Substance P Receptor Expression in Patients with Inflammatory Bowel Disease

Determination by three different techniques, i.e. storage phosphor autoradiography, RT-PCR and immunohistochemistry


W.P. ter Beek
I. Biemond
E.S.M. Muller
M. van den Berg
C.B.H.W. Lamers

Department of Gastroenterology-Hepatology, Leiden University Medical Centre, The Netherlands
Abstract

**Background:** Inflammatory bowel disease (IBD) is characterized by chronic intestinal inflammation accompanied by changes in motility. It is known that regulatory peptides like substance P (SP) are important pro-inflammatory peptides which are also involved in neuronal conduction. **Aim:** To get clues for new diagnostic and therapeutic approaches we describe the SP receptor (NK-1) distribution in IBD compared to control intestinal tissue, on mRNA and protein level by three complementary techniques. **Results:** Autoradiography showed differences within the intestinal wall of control patients; mucosal binding was 17 fmol/g and muscular binding was significantly (p=0.01) higher (98 fmol/g). In inflamed specimens of patients with IBD mucosal SP binding was increased compared to controls (55±10 vs 18±4 fmol/g mucosa, p=0.002). However RT-PCR showed that the mRNA content of the NK-1 receptor in these samples was not increased. In non-inflamed samples of patients with Crohn’s disease (CD) and ulcerative colitis (UC) SP binding was similar as in controls, while mRNA was significantly decreased in CD patients (0.7±0.02 vs 4.4±0.7, p=0.01) but not in UC patients (4.4±0.7 vs 4.1±1.4). Immunohistochemistry identified a broad spectrum of NK-1 receptor locations in control intestine. No aberrant expression in IBD was found. **Conclusions:** This study showed that although there was no difference in location of the SP receptors in IBD patients versus controls, the quantity of SP binding was significantly increased in the inflamed mucosa of IBD patients, while the mRNA level was not increased. Further a difference in mRNA level between non-inflamed tissue of CD and UC patients was shown, with mRNA in CD being lower. These changes in SP receptor expression during chronic inflammation suggest that SP receptors are a potential target for therapeutic regulation of the inflammatory response.
Introduction

Inflammatory bowel disease is characterized by a chronic idiopathic inflammation of the gastrointestinal tract. Despite extensive research and the finding of several factors involved in the pathology of IBD, the aetiology of IBD remains unknown. Two distinct forms of IBD have been described, Crohn’s disease (CD) and ulcerative colitis (UC). The first disease can be present in all of the gastrointestinal tract and both mucosa and smooth muscle can be affected. In this disease ulcerations and transmural inflammation are common and the immune response is dominated by type-1 T-cell activity. On the other hand there is ulcerative colitis, which is mainly characterized by a type-2 immune response and is restricted to the mucosa of the colon and rectum. Clinical features of IBD comprise apart from general manifestations of inflammation a disturbed intestinal and colonic motility, diarrhoea and weight loss [1]. One of the factors that have been proposed to be involved in the pathology of IBD are neuropeptides which are expressed by the dense enteric neuron system in the gastrointestinal tract. Neuropeptides are often involved in the regulation of intestinal motility, fluid secretion and inflammatory processes [2-4].

