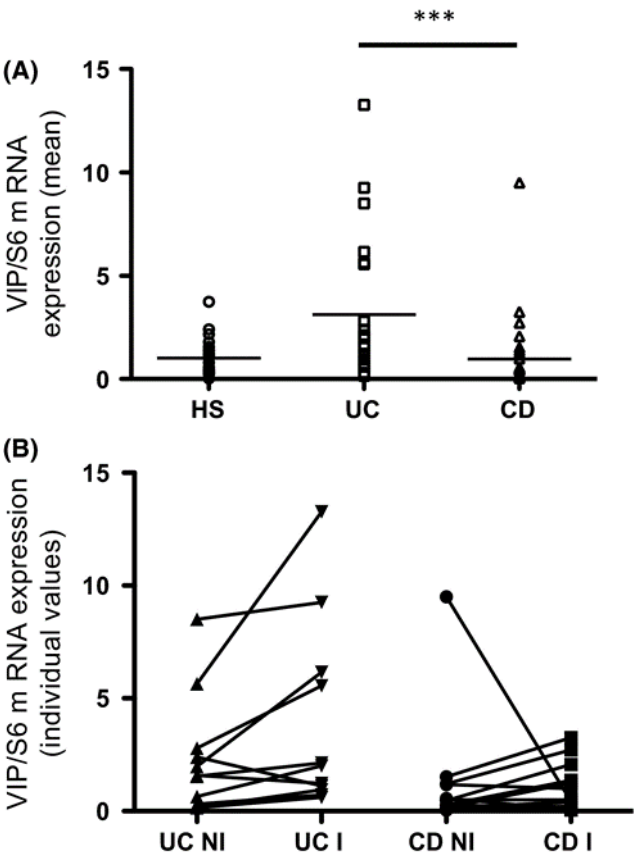

Figure 1
VIP mRNA expression in colonic biopsies of patients with Crohn's disease (CD), ulcerative colitis (UC) and healthy subjects (HS). Biopsies were taken in inflamed (I) and non-inflamed (NI) areas in patients with IBD. Results are expressed as mean and individual values. (A) Pooled biopsies obtained in inflamed and non-inflamed area of patients with UC and CD were compared with those obtained in HS. The mean (±SD) VIP mRNA expression was significantly lower in CD compared to UC: 0.97 ± 1.77 and 3.12 ± 3.49, respectively, (Kruskal–Wallis and Dunns comparison $P < .001$). (B) The VIP mRNA expression was quantified in biopsies obtained in inflamed and non-inflamed areas in patients with UC and CD. There was no difference between inflamed and non-inflamed areas in CD. Conversely, VIP mRNA expression in patients with UC was higher in I compared to NI area (3.90 ± 4.18 and 2.33 ± 2.59, respectively; $P = .02$) and was significantly lower in biopsies obtained from CD NI compared to those obtained in UC I (1.01 ± 2.31 vs 3.90 ± 4.19; Kruskal–Wallis and Dunns comparison $P = .003$). ***$P < .001$

We next evaluated, in UC and CD, the effect of inflammation by comparing VIP mRNA expression (mean (±SD)) in biopsies from inflamed (I) and non-inflamed (NI) areas. In patients with CD, VIP mRNA expression was similar in I and NI areas (0.93 ± 1.02 and 1.01 ± 1.31, respectively; $P = .2$) (Figure 1B). VIP mRNA expression in patients with UC was higher in I compared to NI area (3.90 ± 4.18 and 2.33 ± 2.59, respectively; $P = .02$) (Figure 1B). VIP mRNA expression was not influenced by treatments used in patients with IBD (not shown).

3.2 Effect of the colonic biopsy supernatants on VIP expression by the ENS

We next aimed at determining whether incubation of primary culture of ENS with supernatant (SUP) of biopsies obtained in patients with IBD and HS could modulate VIP expression.

