Neuropeptide receptor expression in inflammatory bowel disease

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Contents

Abbreviations

5-HT	Serotonin
Ach	Acetylcholine
ATP	Adenosine triphosphate
BN	Bombesin
BRS	Bombesin receptor subtype
BSA	Bovine serum albumin
CD	Crohn's disease
cDNA	complementary desoxyribonucleic acid
ENS	Enteric nervous system
GALT	Gut-associated lymphoid tissue
GPR38-A	G-protein coupled receptor 38-A
GRP	Gastrin-releasing peptide
H_2O_2	Hydrogen peroxidase
IBD	Inflammatory bowel disease
IL	Interleukin
Kd	Dissociation constant
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
NK	Neurokinin
NMB	Neuromedin B
NRS	Normal rat serum
NT(R)	Neurotensin (receptor)
RT-PCR	Reverse-transcriptase polymerase chain reaction
SEM	Standard error of the mean
SP	Substance P
TBS	Tris-buffered saline
TNBS	Trinitrobenzenesulfonic acid
UC	Ulcerative colitis

1 Introduction

Inflammatory bowel disease (IBD) is a chronic disease of unknown aetiology. Several factors have been thought to influence the pathogenesis, clinical course and symptoms of the disease. It has become increasingly clear that not one single factor is responsible but that there is an interplay between several factors including the environment, genes, and the immune and the nervous systems. But before the complete picture of the disease can be elucidated, several factors have to be investigated first. One of the factors that is thought to play a role in IBD are the neuropeptides. Neuropeptides are a set of peptides that play a role in neural, endocrine and hormonal pathways. This introduction focuses on the location of these peptides in the gastrointestinal tract and the role they might have in inflammatory responses.

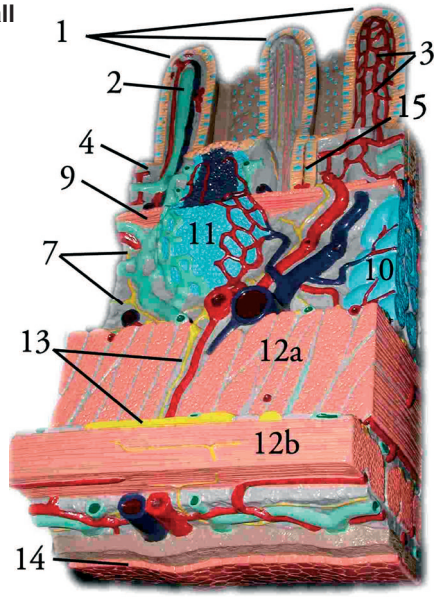
The gastrointestinal wall

The gastrointestinal wall is composed of four layers: mucosa, submucosa, muscularis externa and serosa. The first layer, the mucosa, forms the inner lining of epithelial cells. Below this is the lamina propria, which consists of loose connective tissue containing blood and lymphatic vessels and a dense network of nerve fibres, and on the border with the submucosa a small layer of smooth muscle cells, the muscularis mucosa. The second layer, the submucosa, consists of loose connective tissue with blood and lymphatic vessels, glands and the submucosal neuronal plexus. Further in the ileum the submucosa also contains the Peyer's patches, a large aggregation of lymphoid tissue. The third layer, the muscularis externa, contains two layers of smooth muscle, the thick inner circular muscle and the thinner outer longitudinal muscle, in between which can be found the myenteric plexus. And the fourth and most outer layer, the serosa, consists of mesothelium, which is a continuous sheet of squamous cells (see figure 1). The gastrointestinal tract is innervated by the autonomic nervous system, which is divided into the extrinsic nervous system (sympathic and parasympathic) and the important intrinsic nervous system. The extrinsic system gives its signals to the intrinsic system, which is entirely located within the intestinal wall. The intrinsic nervous system is also called the enteric nervous system (ENS). The ENS contains sensory neurons,

interneurons and motor neurons and provides internal pathways and evokes reflexes within the entire gastrointestinal tract [1].

Figure 1. Plastic Model of the Small Intestinal Wall

1. Villi with epithelium and goblet cells
2. Lacteal
3. Villi capillaries
4. Lamina propria
7. Submucosal plexus
9. Muscualis mucosa
10. Brunner gland
11. Lymphatic nodule
12. Circular muscle
12. Longitudinal muscle
13. Myenteric plexus
14. Serosa
15. Crypts of Lieberkühn



From http://daphne.palomar.edu/ccarpenter/Models/

Enteric nervous system (ENS)

The ENS is a collection of neurons in the gastrointestinal tract that controls motility, exocrine and endocrine secretions, and the microcirculation of the gastrointestinal tract. In addition, it is involved in the regulation of immune and inflammatory processes. Nerve endings are in close contact with the smooth muscle, mucosal secretory cells, endocrine cells, the microvasculature, and the immunomodulatory and inflammatory cells of the gut [2]. Nerve cell bodies of the ENS are mainly located in two major ganglionated plexuses – firstly, the myenteric plexus, which is found between the longitudinal and circular layers of the muscularis externa and, secondly, the submucosal plexus located within the connective tissue of the submucosa. The ENS is connected with the central nervous system via parasympatic and sympatic nerves (figure 2). The enteric neurons have a broad spectrum of chemical mediators, including acetylcholine (Ach), serotonin (5-HT),

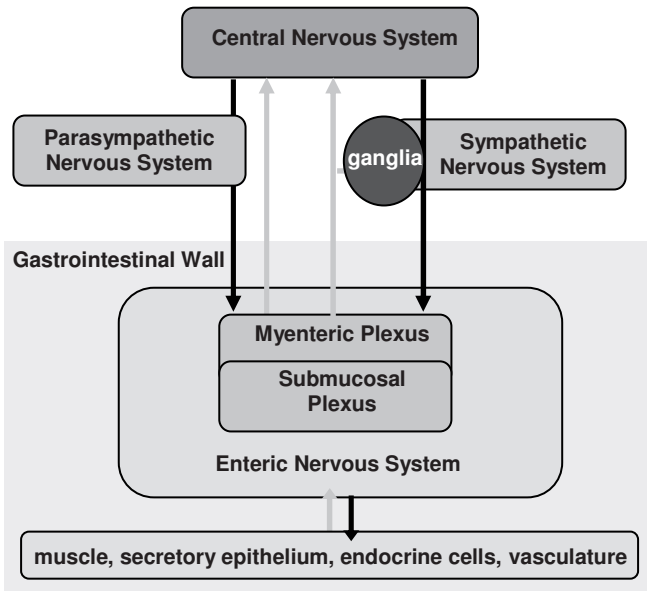


Figure 2. Innervation of the Gastrointestinal Tract

The neural plexuses in the gastrointestinal wall represent an independently functioning network known as the enteric nervous system, which is connected to the central nervous system by para-sympathetic and sympathetic nerves. Afferent connections are indicated with black arrows and efferent connections with grey arrows. The enteric nervous system may influence the effector systems in the gastrointestinal wall directly or indirectly through its action on intermediate cells.

nitric oxide, purines like adenosine triphosphate (ATP), and peptides such as substance P (table 1) [2]. Functionally the enteric neurons can be divided in four groups of neurons, namely sensory neurons, muscle motor neurons, secretomotor neurons and interneurons. The muscle motor neurons supply the longitudinal and circular muscle layers and are important for the peristaltic reflex of the intestine. Ach and substance P are the major excitatory transmitters in the muscle motor neurons. The noncholinergic and nonadrenergic motor neurons are more important for the relaxation of the intestine and are therefore also called the inhibitory neurons. The secretomotor neurons supply the mucosa, especially the crypts, where they stimulate crypt secretion. Interneurons run in aboral direction over long distances, connecting the many ganglia in the intestine. These two groups of neurons (secretomotor neurons and interneurons) contain various transmitters, namely, Ach, substance P, somatostatin, vasoactive intestinal polypeptide, bombesin, neurotensin and motilin. Sensory neurons can be divided into two subgroups: extrinsic (vagal and spinal afferents with their cell bodies outside the