One of these neuropeptides, substance P (SP) is a member of the mammalian tachykinins. Neurokinin A and B (NKA and NKB) are two other members of this family. SP and NKA are derived from the same gene by alternative splicing. There are three different receptors for this peptide family, the NK-1 –2 and -3 receptor with SP, NKA and NKB as its preferred ligands, respectively. All ligands can bind to all receptors [5]. SP and NKA are abundantly expressed in the gastrointestinal tract but NKB much less [6,7]. The main location of the tachykinins is in the enteric nervous system while they are also present in enteroendocrine and immune cells of the intestinal mucosa. The receptors are expressed by enteric neurones, the muscularis mucosa, interstitial cells of Cajal, longitudinal and circular muscle, epithelium, vasculature and the immune system. All three receptors are present in the intestine but there are differences in distribution and between species. A major action of the tachykinins is their effect on the intestinal motility which can be stimulating and inhibiting depending on the type and place of the receptor that is activated. They are also involved in the secretory function of the intestine by
stimulating release of chloride and bicarbonate [6]. Furthermore SP has pro-inflammatory effects, more precisely it is an important mediator in the neurogenic inflammation. In vitro SP exert chemotactic activity on neutrophils and eosinophils, it further influences cytokines synthesis and release (IL-1, -6, -8 and TNF-α) and release of histamine from peritoneal mast cells [8,9]. It has been reported that in the rectum and colon of ulcerative colitis patients the SP levels are increased and correlate with disease activity [10,11]. Furthermore, in inflammatory bowel disease the NK1 receptors are up regulated in the intestinal blood vessels and lymphoid structure as shown by Mantyh et al. with an emulsion autoradiographic method [12,13] and by Renzi et al. with in situ hybridisation and immunohistochemistry [14]. Later it was shown that proinflammatory cytokines induce NK1 receptor expression in colonic epithelial cells [15]. Furthermore, NK1 receptor mRNA is elevated in colonic mucosa of IBD patients as compared with non-inflamed control mucosa [16]. With animal models it was shown that NK1 antagonists can reduce the fluid secretion and intestinal inflammation [17]. In most of the above studies one or two techniques are used to describe the NK receptor expression. In this paper we used a combination of three complimentary techniques i.e., quantitative storage phosphor autoradiography, RT-PCR and immunohistochemistry to study the expression of the SP receptor in patients with IBD versus controls.

**Material and methods**

**Tissue samples**

Intestinal tissue samples (colon and ileum) were collected at the Leiden University Medical Centre, the Netherlands from 28 patients with IBD (16 CD and 12 UC patients), both from inflamed and non-inflamed areas. Intestinal tissue specimens from patients operated for neoplasm’s (n=14) were used as controls. The control tissue was taken at least 10 cm from the affected site. The patients gave consent for the anonymous use of their tissue material. Table one gives an overview of patients characteristics. Full thickness tissue samples were embedded in tissue-tek O.C.T. compound and frozen on dry ice for autoradiography and embedded in
paraffin for immunohistochemistry. For RT-PCR mucosa was snap frozen in isopentane on dry ice. Tissue for RT-PCR and autoradiography was stored at -80°C until use.

### Table 1. Patients Characteristics

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Male/Female</th>
<th>Age (mean)</th>
<th>Age (range)</th>
<th>Inflamed/non-inflamed</th>
<th>Use of corticosteroids</th>
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<tbody>
<tr>
<td>controls</td>
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<td>7/7</td>
<td>59</td>
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<td>3/13</td>
<td>35</td>
<td>18-73</td>
<td>13/4</td>
<td>3/13</td>
</tr>
<tr>
<td>UC patients</td>
<td>12</td>
<td>4/8</td>
<td>38</td>
<td>19-72</td>
<td>9/4</td>
<td>2/10</td>
</tr>
</tbody>
</table>