VIP mRNA expression (mean ± SD) were significantly different in ENS when incubated with SUP-HS, SUP-UC, and SUP-CD (Kruskal–Wallis test, $P = .0002$). In particular, VIP mRNA expression was significantly lower in ENS incubated by SUP-CD as compared to those incubated with SUP-UC (0.40 ± 0.17 and 1.78 ± 1.34, respectively, $P < .0001$) and also with SUP-HS (0.40 ± 0.17 and 1.07 ± 1.04, respectively, $P = .01$) (Figure 2A).

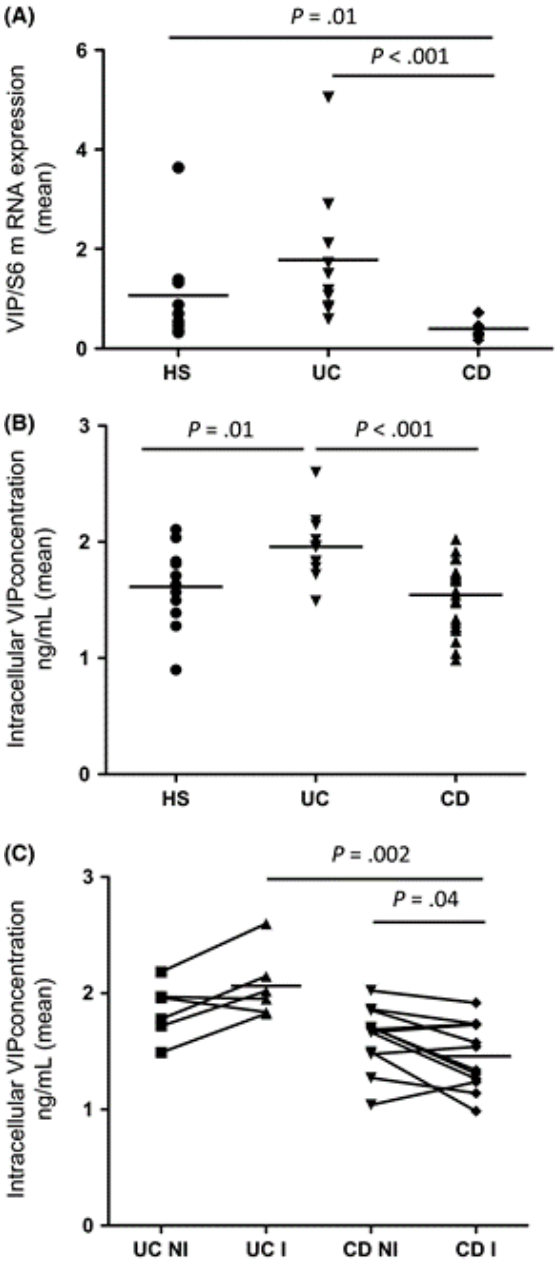

Figure 2
Effect of 48 h incubation of ENS primary culture with supernatants of colonic biopsies of patients with ulcerative colitis (UC), Crohn's disease (CD) and healthy subjects (HS) on mRNA expression (A) and intracellular VIP concentration (B and C). (A) The mean ± SD VIP mRNA expression was significantly lower in ENS incubated by SUP-CD compared to those incubated with SUP-UC (0.40 ± 0.17 and 1.78 ± 1.34, respectively, $P < .0001$) and with SUP-HS (0.40 ± 0.17 and 1.07 ± 1.04, respectively, $P = .01$). (B) The mean ± SD intracellular VIP concentration was significantly lower in primary culture of ENS incubated with SUP-CD than with SUP-UC (1.55 ± 0.27 ng mL^{-1} and 1.96 ± 0.28, respectively, Mann–Whitney test $P = .0003$). In contrast, intracellular VIP concentration was significantly larger in ENS incubated with SUP-UC compared to HS, (1.96 ± 0.28 and 1.62 ± 0.33 ng mL^{-1}, respectively, $P = .01$). (C) Effect of inflammation and diseases upon changes in VIP expression induced in the ENS by the SUP of biopsies. Intracellular VIP expression in ENS incubated with SUP-I CD was significantly lower as compared to SUP-NI (1.46 ± 0.29 and 1.62 ± 0.27 ng mL^{-1}, respectively, $P = .04$). The VIP expression in ENS incubated with SUP-I CD was significantly lower compared to SUP-I UC (1.46 ± 0.29 and 2.06 ± 0.29 ng mL^{-1}, respectively; $P = .002$)