Table 1. Putative Neurotransmitters Found in the Enteric Nervous System

Amines	**Peptides**
Acetylcholine	Calcitonin gene-related peptide
Norepinephrine	Cholecystokinin
Serotonin (5-hydroxytryptamine)	Galanin
Amino acids	Gastrin-releasing peptide
γ-Aminobutyric acid	Neuromedin U
Purines	Neuropeptide Y
ATP	Neurotensin
Gases	Opioids
Nitric oxide	Dynorphin
Carbon monoxide	Enkephalins
	Endorphins
	Peptide YY
	Pituitary adenylyl cyclase-activating peptide
	Somatostatin
	Substance P
	Thyrotropin-releasing hormone
	Vasoactive intestinal contractor
	Vasoactive intestinal polypeptide

gut wall) and intrinsic primary afferent neurones (cell bodies within the gut wall). Both subgroups include mechano-, chemo- and thermoreceptors, which control their activation. They express a wide range of receptors, mostly G-protein-coupled receptors, on their cell membranes (substance P, vasoactive intestinal polypeptide, calcitonin gene-related peptide and others) that modulate their sensitivity. In response to changes in the lumen the entero-endocrine cells in the mucosa secrete peptides that can bind to the receptors on the sensory neurons. The other way round is also possible; entero-endocrine cells become activated by the sensory neurons in order to release their compounds, e.g. the release of gastrin from G-cells under the influence of the ENS is best known [1-4].

G-protein coupled receptors

As mentioned above, G-protein coupled receptors form the most important receptor group in the signalling process of the neuropeptides of the ENS. The receptors consist of 7 transmembrane alpha-helical structures and intracellular and extracellular domains. The G-protein coupled receptors can be divided into three families, A, B and C. The rhodopsin-like family A is the largest subgroup and the ligand binding site of the A family is primarily located in the transmembrane region. The secretin-like receptor family B can bind several neuropeptides and peptide hormones. For this receptor family the binding sites are located at the relatively long NH_2-terminus, sometimes in combination with the extracellular transmembrane regions. The third group, the metabotropic glutamate receptor-like family C, is the smallest group with only 17 members; they have both a long NH2-terminus and COOH- terminus, with the binding site in the NH2-terminus. Intracellularly a G-protein, which consists of an α-, β-, and γ-subunit, is connected with the receptor. Binding of an agonist to the receptor's active site induces a conformational change that converts the receptor to its active state. This leads to the exchange of G-protein-bound GDP for GTP, after which the G-protein is disconnected from the receptor and the α-subunit dissociates from the βγ-dimer. The α-subunit can subsequently activate several second messenger pathways [5]. In figure 3 a schematic diagram of a G-protein coupled receptor is given.

Neuroendocrine-immune interactions

Lymphoid cells are found in three distinguishable compartments in the intestine: the specialized CD8 T-cells in the intra-epithelial compartment (5% to 15% of the epithelium in normal intestine), the effector cells (e.g. plasma cells, cytotoxic T lymphocytes and macrophages) in the lamina propria and, thirdly, the B-lymphocytes and to a lesser extent the T-lymphocytes, macrophages and dendritic cells in the gut-associated lymphoid tissue (GALT) such as Peyer's patches and solitary lymphoid nodule-follicles, where immune cells first encounter environmental antigens [6]. Nowadays it is well known that there is a bilateral

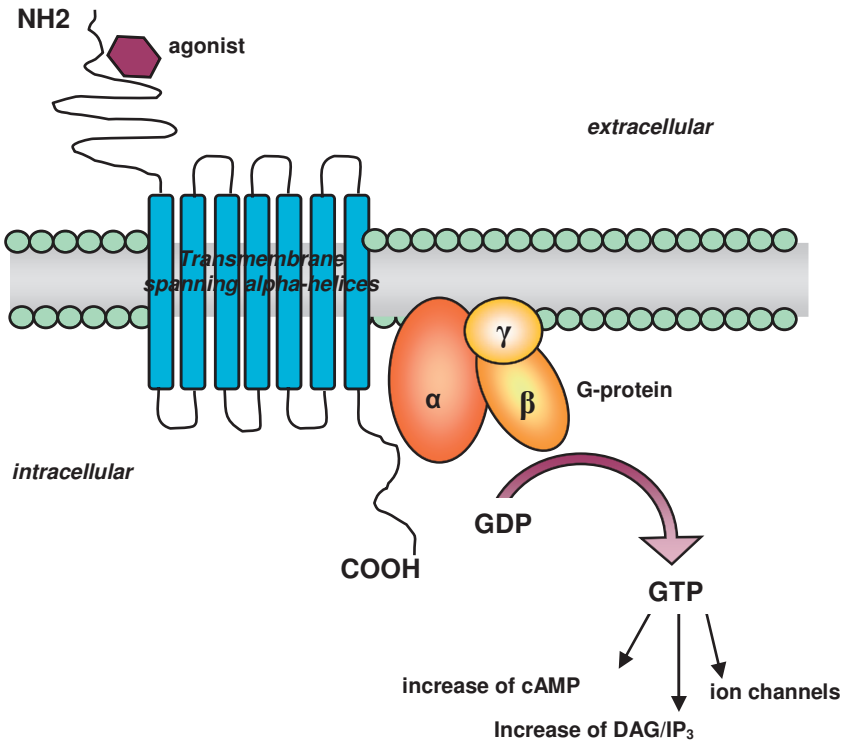


Figure 3. Schematic diagram of a G-protein Coupled Receptor

The receptor consists of an extracellular NH2-terminus, seven transmembrane alpha- helix domains and the intracellular COOH-terminus. The G-protein is located in close proximity to the receptor and consists of an α-, β-, and γ-subunit. Upon binding of an agonist to the receptor, the COOH- terminus associates with the G-protein and GDP is replaced with GTP. This leads to the dissociation of the α -subunit which provokes an increase of several second messengers such as cAMP and DAG/IP3 or the activation of ion channels.

interaction between the immune system and the nervous system. Receptors for enteric neurotransmitters are expressed on lymphocytes and mast cells located in the lamina propria with nerve endings in close proximity. Neurons, on the other hand have receptors for neuropeptides released by the lymphoid cells. Another example of the involvement of the ENS in inflammation is the fact that enteric glial cells produce interleukins and express MHC class II antigens in response to stimulation by cytokines [2]. There is variety in the number and diversity of receptors for neuropeptides on the different lymphoid subsets. For example, only a

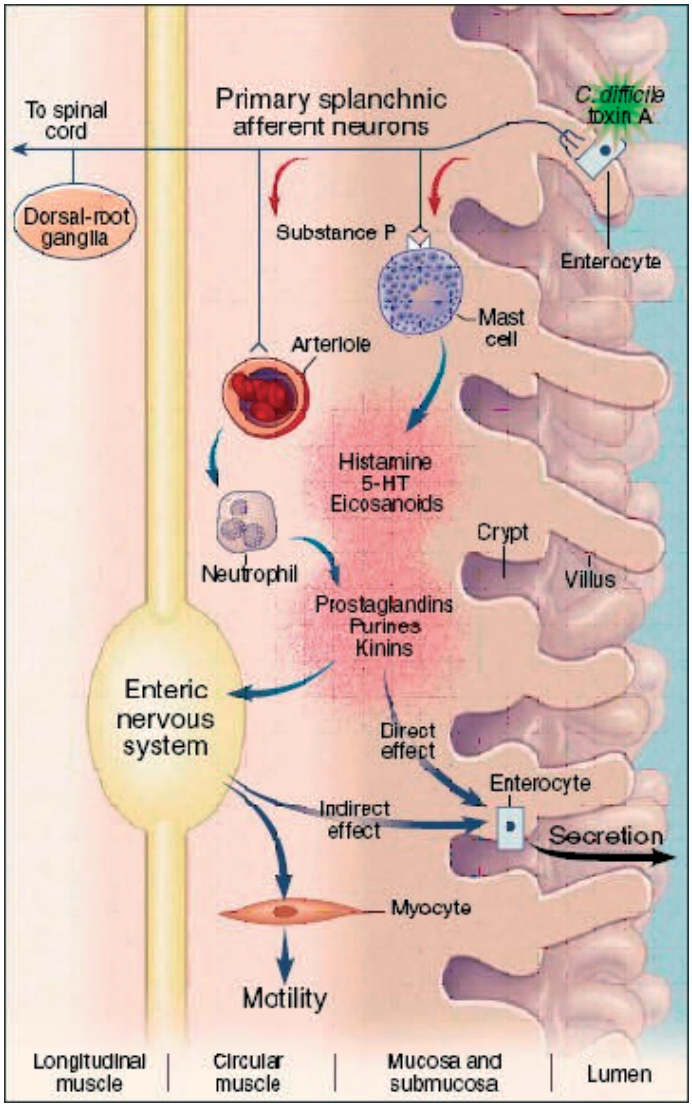


Figure 4. Secretomotor and Inflammatory Actions of *Clostridium difficile* Toxin A.