### Storage phosphor autoradiography

Cryostat tissue sections (14 μm) were cut at -20°C, mounted on gelatin-coated glass slides and stored overnight at -80°C. Storage phosphor autoradiography was performed by the following protocol which was optimized in our laboratory. Slides were air dried for 30 min and pre-incubated in 50 mM Tris-HCl (pH 7.4) containing 0.5% BSA and 2.5 mM EDTA for 20 min. For total binding, slides were incubated with 50 mM Tris-HCl, 40 μg/ml bacitracin, 4 μg/ml leupeptin, 2 μg/ml chymostatin, 3 mM MnCl₂ and 75 pM ¹²⁵I-SP (Amersham Pharmacia Biotech, UK) at pH 7.4 for 180 min at room temperature. Alternate serial sections were incubated with addition of 1 μM nonradioactive SP (Bachem AG, Switzerland) to determine non-specific binding. After incubation sections were washed five times for 5 min with 50 mM Tris-HCl pH 7.4 4°C. Washed slides were rapidly dried with a stream of cold air. Slides were placed in a storage phosphor cassette for 40 h at room temperature. Laser scanning of the screen in the Phosphor Imager® (Molecular Dynamics, Sunnyvale, CA, USA) visualized the latent image stored in the storage phosphor screen. The data of the digitized image were processed with ImageQuant® software (Molecular Dynamics, Sunnyvale, CA, USA). Slides with 10 μl drops of different concentrations of radiolabelled ligand were used for standardization. Rat brain sections act as positive control. Binding is expressed as
fmol/g tissue. Serial sections were stained with hematoxylin/eosin to distinguish between the smooth muscle and mucosa.

**Quantitative RT-PCR**

The expression of NK-1 mRNA was determined with RT-PCR. Total RNA from the mucosal tissue samples was isolated by phenol chloroform extraction of guanidinium isothiocynate lysates [18]. RNA of human duodenal mucosa was used as positive control. RNA (2 μg) was used to synthesise cDNA with M-MLV reverse transcriptase (Invitrogen, USA) and a random primer mix (Hoffmann-La Roche, Switzerland). The obtained cDNA was serial diluted from 1:4 to 1:1024. The diluted cDNA served as a template for the PCR using REDTaq™ DNA polymerase (Sigma-Aldrich, USA) and primers for NK-1 (forward, TGACCGCTACCACGAAGTCTC; reverse, ATAGTCGCCGGCGCTGATGAAG; Sigma-Genosys, UK). The amount of β-actin (primers: forward, GGTCAGAAGGATTCTATG; reverse, GGTCTCAACATGATCTGGG; Sigma-Genosys, UK) mRNA expression was used for standardization of the amount of used cDNA in the different samples. Following amplification programs were used 1 min 95°C; 1 min 53°C; 1 min 72°C 36 cycles and 30 sec 94°C; 45 sec 56°C; 1 min 72°C 30 cycles for NK-1 and β-actin respectively. PCR fragments were loaded on a 1.5% agarose gel and after electrophoresis DNA were visualized with ethidium bromide under UV light and a digital picture was made. Intensity of the bands was measured with SCION software (Scion Corporation, Washington D.C., USA) and plotted against starting amount of cDNA on log scale. Ratios of intensity between samples and positive control were calculated.

**Immunohistochemistry**

Immunolocalization of NK-1 receptors was assessed by an indirect peroxidase-labelled antibody method [19] in a subset of 8 control patients and 15 IBD patients. In brief, paraffin embedded tissue sections (4 μm) were cut, mounted on poly-L-lysine coated glass slides and dried overnight by 37°C. Tissue was deparaffinized and rehydrated. Antigen retrieval was assed by heating the slides for 4 min in the microwave by 450 W and thereafter rinses them in TBS with 0.001% saponin.
Slides were incubated for 20 min with 1.5% normal rabbit serum (NRS) to block non-specific binding. Excess serum was drained off, and sections were incubated overnight at 4°C with goat anti-human NK-1 polyclonal antiserum (Santa Cruz Biothechnology, USA), appropriately diluted (1:200) in TBS containing 1.5% NRS. The next day sections were rinsed thoroughly in TBS and incubated with biotinated rabbit anti-goat Ig (Dako A/S, Denmark; 1:200 in TBS) and peroxidase-labelled streptavidin (Dako A/S, Denmark; 1:100 in TBS) for 45 min each. Sections were stained by incubation in 0.1 M acetate buffer (pH 5.2) containing 0.03% 3-amino-9-ethylcarbazole and 0.03% H2O2 for 10 min, resulting in a red staining product. Finally, sections were lightly counterstained in Mayer’s hematoxylin and mounted in Aquamount™ (BDH, Germany). Control of specificity of the staining was assed by incubating the primary antibody with its blocking peptide for 4 hours at room temperature prior to incubation on the slides. Negative control sections, in which the primary antibody was replaced by TBS, were negative. Two observers analysed the sections.