In addition, intracellular VIP concentration (mean ± SD) was significantly different in ENS when incubated with SUP-HS, SUP-UC, and SUP-CD (Kruskal–Wallis test, $P = .001$) (Figure 2B). In particular, intracellular VIP concentration was significantly lower in ENS incubated with SUP-CD than with SUP-UC (1.55 ± 0.27 ng mL^{-1} and 1.96 ± 0.28, respectively, Mann–Whitney test $P = .0003$) but not different compared to HS. In contrast, intracellular VIP concentration was significantly larger in ENS incubated with SUP-UC compared to HS, (1.96 ± 0.28 and 1.62 ± 0.33 ng mL^{-1}, respectively, $P = .01$) (Figure 2B).

We next evaluated in CD and UC, the effect of inflammation by comparing the effects of the incubation of SUP from biopsies from inflamed (I) and non-inflamed (NI) areas upon VIP expression in the ENS (Figure 2C). Intracellular VIP expression in ENS incubated with SUP-I

CD was significantly lower as compared to SUP-NI CD (1.46 ± 0.29 and 1.62 ± 0.27 ng mL^{-1}, respectively, $P = .04$). In contrast with CD, intracellular VIP concentration in ENS incubated with SUP-I-UC was similar to SUP-NI-UC (2.06 ± 0.28 and 1.85 ± 0.24 ng mL^{-1}, $P = .2$). In addition, intracellular VIP concentration was significantly lower in ENS incubated by SUP-I-CD compared to SUP-I-UC: 1.46 ± 0.29 and 2.06 ± 0.28 ng mL^{-1}, respectively; Mann–Whitney test, $P = .002$) (Figure 2C). Interestingly, extracellular VIP concentration in ENS incubated with SUP-I CD was also lower as compared to SUP-I UC (0.05 ± 0.03 and 0.09 ± 0.03 ng mL^{-1}; $P = .03$). Finally, SUP from colonic biopsies of patients with IBD (from I and NI) did not change extracellular or intracellular NSE levels (data not shown).

3.3 Cytokine expression in colonic biopsies of IBD patients

We next aimed to identify putative cytokines involved in SUP-induced neuroplastic changes in IBD by characterizing changes in mRNA expression of key cytokines in colonic biopsies.

We characterized the mRNA expression of cytokines such as IL-5, IL-6, IL-10, IL-17, TNF-α, IFN-γ in colonic biopsies (Table 3). In I areas, the expression of mRNAs of all cytokines, except IL-5, was significantly increased as compared to HS, independently of the disease. In NI areas of patients with CD, the expression of IL-6, IL-10, and IL-17 mRNAs was significantly increased as compared to HS. In NI areas of patients with UC, the expression of IL-10, IL-17, TNF-α, and IFN-γ but not IL-6 mRNAs was significantly higher than in HS. No difference was observed between biopsies of patients with UC and CD in I and NI areas. The treatment used by patients had also no influence on cytokines mRNA expression.

Table 3. Cytokine mRNA expression in colonic biopsies

	HS	UC		CD	
		NI	I	NI	I
IL-6 mean (SD)	0.66 ± 0.38	1.28 ± 0.90[b]	2.27 ± 0.38[a]	1.35 ± 0.66[a,c]	2.33 ± 0.92[a]
IL-10 mean (SD)	0.32 ± 0.09	1.70 ± 2.11[a]	0.70 ± 0.41[a]	0.65 ± 0.32[a]	1.19 ± 0.91[a]
TNF-α mean (SD)	0.10 ± 0.03	0.17 ± 0.08[a]	0.17 ± 0.08[a]	0.12 ± 0.05[c]	0.24 ± 0.15[a]
IL-5 mean (SD)	1.18 ± 0.41	1.32 ± 0.44	0.11 ± 0.05	0.49 ± 0.33	1.27 ± 0.39
IL-17 mean (SD)	1.42 ± 1.24	13.67 ± 18.14[a]	15.83 ± 13.53[a]	6.04 ± 6.02[a,c]	16.03 ± 12.51[a]
IFN-γ mean (SD)	4.88 ± 5.12	25.46 ± 23.02[a]	22.87 ± 12.97[a]	4.45 ± 4.36[c]	39.91 ± 39.53[a]