C. difficile toxin A causes injury to and necrosis of enterocytes. The necrotic enterocytes release noxious substances that stimulate primary splanchnic afferent neurons. Neural impulses are transmitted up and then back down a separate branch of the bifurcated axon in the axon reflex (red arrows), which stimulates the release of substance P around adjacent mast cells and submucosal arterioles. Substance P stimulates the release of a wide variety of chemical mediators from mast cells. The mediators recruit neutrophils (and eosinophils, not shown), which augment the inflammatory process by releasing additional inflammatory mediators. These mediators cause intestinal secretion through direct effects on enterocytes and indirect effects through the ENS. *C. difficile* toxin A also stimulates motility by inducing repetitive bursts of action potentials. The symbol Ψ represents afferent-nerve endings, and Y efferent-nerve endings. *From Goyal and Hirano (1996) [2]*

small number of circulating T-cells and B-cells show binding to substance P, whereas a high proportion of the lymphocytes in the Peyer's patches has binding sites for substance P. Therefore, several regulatory effects can be expected. For several neuropeptides functional studies with lymphoid cells have been carried out.

These studies show that calcitonin gene-related peptide, somatostatin and vasoactive intestinal polypeptide are mainly inhibitors of lymphocyte proliferation, but their effects depend on co-activation, the stage of differentiation of the lymphocytes and the cytokine milieu. Other studies have shown that somatostatin suppresses IgA production by lymphocytes of the Peyer's patches, enhances natural killer cell activity and stimulates secretion from peritoneal mast cells but not from lamina propria mast cells. Substance P, on the other hand, stimulates histamine secretion from both types of mast cells and it has a stimulating effect on lymphocyte proliferation. Substance P is also a potent chemotactic stimulus for human monocytes and it is involved in the regulation of the production and release of some cytokines [7;8]. The inflammatory reaction in response to administration of toxin A is well studied and it has been shown that it is also controlled by the ENS. In figure 4 an overview of the processes and interactions that take place in response to *Clostridium difficile* toxin A is shown. The ENS may also have a role in the pathogenesis of IBD. One example of the influence the nervous system exerts on IBD is the effect that stress has on the activity of IBD [2].

Inflammatory Bowel Disease

Ulcerative colitis (UC) and Crohn's disease (CD), together referred to as inflammatory bowel disease (IBD), are two chronic idiopathic inflammatory diseases of the gastrointestinal tract. Both are characterized by chronic, uncontrolled inflammation of the intestine which relapses and remits throughout its course. There is a difference in the area of the gastrointestinal tract affected in UC and CD. UC is mostly confined to the rectum, but may occur in the entire colon, CD, on the other hand, can appear in the entire gastrointestinal tract and at more than one location at the same time, with the ileocecal region being the most frequent one. Another difference between UC and CD is the depth of penetration of the inflammatory infiltrate within the gut wall; inflammation in UC is superficial and in CD it is more transmural. Also the presence of fibrosis, strictures, fistulae and granulomas in CD but not in UC distinguishes the two diseases [9]. Despite the above-mentioned differences, in about 10% of patients with colitis due to IBD no

distinction can be made between CD and UC. In this case the disease is called indeterminate colitis. Clinical symptoms of IBD include diarrhoea, cramping and pain. The ENS may play a role in the regulation of these processes [10;11]. In the Netherlands approximately 8,000 people suffer from CD and 14,000 from UC. The combined incidence of the two diseases was 16 per 100,000 inhabitants in Europe in the nineties of last century [12]. The aetiology of IBD is unknown. It is a complex disease and several interacting elements contribute to the development of the inflammation. Firstly, environmental factors like smoking, hygiene, diet and others appear to be involved, as indicated by the higher incidence of IBD in well-developed countries and the incomplete concordance rate within monozygotic twins [10;11;13]. Secondly, genetic factors are involved as is illustrated by the fact that the frequency of IBD in first-degree family members can be as high as 30%, the concordance rate in monozygotic twins is higher than in dizygotic twins, and a difference in prevalence between different ethnic groups is seen. Further, genomic analyses have showed that UC and CD are heterogeneous polygenic disorders sharing some, but not all, susceptibility loci. One of the clearest links found is the one between the NOD2/CARD15 gene and susceptibility for CD, but mutations in this gene account for only about 20% of CD, indicating that more genes/factors must be involved [10;11;13;14]. Thirdly, changes in microbial exposure can be of influence on the onset of inflammation in IBD. This can be due to increased intestinal permeability, as is seen in IBD, or to the exposure of some specific pathogens, with *mycobacterium paratuberculosis* being most mentioned [11;13;15]. The last of the factors contributing to the inflammation in IBD are immunoregulatory defects, but there is still debate on whether these are primary defects or responses to one of the other factors. The normal intestine is continuously in a basal state of inflammation, where a high number of immunoregulatory cells are present to defend the intestine from toxic or infectious agents. This defence mechanism seems to be exaggerated in IBD [10;13;16]. In the IBD-affected intestine a massive change in number and type of immunoregulatory cells is seen. There is an especially large increase in the number of IgG plasma cells (IgG1 and IgG2 for UC and CD, respectively), but also the number of other plasma cells – activated T-cells, macrophages, mast cells and polymorphonuclear leukocytes – is greater in

the lamina propria of patients with IBD. As a result of these increased numbers of inflammatory cells there are also increased cytokine levels in the mucosa and the normal architecture of this layer is destroyed with changes in all cell types present in the mucosa [13;15;16]. Also the ENS shows abnormalities in IBD, i.e. hypertrophy and hyperplasia of nerve fibres, and alteration in neuronal cell bodies and enteric glial cells. There is an increase in the number of enteric glial cells and an increased expression of MHC class II. Hypertrophy of the nerve fibres correlates with the degree of inflammation and is mainly seen in CD, and not in UC. Alterations (number, damage and hypertrophy) in neuronal cell bodies are seen in both CD and UC. Not only does the structure of the ENS change, but also the expression of neuropeptides and their receptors by the neurons and entero-endocrine cells. There is an increase in neurons containing nitric oxide synthase, vasoactive intestinal polypeptide and substance P, although the literature shows some confilicting results on this issue. Responses of the neurons to vasoactive intestinal polypeptide and substance P are increased and decreased, respectively. In addition, the number of somatostatin-containing D cells is reduced. It is not certain whether the above-mentioned abnormalities are primary or secondary to the inflammation process [17-20].

Neuropeptides

Neuropeptides are peptidergic neurotransmitters, which are produced by neurons. Most neuropeptides were initially discovered in the brain, but later it became clear that a large number of these peptides is also present in secretory vesicles of unmylinated sensory nerve endings of the ENS. Neuropeptides described to be present in the ENS include calcitonin gene-related peptide, vasoactive intestinal polypeptide, somatostatin, and substance P, but also neuropeptide Y, gastrin-releasing peptide, cholecystokinin, neurotensin, motilin and galanin. Many of these peptides are also found in enterocytes in the mucosa. These neuropeptides are released from the enterocytes as a paracrine or endocrine substance under the influence of the ENS [1]. Immune cells like lymphocytes and macrophages are also capable of synthesizing some of these peptides (i.e. substance P, calcitonin gene-

related peptide, vasoactive intestinal peptide, somatostatin, cholecystokinin and neuropeptide Y) [7;8;21]. On the basis of their chemical structures these peptides can be divided into several families. Table 2 gives an overview of the different peptides of each family and the receptors to which they can bind. In the following paragraphs five neuropeptides and their roles in inflammatory processes are discussed in more detail.