Data-analysis
Data is expressed as mean ± SEM (standard error of mean). Paired an unpaired Student’s t-test were used to infer significant differences between groups. Values of p < 0.05 were considered significant.

Results
Storage phosphor autoradiography enabled the visualization and quantification of SP binding sites in human intestine. In control patients the amount of binding sites found in the smooth muscle was significantly higher than in the mucosa (17±5 vs 98±20, p=0.01). This was also seen in the tissue of IBD patients although the difference was not significant. For the IBD patients an increase was seen in the amount of binding sites in the mucosa as compared with the control patients. In the non-inflamed mucosal areas of IBD patients there was already an increase but in the inflamed areas this increase was more pronounced and statistically significant (p = 0.002; table 2). Within the IBD group there were no differences between CD
and UC patients. Binding sites in the smooth muscle were similar in IBD and controls.

Quantification of the mRNA amount of the NK-1 receptor in the mucosal samples showed different results. The increase seen with the receptor binding study was not seen on mRNA levels (figure 1). Levels in control and inflamed IBD tissue were comparable and in non-inflamed IBD tissue a small decrease was seen in de mRNA levels but this was not significant. This decrease was mainly due to a significant decrease in the 3 CD patients (0.7±0.02; p=0.01), while this decrease was absent in the 4 UC patients (4.1±1.4) versus controls (4.4±0.7).

Immunohistochemical staining was performed on a subset of samples. This non-quantitative technique enables to visualize the location of the NK-1 receptors but was not quantitative. Figure 2 shows a representative sample of NK-1 receptor

Table 2. SP Binding Sites (fmol/g tissue) in Inflammatory Bowel Disease and Controls as Measured by Storage Phosphor Autoradiography

<table>
<thead>
<tr>
<th></th>
<th>Mucosa</th>
<th>Smooth muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>control</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>Noninflamed IBD</td>
<td>43</td>
<td>16</td>
</tr>
<tr>
<td>Inflamed IBD</td>
<td>55*</td>
<td>10</td>
</tr>
</tbody>
</table>

* p<0.01 vs control

Figure 1. Relative NK-1 Receptor mRNA Expression in Human Intestinal Mucosa from IBD Patients and Control Subjects
staining in a control colon and colon of an UC patient. Staining in the ileum was comparable. Strong NK-1 receptor staining was demonstrated in the epithelial cells, lamina propria, muscularis mucosa, veins in submucosa, submucosal plexus, myenteric plexus, and longitudinal and circular smooth muscle. Slides in which the primer antibody was pre-incubated with the blocking peptide did not show this staining confirming its specificity. No differences in location between control and IBD tissue was seen.

![Figure 2. Immunohistochemical Staining of NK-1 Receptor in Human Colon](image)

**Figure 2.** Immunohistochemical Staining of NK-1 Receptor in Human Colon
A: control mucosa, B: UC mucosa and C: control smooth muscle. Notice the strong epithelial staining, the staining of infiltrated inflammatory cells and the smooth muscle with myenteric plexus.