- [a] $P < .05$ vs healthy controls.
- [b] $P < .05$ vs inflammatory area of ulcerative colitis.
- [c] $P < .05$ vs inflammatory area of Crohn's disease.
- UC, ulcerative colitis; CD, Crohn's disease; HS, healthy subjects; NI, non-inflamed; I, inflamed.

3.4 Effect of IL-6 and blocking-IL-6-antibody on VIP expression by the ENS

Primary culture of ENS were incubated with the same cytokines at different concentrations. Among all cytokines tested, only IL-6 (which was increased in NI areas of CD but not UC) significantly modified intracellular VIP concentration in the ENS (Table 4). Indeed, IL-6 significantly decreased intracellular VIP concentration (mean ± SD) in the ENS (1.2 ± 0.3 vs 0.93 ± 0.1 and 0.8 ± 0.2 ng mL^{-1} in the presence of IL-6 at 10, 50 and 100 ng mL^{-1},

respectively; Kruskal–Wallis test, $P = .01$) (Figure 3A). IL-6 also significantly decreased VIP mRNA expression in ENS primary culture (data not shown).

Table 4. VIP intracellular concentration in primary ENS culture in the presence of cytokines at different concentrations

	IL-5 concentrations (ng/mL)			IL-10 concentrations (ng/mL)		
	0.1	**1**	**10**	**0.5**	**5**	**50**
Intracellular VIP (ng mL^{-1})	0.85 ± 0.22	0.85 ± 0.33	1.06 ± 0.25	0.95 ± 0.24	0.85 ± 0.26	0.91 ± 0.28
	INF-γ concentrations (ng/mL)			TNF-α concentrations (ng/mL)		
	10	100	1000	1	10	100
Intracellular VIP (ng mL^{-1})	0.94 ± 0.33	0.86 ± 0.37	0.91 ± 0.23	0.88 ± 0.37	0.74 ± 0.24	0.72 ± 0.27
	IL-6 concentrations (ng/mL)			IL-17 concentrations (ng/mL)		
	10	50	100	1	25	50
Intracellular VIP (ng mL^{-1})	1.15 ± 0.30[a]	0.93 ± 0.14[b]	0.81 ± 0.16	0.88 ± 0.16	0.97 ± 0.31	0.85 ± 0.25

- [a] 10 ng mL^{-1} vs 100 ng mL^{-1}, $P = .01$.
- [b] 50 vs 100 ng mL^{-1}, $P = .04$.