Substance P and calcitonin gene-related peptide

Substance P belongs to the family of mammalian tachykinins and it is present in the ENS, in enteroendocrine and immune cells of the intestinal mucosa [22]. Three different receptors for substance P have been described, the NK-1, -2 and -3 receptor (table 2). Substance P binds with high affinity to the NK-1 receptor but it can bind to the other two receptors as well [23]. Calcitonin gene-related peptide belongs to the calcitonin family and has an alpha and beta isoform. Several receptors for this peptide family have been identified (table 2) [24]. Sensory peripheral nerve fibres release neuropeptides to initiate and modulate an inflammatory response in the periphery. This response is predominantly mediated by the two neuropeptides substance P and calcitonin gene-related peptide, and can take place in several organs in the periphery, namely skin, the joint [25;26], and also in the intestine. Experimental studies in which colitis is induced in rats using trinitrobenzenesulfonic acid (TNBS) have shown that depletion of the sensory nerves before induction of the colitis or the administration of an antagonist for calcitonin gene-related peptide leads to a more severe form of colitis. This indicates that calcitonin gene-related peptide has a protective effect during the induction of colitis [27]. It was suggested that this effect is mediated by enhancement of the mucosal blood flow and the effects of calcitonin gene-related peptide on monocytes, macrophages, lymphocytes and neutrophils [28]. In patients with IBD a decreased concentration of calcitonin gene-related peptide was found in the muscle layer and mucosal nerve fibres [29]. Also substance P is an important mediator in neurogenic inflammation, where substance P has a pro-inflammatory function. In experimental colitis the administration of a substance P antagonist reduces the effects of the inflammation [30;31]. In vitro substance P exerts

Table 2. Gastrointestinal Peptide Families and their Receptors

Family	Members	Receptors
Cholecystokinin family	Cholecystokinin	CCK-A receptor
	Gastrin	CCK-B receptor
Secretin-glucagon family	Secretin	Secretin receptor
	Glucagon	Glucagon receptor
	Vasoactive intestinal polypeptide	VPAC-1R, VPAC-2R
	Glucagon-like peptides	GLP-1R, GLP-2R
	Gastric-inhibitory polypeptide	GIP receptor
	Pituitary adenylate cyclase-activating polypeptide	PAC1 receptor
Pancreatic polypeptide	Neuropeptide Y	Receptor Y1
	Peptide YY	Receptor Y2
	Pancreatic polypeptide	Receptor Y4
		Receptor Y5
Tachykinin family	Substance P	NK-1 receptor
	Neurokinin A / Substance K	NK-2 receptor
	Neurokinin B	NK-3 receptor
Bombesin family	Neuromedin B	NMB receptor
	Gastrin-releasing polypeptide	GRP receptor
		BRS-3
Calcitonin family	Calcitonin	Calcitonin receptor
	Amylinon	Amylin receptor
	Calcitonin gene-related peptide	CLR-1
	Adrenomedulin	CLR-2
Other peptides	Somatostatin	SST receptor 1, 2a, 2b, 3, 4 and 5
	Motilin	GPR38-A
	Neurotensin	NTR-1, -2 and -3
	Endothelin	ETa, ETb

stimulatory effects on monocytes, macrophages, lymphocytes, neutrophils and mast cells [28], increases the cytokines and histamine release and has chemotactic activity on neutrophils and eosinophils [32;33]. Studies of the rectum and colon of IBD patients have shown increased substance P levels, which correlated with disease activity [34;35], while there was also an up regulation of NK-1 receptors in the intestinal blood vessels and lymphoid structures [36-38]. Furthermore, NK-1 receptor mRNA is elevated in colonic mucosa of IBD patients as compared with non-inflamed control mucosa [39].

Neurotensin

Neurotensin is a tridecapeptide first isolated from bovine hypothalamus [40] and later from bovine intestine [41]. In the intestine, neurotensin is released by neuroendocrine cells (N cells) and enteric neurons [42]. Higher concentrations of neurotensin are found in the ileum than in other parts of the gastrointestinal tract [43]. Neurotensin exerts its effect by interacting with three different receptors: NTR-1, NTR-2 and NTR-3. NTR-1 and NTR-2 are seven transmembrane G-protein coupled receptors; while the third receptor is a single transmembrane protein with an extracellulair cysteine-rich domain and a furin cleavage site [44]. Neurotensin binds with high affinity to NTR-1 and NTR-3, and with low affinity to NTR-2. NTR-1 mediates most of the effects of neurotensin in the intestine [45;46]. Within the gastrointestinal tract neurotensin is involved in the regulation of motility. Neurotensin decreases gastrointestinal motor activity after being released from the distal gut in response to fat intake. However, in the rectosigmoid area a neurotensin-dependent prolongation of contractions was seen. Thus, neurotensin can induce both excitatory and inhibitory motor responses. Furthermore, neurotensin influences pancreatic and gastric acid secretion, and initiates hormone release [42;47;48]. Neurotensin is a pro-inflammatory mediator in acute inflammation. It increases vascular permeability, stimulates mast cell degranulation, histamine and chloride secretion. In addition, the chemotaxic capacity and phagocytic activity of peritoneal lymphocytes and macrophages, respectively, are also increased by neurotensin [49-53]. Castagliuolo *et al*. have shown that pre-treatment with a neurotensin antagonist reduces the acute

symptoms of inflammation in the *Clostridium difficile* toxin-A inflammation model in rats [49]. On the other hand, in chronic inflammation neurotensin promotes mucosal healing by causing proliferation of intestinal epithelial cells. These two different effects of neurotensin in acute and chronic inflammation are mediated via two distinct pathways. The first one is the PKC-mediated NF-kβ and cytokine activation pathway and the second one is the metalloproteinase-dependent activation of the EGF receptor pathway. The first leads to an inflammatory response, whereas the second increases cell proliferation and tissue repair [45]. In the normal intestine, receptors for neurotensin were shown to be present on the smooth muscle [54;55] and on the plexuses of the small intestine [43;56-58] and colon [59;60]. Also inflammatory cells (mast cells, neutrophils, lymphocytes and macrophages) express the neurotensin receptors [51]. Studies on the location of neurotensin binding sites in the mucosa are contradictory; Riegler *et al*. found neurotensin receptors at the bottom of the crypts and in the lamina propria of human colonic mucosa [52], but other studies did not show binding of neurotensin to the mucosa. The NTR-1 was ectopically expressed on human colonic microvascular epithelial cells in inflammation and an increased expression of NTR-1 mRNA was found in colonic tissue of patients with UC [45].