### Discussion

In this paper three different techniques were applied to describe the distribution of the neuropeptide substance P receptor. The first technique, quantitative storage phosphor autoradiography is a method which detects all functional receptors to which SP can bind. The results of this technique are comparable with data reported in literature using film and emulsion autoradiography, but storage phosphor autoradiography has two major advantages over film autoradiography as the
exposure time is shorter and the dynamic range is larger, which make this technique quantitative over a wide range of receptor amounts. A small disadvantage of storage phosphor autoradiography over the emulsion radiography is the lower resolution [20]. But in case of the human intestine the mucosa can still be measured separately from the smooth muscle, but individual cells in both compartments can not be distinguished. This study showed that in control intestine the most important binding site for SP was the smooth muscle with its enteric nervous system, but also the mucosa expressed SP binding sites. In the inflamed mucosa of IBD patients significant more SP binding sites were expressed as in controls. This finding was in agreement with results from others using film autoradiography [12,13,21,22]. Furthermore we found that this increase was already seen in the non-inflamed tissue of IBD patients (both CD and UC) but to a lower degree.

The second technique used was RT-PCR. The mRNA content of the NK-1 receptor for SP in inflamed IBD mucosa was similar to that in control mucosa and in non-inflamed mucosal areas of UC patients. However in CD patients the mRNA content was significantly decreased compared to controls. Goode et al. [16] showed an increase of NK-1 mRNA found in inflamed IBD tissue as compared to controls. The difference between their and our study may be due to difference in study population and collection of tissue. Goode et. al. studied endoscopic specimens for mRNA quantification in contrast to surgical specimen in our study. These investigators used biopsies from active IBD lesions and as controls biopsies from IBD patients without inflammatory activity, while no patient without IBD were studied. As is seen in our study the mRNA content in patients with CD can be decreased in the areas without inflammatory activity. The apparent increase in mRNA in active disease found in their study [16] may in fact be due to a decrease in IBD without active disease. When compared to controls without an inflammatory disease, as is done in our study, there was no change in the mRNA content of the NK-1 receptor in the inflamed mucosa of IBD patients. The discrepancy between the increase in binding sites for SP and the similarity of NK-1 receptor mRNA in inflamed IBD mucosa compared to controls could be explained by receptor recycling. It is known that in control neurons approximately 12-18% of the NK-1
receptors are present in the cytoplasm, and that after ligand binding receptors from the plasma membrane are internalized and that NK-1 receptors from the cytoplasm can be recycled to the plasma membrane again [23]. A change in this equilibrium can increase the amount of binding sites on the plasma membrane without an increase in mRNA.

As third technique we used immunohistochemistry. While the other two techniques were quantitative and a difference in the amount of receptors could be detected immunohistochemistry is a qualitative technique for location of the receptors. With immunohistochemistry all individual cells that express the NK-1 receptor can be visualized. This gives the opportunity to detect if there is an aberrant expression of the SP receptor in diseased intestine. We found that the expression pattern of the NK-1 receptor was not changed in IBD patients as compared with controls. This is also in agreement with literature [14-16]. The location of the NK-1 receptor as was shown with immunohistochemistry was in agreement with the result found with storage phosphor autoradiography. Both compartments mucosa and smooth muscle express the SP receptor, with expression in the mucosa being patchy and in the smooth muscle being more diffuse but reaching a higher quantity. In addition to storage phosphor autoradiography immunohistochemistry showed that within the mucosal compartment NK-1 receptors are present in epithelial cells, lamina propria, muscularis mucosa, veins in the submucosa, and the submucosal plexus. In the muscular compartment receptors where shown in both the longitudinal and circular smooth muscle as in the myenteric plexus. The knowledge of which cell types do express the NK-1 receptor is important in the search for antagonists and agonist as therapeutic or diagnostic compound.

In this paper the above mentioned three complementary techniques were used for the first time together to study the SP receptor. All three techniques have their own advantages and shortcomings. Combining these three techniques did give a total view of the expression of the neuropeptide SP receptor in the human intestine. From our study it can be concluded that the SP receptor is broadly expressed in the humane intestine and that SP can have effects both via neurons and directly on the smooth muscle and mucosal cells. In patients with IBD the mucosal SP binding is significantly increased in the inflamed areas, while the mRNA level was not
changed. In patients with CD the mRNA content in the non-inflamed areas was decreased while the binding sites already showed a small but not significant increase.

References