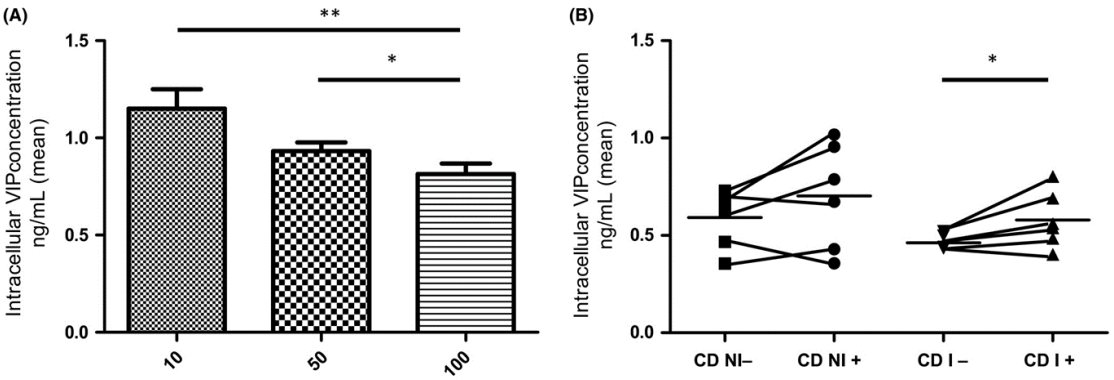

Figure 3
Effect of IL-6 and blocking-IL-6-antibody on the intracellular VIP concentration of primary culture of the ENS. (A) Intracellular VIP concentration after 48 h incubation of primary culture of the ENS decreased significantly with increasing doses of IL-6 (10, 50 and 100 ng mL^{-1}). (B) Intracellular VIP concentration after 48 h incubation of primary culture of the ENS with SUP of patients with Crohn's disease (CD) in absence (−) or presence of blocking polyclonal antibody anti-IL6 (+). The intracellular VIP concentration was significantly increased by the addition of neutralizing IL-6 antibody to SUP-CD-I but there was no difference by the addition of neutralizing IL-6 antibody to SUP-CD-NI. Results are expressed as mean and individuals values. *$P < .05$ and **$P < .01$

We next determined whether incubation of SUP of patients with CD with a blocking anti-IL-6 antibody prevented its ability to induced changes in VIP expression in primary ENS culture. Intracellular VIP concentration in ENS cultured with SUP-I CD was significantly increased by preincubation of SUP-I CD with anti-IL-6 blocking antibody (0.4 ± 0.03 vs 0.6 ± 0.1,

respectively; $P = .03$). However, intracellular VIP concentration in ENS cultured with SUP-NI CD was similar after preincubation of SUP-NI CD with anti-IL-6 blocking antibody ($P = .11$) (Figure 3B).

4 DISCUSSION

The current study identified changes of VIP expression in colonic biopsies from IBD patients. In particular, VIP mRNA expression was significantly reduced in CD as compared to UC. Next, we were able to reproduce part of these neuroplastic changes by treating primary culture of ENS with SUP of colonic biopsies. In particular, VIP mRNA expression and intracellular protein level were significantly lower in the ENS incubated with SUP-CD than those incubated with SUP-UC. We also found that in CD but not in UC, SUP-I reduced intracellular VIP concentration in the ENS as compared with SUP-NI. We further showed that (i) IL-6 mRNA expression is increased in NI area of CD but not UC patients as compared to HC, (ii) IL-6 reduced VIP mRNA and protein expression in ENS and (iii) in CD, the effect of SUP upon VIP expression was blocked by anti-IL-6 antibody. Our data demonstrate that in CD colonic soluble mediators regulate VIP expression in part via IL-6.

A major finding of our study was to demonstrate that soluble factors derived from colonic biopsies reproduce in part changes in VIP observed in colonic biopsies from patients with IBD. Our study extends to IBD similar findings observed in a low-grade inflammatory bowel disease, ie, IBS. In particular, SUP from IBS patients acutely increased the excitability of enteric neurons.17 Our present study also suggests that besides altering neuronal functions over short term, SUP of colonic biopsies can also impact upon the ENS by inducing long-term effects characterized by regulation of gene expression. Similar long term changes induced by SUP of biopsies has been reported in IEC where SUP of IBS patients increased intestinal paracellular permeability by modulating ZO-1 mRNA and protein expression18 and also in the ENS where SUP of IBS enhanced enteric neuronal sprouting. This further highlights the capacity of SUP of intestinal biopsies to adoptively transfer disease symptoms/phenotype in a healthy system.