Bombesin

Bombesin is a 14-amino-acid peptide which was originally isolated from the skin of the amphibian *Bombina bombina* [61]. In mammals two counterparts of bombesin were found, namely gastrin-releasing peptide and neuromedin B [62]. Both are processed from a precursor comprising of an NH2-terminal signal sequence, the active peptide and a COOH-terminal extension peptide. Peptides from the bombesin-like family are abundantly expressed in the brain, but they are also expressed in the periphery [63]. In the gastrointestinal tract gastrin-releasing peptide is found in neurons of the intestine, whereas in the stomach gastrin-releasing peptide is mainly found in endocrine cells and hardly in neurons. On the other hand, neuromedin B-like immunoreactivity is mostly found in the nerves of the circular smooth muscle in the oesophagus and rectum. Three human receptors for the bombesin-like peptide family were cloned. The first one is the gastrin-

releasing peptide receptor with a high affinity for gastrin-releasing peptide [64-66], the second one is the neuromedin B receptor with a high affinity for neuromedin B [67], and the third one is the bombesin-like peptide receptor subtype 3 (BRS-3) [68;69]. For the latter receptor a high-affinity endogenous ligand still has to be found; both gastrin-releasing peptide and neuromedin B have a low affinity for this receptor. All three receptors belong to the family of seven transmembrane G-protein coupled receptors. The distribution of the receptors in the gastrointestinal tract is somewhat different in several species. In rats the presence of receptors was described in the circular muscle of the gastric fundus and antrum, the submucosal layer of the small intestine, and the longitudinal and circular muscles and submucosal layers of the colon [70]. In humans, the neuromedin B receptor is found in the muscularis mucosa of the oesophagus, whereas the gastrin-releasing peptide and the BRS-3 receptor are present in the pancreatic acini [71;72]. Besides the abundant expression in the oesophagus and pancreas, bombesin binding sites have also been reported in ileal and colonic smooth muscle. So far no bombesin-like receptors have been described to be present in the epithelial cells of the intestine [73-75]. Bombesin and the related peptides gastrin-releasing peptide and neuromedin B have a variety of central and peripheral functions. In the central nervous system the peptides are thought to play a role in the regulation of homeostasis, thermoregulation, metabolism and behavior. In the gastrointestinal tract they stimulate secretion from various endocrine and exocrine cells including G, I, L and N cells (gastrin, cholecystokinin, enteroglucagon/peptide YY and neurotensin, respectively), exert direct effects on smooth muscle and have mitogenic effects [47;76;77]. *In vitro* studies have shown that bombesin-like peptides also have immunoregulating functions. Gastrin-releasing peptide is a potent chemoattractant for macrophages and lymphocytes [78]. In addition, it is able to enhance the phagocytic process in macrophages [79] and to stimulate cellular cytotoxicity and natural killer cell activity in human peripheral blood and lamina propria mononuclear cells [80-82]. Furthermore, it increases IgA and IgG antibody secretion, inhibits IL-2 induced proliferation [78], and increases secretion of colonic mucins and intestinal trefoil factor from goblet cells through the enteric

nerves [83]. Inflammation models in rats and rabbits showed that bombesin attenuated the colonic damage by stimulating mucosal proliferation [84;85].

Motilin

Motilin, a 22 amino-acid peptide, was first isolated from the duodenal mucosa, but the peptide is released by endocrine cells of the entire upper small intestinal mucosa. Motilin immunoreactivity was also seen in the nerve fraction of the smooth muscle [86]. For a long time the receptor for motilin was unknown, although affinity studies pointed to two different motilin-receptor subtypes [87]. Nowadays the orphan human G-protein receptor (GPR38-A) is recognized as a motilin receptor [88]. In humans, most motilin binding was found in nerve fractions of antrum, but binding sites were also detected in duodenum and colon, but not in ileum and jejunum [87;89;90]. On the other hand, GPR38-A mRNA was detected in enteric neurones of both the human colon and ileum [88]. Studies in animals showed some difference in the distribution of the motilin receptor. For instance, in the rabbit most receptors for motilin were found in the colon instead of in the antrum, as was the case in humans. Furthermore, in the rabbit receptors were present in the small intestine, decreasing in number aborally [91-94]. Studies of the antrum and colon of the rabbit to locate the receptors in nerve and/or muscle cells are contradictory [91;92;95-97]. In the small intestine of the rabbit, receptors are solely found in the smooth muscle fraction, but in the guinea pig they are detected in the ileal myenteric plexus [98]. Furthermore, in the guinea pig, and also in the cat, the colon did not express motilin receptors [99-101]. The best described effect of motilin is the regulation of the contractility of the antrum and duodenum. Motilin has an important role in the initiation of the interdigestive migrating motor complex. Infusion of motilin in humans induced an increased frequency of antrum contraction [102-104]. *In vitro* studies further showed that motilin has a direct excitatory effect on the colonic circular smooth muscle [89]. Besides the regulation of motility, for example gallbladder contraction, motilin is able to stimulate enzyme secretion in the stomach and pancreas [86]. Recently, it became clear that the inflammatory processes in the intestine also affect motilin functions. In a TNBS-colitis model in rabbits the motilin receptor expression in the colonic smooth muscle was

decreased and there were milder contractions in response to motilin. On the other hand, in the antrum the motilin receptor expression was increased, as was the motilin content in the duodenal mucosa. These effects could be reversed by the administration of IL-11 [105-107].

Conclusion

It is evident from the information on neuropeptides presented above that our knowledge of neuropeptides and neuropeptide expression in the gastrointestinal tract is far from complete and that the role of neuropeptides in IBD and other intestinal inflammatory disorders is largely unknown. In this thesis studies were performed to further characterize intestinal neuropeptide receptor expression, particularly in IBD.

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2

Outline and Aims of this Thesis

Outline and aims of this thesis

Neuropeptides are important biologically active peptides, which are found in abundance in the gastrointestinal tract. They are involved in the regulation of the inflammatory response and gastrointestinal motility. Both processes are disturbed in patients with inflammatory bowel disease (IBD). This has led to the hypothesis that these peptides are involved in the pathology of IBD. The effects of neuropeptides are caused by interaction with specific cell surface receptors. Lately the interest in the receptors for these gastrointestinal neuropeptides has grown considerably as a result of the ongoing increment in the availability of diverse antagonists and agonists for these receptors in patient care. Although knowledge on the expression pattern of these receptors is growing, it is still incomplete, especially with regard to the human situation. A better understanding of the receptor expression pattern in healthy and diseased intestine may lead to the development of new diagnostic and therapeutic approaches.

The aim of this thesis is to establish whether it is worthwhile setting up studies to investigate the use of agonists or antagonists in IBD patients by increasing our knowledge on the expression patterns in control and inflamed human intestine of the receptors for four important gastro-intestinal neuropeptides. These four neuropeptides are substance P, neurotensin, bombesin/gastrin-releasing peptide and motilin. Three complementary techniques were used to describe the receptor expression patterns. First, the active binding sites for the examined neuropeptides were quantified by autoradiography and subsequently identified. Then the precise location of the receptors in the intestinal tissue was shown immunohistochemically. Thirdly, in addition to the information on the protein expression level gained by autoradiography and immunohistochemistry, information on the mRNA expression levels was obtained using the RT-PCR method.

In the first part of the study (chapter three) substance P receptor expression was investigated. Substance P is one of the most important pro-inflammatory neuropeptides to be described in the gastrointestinal tract. In animal studies the administration of a substance P antagonist reduced the inflammatory response in the intestine. Furthermore, a small number of papers has described the expression pattern of the receptor for substance P in humans, but in none of these studies the

combination of three techniques was used to investigate the expression pattern of this receptor.

The fourth and fifth chapters describe the expression pattern of the receptor of the neuropeptide neurotensin in the gastrointestinal tract. Firstly, differences in neurotensin binding sites between control and IBD intestine are described (chapter four). Neurotensin is known to exert both a stimulating and inhibiting effect on motility depending on the location and the type of receptor. Chapter five therefore, describes a study on the differences between the three known receptors for neurotensin.

In chapter six the receptors of the bombesin like-peptide family are studied. Bombesin-like peptides belong to a well-known peptide family in the gastrointestinal field, but their role in the intestine in the inflammatory process and under normal circumstances is not known. Most studies concentrated on its role in gastric secretion and motility.

Finally, in chapter seven a receptor for another well-known gastrointestinal peptide, motilin, is studied. An agonist for this receptor (erythromycin) is already used to treat non-inflammatory diseases affecting motility. This approach opens the field for studying agonists and antagonists in IBD patients. But before administration of agonists or antagonists is warranted, more knowledge is required on the expression of the receptor for this peptide in colons and ilea of both control subjects and patients with IBD.

In the final chapter of this thesis all results of the above mentioned studies are discussed and summarized.

Substance P Receptor Expression in Patients with Inflammatory Bowel Disease

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

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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 form 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

	N	Male/ Female	Age (mean)	Age (range)	Inflamed/ non-inflamed	Use of corticosteroids yes/no
controls	14	7/7	59	34-73	0/14	0/14
CD patients	16	3/13	35	18-73	13/4	3/13
UC patients	12	4/8	38	19-72	9/4	2/10

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 $MnCl_2$ and 75 pM ^{125}I-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, TGACCGCTACCACG-AGCAAGTCTC; reverse, ATAGTCGCCGGCGCTGATGAAG; Sigma-Genosys, UK). The amount of β-actin (primers: forward, GGGTCAGAAGGATTCCTATG; reverse, GGTCTCAAACATGATCTGGG; 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 deparafinized 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 biotinylated 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% H_2O_2 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

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

	Mucosa			Smooth muscle		
	Mean	SEM	n	Mean	SEM	n
control	18	4	8	83	19	9
Noninflamed IBD	43	16	5	95	26	5
Inflamed IBD	55*	10	19	79	13	17

* p<0.01 vs control

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 decease 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

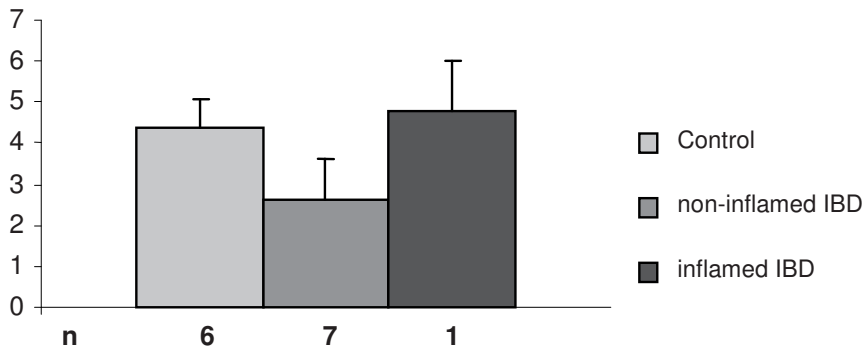

Figure 1. Relative NK-1 Receptor mRNA Expression in Human Intestinal Mucosa from IBD Patients and Control Subjects

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.

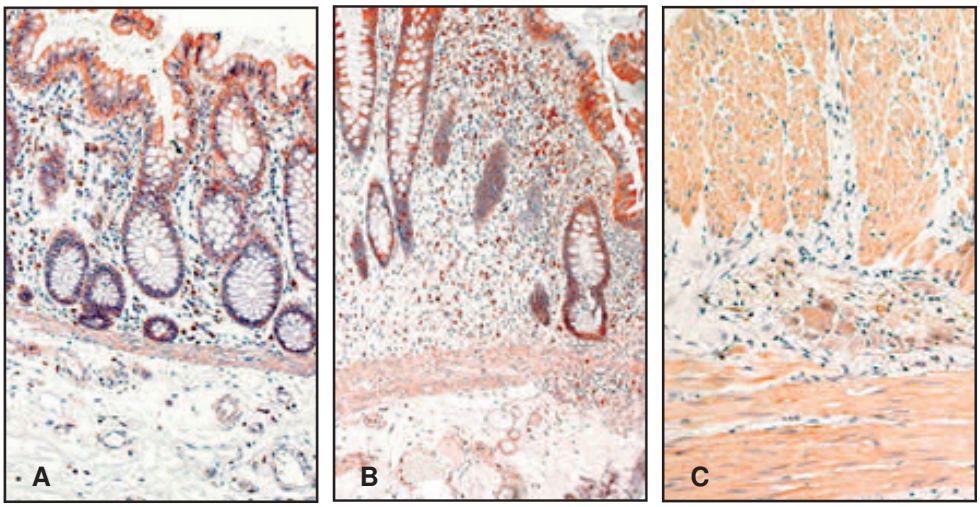

Figure 2. Immunohistochemical Staining of NK-1 Receptor in Human Colon A: control mucosa, B: UC mucosa and C: control smooth muscle. Notice the strong epithelial staining, the staining of infiltrated inflammatory cells and the smooth muscle with myenteric plexus.

Discussion

In this paper three different techniques where 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.

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Quantification of Neurotensin Binding Sites at Different Locations in Inflammatory Bowel Disease and Control Human Intestine

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Abstract

Background: Recently, interest in gastro-intestinal neuropeptides and their receptors has greatly increased because of the possibility of using agonist or antagonists in patient care. Neurotensin is involved in intestinal processes such as inflammation, secretion and motility by interacting with specific cell-surface receptors. The knowledge of intestinal neurotensin receptor expression is growing, but it is still incomplete, especially with regard to the human situation. **Aim:** To further explore the localization and number of neurotensin binding sites in mucosa and muscle of control human colon and ileum and in tissue of patients with inflammatory bowel disease (IBD). **Methods:** Full thickness intestinal tissue samples were collected from 23 control patients and 28 patients with IBD (11 Crohn's disease (CD) and 17 ulcerative colitis (UC)). The tissue of patients with IBD was categorized on the basis of macroscopic appearance and myeloperoxidase (MPO) expression in inflamed and noninflamed samples. For detection of neurotensin binding sites a quantitative autoradiographic method was used. **Results:** Neurotensin binding to ileal muscle of control patients (22±6 fmol/g) was significantly lower than the binding to control colonic muscle (120±16 fmol/g), whereas mucosal binding was even less (7±4 and 13±4 fmol/g for ileum and colon, respectively). There was a significant inverse correlation ($r = -0.68$) between inflammation as quantified by the MPO method and neurotensin binding to colonic muscle in patients with IBD. In ileum the number of binding sites decreased significantly in muscle of inflamed tissue samples as assessed macroscopicly by the pathologist compared to muscle in noninflamed samples of patients with CD (6±2 vs.18±4 fmol/g). **Conclusions:** Neurotensin binding was strongly present in colonic muscle of controls and significantly less in the ileal muscle. In patients with IBD a decrease in neurotensin binding sites was seen in intestinal smooth muscle, which is correlated with the degree of inflammation. The control intestinal mucosa expressed a small number of neurotensin binding sites, while IBD patients even had a slightly lower neurotensin binding.

Introduction

The tridecapeptide neurotensin was first isolated from bovine hypothalamus [1] and later from bovine intestine [2]. In the intestine, neuroendocrine cells (N cells) in the mucosa and enteric neurons release neurotensin [3]. The highest concentration of neurotensin-like immunoreactivity is found in the ileum [4]. Within the gastrointestinal tract neurotensin has many different functions; this peptide acts on pancreatic and gastric acid secretion, it is involved in the initiation of hormone release, and it plays a role in motility and chloride secretion [3,5,6].

In animals several studies have been carried out on the distribution of neurotensin receptors in the control intestine, showing neurotensin binding to the smooth muscle and to the plexuses of the small intestine [4,7-9] and colon [10]. In contrast, the knowledge of neurotensin receptor distribution in humans is still incomplete. Reubi *et al.* showed neurotensin binding sites in colonic muscle and nerves surrounding adenocarcinoma [11]. Other studies have also shown binding sites in the colonic muscle [12,13]. To our knowledge there are no data on the distribution of neurotensin receptors in human ileum and the reports on neurotensin receptors in the mucosa are contradictory. Using immunohistochemistry Riegler *et al*. have detected neurotensin receptors at the bottom of the crypts and in the lamina propria of human colonic mucosa [14], but other studies did not show mucosal binding [12,15,16].

It recently became clear that neurotensin also plays a role in the inflammatory response. *In vitro* studies have shown that inflammatory cells express the neurotensin receptor and that neurotensin increases vascular permeability, stimulates mast cell degranulation, phagocytosis, and histamine and chloride secretion [14,17-19]. Castagliuolo *et al*. have shown that pre-treatment with a neurotensin antagonist reduces the acute symptoms of inflammation in the *Clostridium difficile* toxin-A inflammation model in rats. They concluded that neurotensin and its receptor are important in the acute inflammatory response in the colon [17]. However, little is known about the role of neurotensin and its receptors in the inflammatory process in the human intestine. Ulcerative colitis (UC) and Crohn's disease (CD), both forms of inflammatory bowel disease (IBD), are characterized by chronic inflammation accompanied by changes in motility and

diarrhoea. But to the best of our knowledge, no studies have examined the neurotensin receptor expression in IBD.