Concerning our study, SUP reproduced part of changes in VIP expression observed in biopsies between UC and CD. However, it allowed new changes to be observed, in particular differences in VIP expression as a function of inflammation for CD and also compared to healthy subjects (HS). These differences could be due in part to the fact that when using ENS primary cultures as compared with biopsies, we analyzed on a more specific cell population than that present in a biopsy (containing not only ENS but also immune, epithelial cells, fibroblasts..). Indeed, primary ENS cultures are obtained using a well-established protocol and the proportions of cells are stable between experiments.19 Furthermore, in our primary culture of ENS, VIP expression is exclusively observed in neurons but not in other cells types (ie, enteric glial cells or smooth muscle cells). However, in biopsies, VIP is also expressed in other cell types such as immune cells and changes in VIP could, therefore, also reflect changes in infiltrate or differential regulation between cells types. However, differences in VIP expression between biopsies and the ones reported in primary culture of ENS treated with SUP, could be due to labile soluble factors whose effects are not observed in SUP treated ENS.

Changes in VIP expression during IBD are often conflicting. Some studies have reported increased VIP in CD13, 20-22, while other studies have shown a decrease.23, 24 Similarly, in UC some studies have reported increased VIP, while others have indicated no difference.14-

16, 25, 26 Such findings could be due to differences in methods used (immunohistochemistry, radioimmunoassay, PCR) or site studied (ie, ileum vs colon). As suggested by our study, such differences might also be due in addition to changes in local intestinal inflammation, independently of the disease clinical activity which does not necessarily reflect the presence or absence of significant lesions. Aside from interindividual variability that could account for differences with previous studies, discrepancies could also result from the variability in the proportion of different cell types contained in different biopsies (based on the depth, size…) while other studies analyzed specific cell types, ie, enteric neurons.13, 20-24

A key finding of our study was the identification of IL-6 as a putative mediator involved in part, in the regulation of VIP expression during CD. We also observed that cytokines expression in UC and CD colonic biopsies were similar, except for IL-6 mRNA that was upregulated in NI areas for CD but not UC and for TNF-α and IFN-γ that was upregulated in NI areas for UC but not CD. Overall, these findings are consistent with studies showing no major differences in cytokine profiles between these diseases.27-29 Concerning the involvement of IL-6 in VIP regulation, we first showed that IL-6 significantly reduced VIP mRNA and protein expression. Secondly, we showed that, in CD-I but not CD-NI, SUP effects were inhibited by treatment with anti-IL-6 antibody. The absence of effects using anti-IL-6 antibody in SUP-NI CD might be due to the lower levels of IL-6 in NI areas. Previous studies have shown a role for IL-6 in the regulation of ENS functions. Indeed, in an animal model of IBS, increased excitability of enteric neurons was shown to be mediated by IL-6, as (i) this activation was decreased by IL-6 neutralizing antibody30 and (ii) IL-6 increased neuronal excitability in the ENS.31 Concerning the regulation of VIP expression by IL-6, our study is the first to show such an effect on enteric neurons. This effect of IL-6 is consistent with a previous study showing that high concentration of IL-6 reduces VIP mRNA expression in sympathetic neurons.32

The functional consequences for the differential changes in VIP expression in the colon of CD and UC patients remain unknown. Reduced VIP expression in CD could participate in the altered barrier and immune functions observed in this disease. VIP has been shown to reduce intestinal barrier permeability and enhance barrier resistance to pathogen aggression.7, 33 In addition, VIP has also been shown to reduce cytokine production in an animal model of colitis induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS).10, 11, 34

In conclusion, our study demonstrates that colonic mucosal soluble factors derived from IBD patients differentially regulate VIP expression in the ENS as a function of diseases and inflammation. IL-6 was identified to be in part responsible for reduced VIP expression in CD. Therefore, approaches aimed to restore normal VIP level might enhance barrier functions and reduce inflammation in CD.

ACKNOWLEDGMENTS

Florence Soufflet, Mandy Biraud, Bernard Lardeux, and Malvyne Derkinderen did the experiments, Arnaud Bourreille, Michel Neunlist, and Malvyne Derkinderen designed the study and analyzed the results, Arnaud Bourreille, Malvyne Derkinderen, Michel Neunlist, and Stanislas Bruley des Varannes wrote the manuscript, Caroline Trang and Emmanuel Coron included the patients.

CONFLICT OF INTERESTS

No competing interests declared.

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