The aim of this study was to describe the distribution and number of neurotensin binding sites in mucosa and muscle of control human intestine. In addition, neurotensin binding sites in tissue (both inflamed and noninflamed) of patients with IBD were studied and compared with control tissue. To address these issues storage phosphor autoradiography was used, a technique to quantify and locate peptide binding to frozen tissue sections.

Materials and methods

Tissue sampling

Full thickness intestinal tissue specimens were obtained within 30 minutes after surgery from 11 patients with CD (mean age 38 years; range 18-73 years) and 17 patients with UC (mean age 38 years; range 19-72 years), both from macroscopically inflamed and/or noninflamed areas as assessed by the pathologist. Samples of patients with CD included colonic and ileal tissue, whereas only colon specimens were taken from the patients with UC. For controls, tissue was taken at least 10 cm from the affected site from 23 patients with non-inflammatory diseases (mean age 56 years; range 34-74 years). The tissue was embedded in Tissue-Tek® O.C.T. compound, rapidly frozen on dry ice and stored until use at 80°C.

Storage phosphor autoradiography

Cryostat tissue sections (14 µm) were cut at -20°C, mounted on gelatine-coated glass slides and stored overnight at -80°C. Several methods [20] of ^{125}I-neurotensin binding to tissue sections were tested and optimised resulting in the following protocol. Slides were air dried for 30 min and pre-incubated in 50 mM Tris-HCl (pH 7.0) containing 0.5% BSA for 20 min. For total binding, slides were incubated with 50 mM Tris-HCl, 0.25 mg/ml bacitracin, 4 µg/ml leupeptin, 2 µg/ml chymostatin, 130 mM NaCl, 7.7 mM KCl, 5 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-

aminoethylether)N,N,N',N'-tetraacetic acid (EGTA), and 75 pM ^{125}I-neurotensin (Perkin Elmer Life Science, Boston, Massachusetts, USA) at pH 7.0 for 180 min at room temperature. Alternate serial sections were incubated with addition of 1 µM non-radioactive neurotensin (Bachem AG, Switzerland) to determine non-specific binding. After incubation, sections were washed five times for 5 min with 50 mM Tris-HCl pH 7.0 containing 0.5% BSA at 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 the screen in the Phosphor Imager® (Molecular Dynamics, Sunnyvale, California, USA) visualized the latent image stored on the storage phosphor screen. The data of the digitised image were processed with ImageQuant® software (Molecular Dynamics, Sunnyvale, California, USA). Slides with 10 µl drops of different concentrations of radiolabeled ligand were used for standardization. Rat brain sections acted as positive control. Binding was expressed in fmol/g tissue. To determine the specific binding, the non-specific binding was subtracted from the total binding. Serial sections were stained with hematoxylin and eosin to distinguish between smooth muscle and mucosa.

MPO-assay

The extent of neutrophil infiltration was quantified by measuring myeloperoxidase activity (MPO) to confirm the macroscopic classification of inflammation and to grade the extent of inflammation. Tissue was homogenized and 25 µl of the homogenate was used in an assay described by Krawisz *et al.* [21] to detect MPO activity. The reactions were followed kinetically for 30 minutes and a sample of human polymorphonuclear neutrophils was used for standardization. MPO activity was expressed in arbitrary units.

Data-analysis

Data were expressed as mean ± SEM (standard error of mean). Unpaired Student's t-tests were used to infer significant differences between groups. Pearson correlation was calculated between ligand binding and MPO activity. Values of $p < 0.05$ were considered significant.

Results

Neurotensin binding to control intestine

The distribution of neurotensin binding in control human colon and ileum is shown in figure 1. Strong neurotensin binding is observed to colonic smooth muscle of control patients (figure 1A-C), while binding to ileal muscle is lower (figure 1D-F). In mucosa of control patients, only weak binding of neurotensin is observed to both colon and ileum. Using the ImageQuant® software neurotensin binding sites were quantified and neurotensin binding is expressed in fmol per gram tissue. In control tissue neurotensin binding is significantly higher to colonic muscle than to ileal muscle (120±16 vs 22±6; figure 2). Neurotensin binding to mucosa is low (13±4 and 7±4 fmol/g for colon and ileum, respectively) and this is significantly different from the binding to muscle (figure 2).

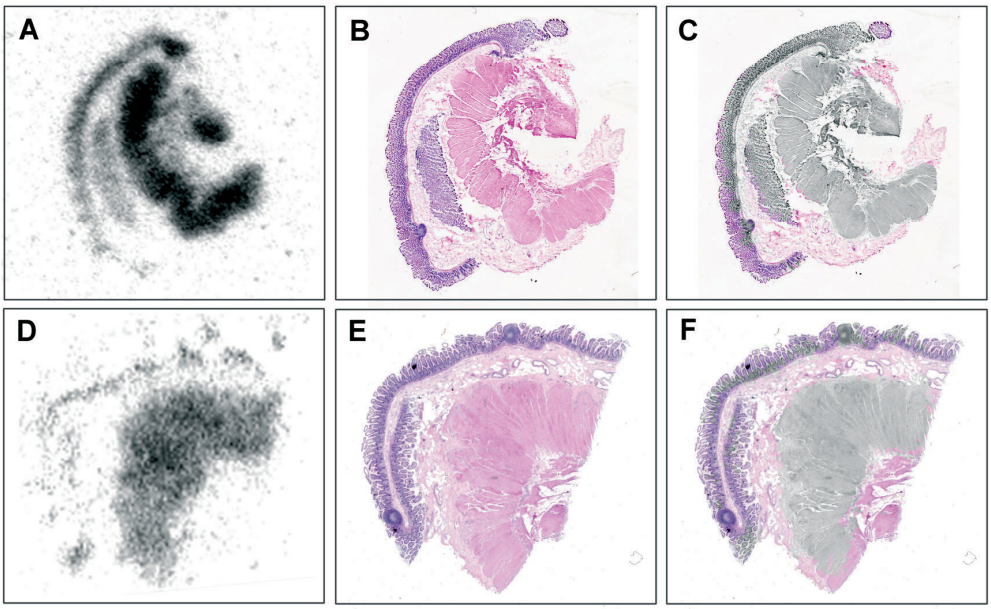

Figure 1. ^{125}I-neurotensin Binding to Control Human Colon (A-C) and Ileum (D-F)

A, D: Specific binding of ^{125}I-neurotensin, the intensity of the greyscale is proportional to the number of binding sites. B, E: Hematoxylin/eosin staining of the serial sections, the pink coloured tissue is smooth muscle and the purple tissue is mucosa. C, F: The precise location of neurotensin-binding is shown by merging the binding image with the hematoxylin/eosin staining, which gives a qualitative result.

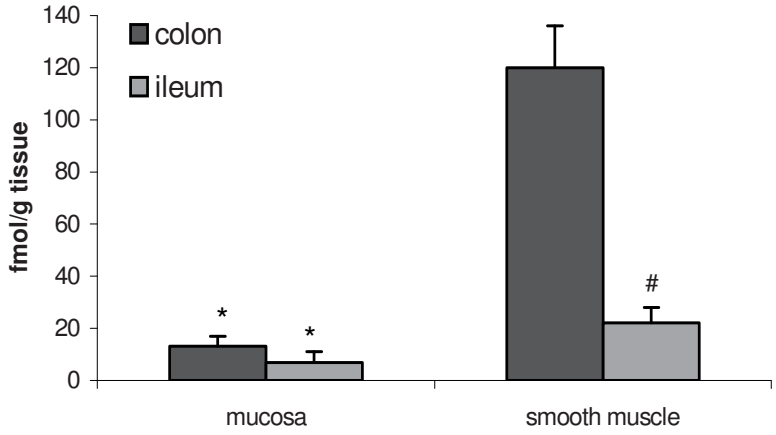

Figure 2. ^{125}I-neurotensin Binding in Human Intestine Binding in 19 colon and 7 ileum samples is measured with autoradiography and expressed in fmol/g tissue and measured. * $p<0.05$ versus smooth muscle, # $p<0.05$ versus colon.

Neurotensin binding to inflamed intestine

The MPO-assay showed that the classification in macroscopically inflamed and noninflamed areas is correlated with the degree of neutrophil infiltration ($r=0.42$, $p=0.004$). MPO values in the inflamed IBD group were significantly higher than those in the noninflamed IBD group (9.7±0.8 vs. 5.5±1.3 U/mg tissue; $p<0.02$). Neutrophil infiltration in control tissue was comparable to the infiltration in the noninflamed IBD group (5.1±0.7 vs. 5.5±1.3 U/mg tissue). In tissue samples of patients with IBD, the same distribution pattern of neurotensin binding is seen as in controls. Most binding is found to colonic smooth muscle and mucosal neurotensin binding is low (figure 3).

Comparing the quantity of neurotensin binding in patients with IBD and controls showed a decrease of the binding in both mucosal and muscular inflamed and noninflamed IBD tissue, but the difference was not statistically significant (table 1). Within the IBD group there was no significant difference between the neurotensin binding in CD and UC patients, although there was a tendency of lower neurotensin binding to colonic muscle of inflamed CD samples compared to UC samples (42±11 vs. 88±21 fmol/g). Neurotensin binding to ileal muscle, all from CD

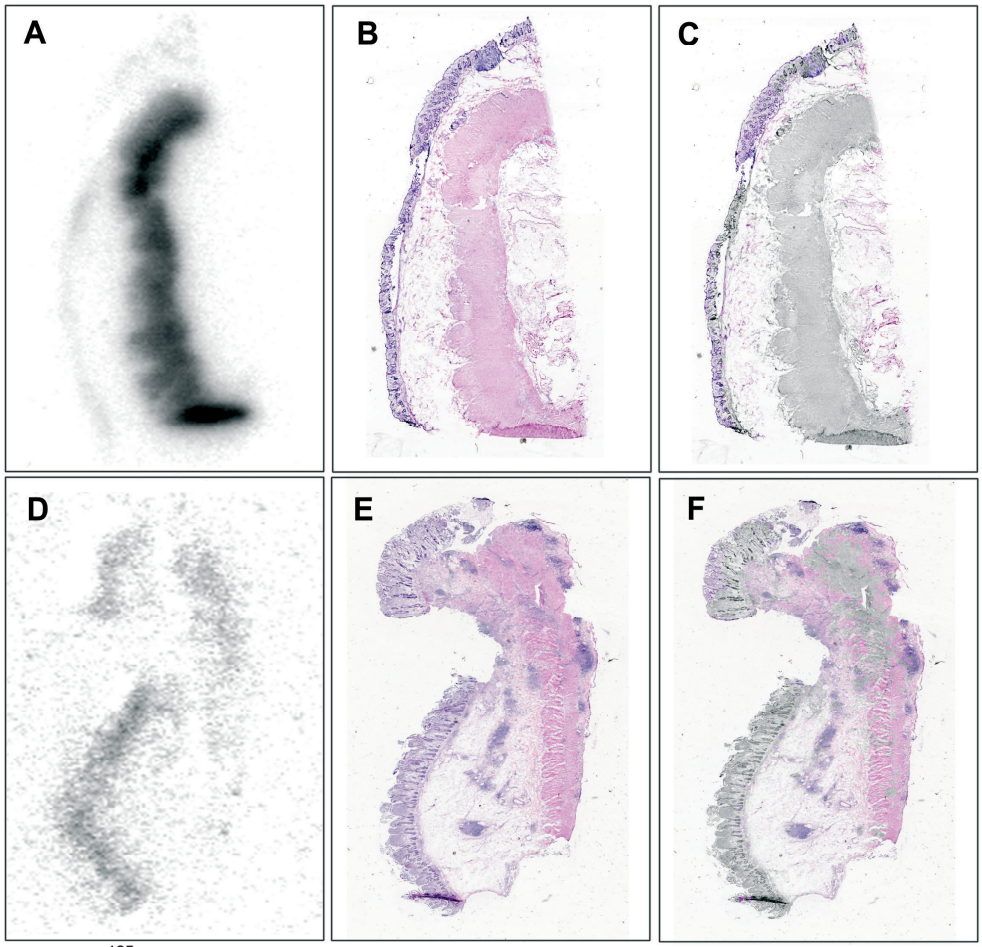

Figure 3 ^{125}I-neurotensin Binding to Macroscopic Inflamed UC Colonic Tissue (A-C) and CD Ileal Tissue (D-F). A, D: Specific binding of ^{125}I-neurotensin, the intensity of the greyscale is proportional to the number of binding sites. B, E: Hematoxylin/eosin staining of the serial sections, the pink coloured tissue is smooth muscle and the purple tissue is mucosa. C, F: The precise location of neurotensin-binding is shown by merging the binding image with the hematoxylin/eosin staining, which gives a qualitative result.

patients, significantly decrease in the muscle of inflamed tissue samples (p=0.02 vs. noninflamed muscle; table 1). If the degree of inflammation (MPO-activity) in patients with IBD is correlated with neurotensin binding, a significant negative correlation (r=-0.68; p=0.03) is seen in the colonic muscle. In the mucosa, no

differences are found between inflamed and noninflamed areas (table 1) of patients with IBD and there is no correlation with MPO activity.

Table 1. Quantity of ^{125}I-neurotensin Binding (fmol/g tissue) in Human Colon and Ileum as Measured by Autoradiography

	colon				ileum			
	mucosa		muscle		mucosa		muscle	
	Mean (SEM)	n	Mean (SEM)	n	Mean (SEM)	n	Mean (SEM)	n
control	13 (4) †	18	120 (16)	19	7 (4)	5	22 (6) #	7
noninflamed IBD	6 (2) †	4	93 (25)	8	2 (1) †	4	18 (4) #	4
inflamed IBD	6 (3) †	15	82 (16)	19	7 (3)	5	6 (2) #*	5

† $p<0.05$ vs. muscle; # $p<0.05$ vs. colon; * $p<0.05$ vs. noninflamed IBD

Discussion

This study describes the distribution of neurotensin binding sites in human colon and ileum of patients with IBD and control samples.

Neurotensin binding in control intestine

Previous studies on the distribution of neurotensin receptors in human control colon by autoradiography have only shown neurotensin receptor expression in the muscular and neuronal compartments of the colon [11,12]. However, Riegler *et al*. have detected neurotensin receptors in human colonic mucosa using immunohistochemistry [14]. In agreement with that study [14], our study has shown neurotensin binding to the mucosa of control human colon and the binding was subsequently quantified. The binding in the mucosa appeared to be lower than in the muscle. As it is generally accepted that neurotensin is involved in the secretion of chloride and fluid [14], both of which are regulated in the mucosa, the finding of neurotensin receptors in the mucosa was not totally unexpected. A possible

explanation for the fact that previous studies did not find neurotensin binding to the mucosa could be that neurotensin receptor expression in tissue surrounding adenocarcinomas as studied by Reubi *et al.* [11] is different from tissue further away from the affected area. Furthermore, it should be noted that Azriel and Burcher [12], who were unable to demonstrate mucosal neurotensin binding, applied a photographic emulsion technique which is not as sensitive as the storage phosphor autoradiography used in our study [20].

To the best of our knowledge, no studies have described the neurotensin receptor expression in human ileum. From animal studies [4,7-9], it is known that the ileum expresses neurotensin receptors, however, the level of expression is less than that found in the colon [10]. Our results on neurotensin binding sites in human ileum are in agreement with these ealier animal studies.

Neurotensin binding to inflamed intestine

Castagliuolo *et al*. have shown that the mRNA content of the neurotensin receptor in rats increases in the mucosa immediately after *Clostridium difficile* toxin A injection [17]. This suggests that neurotensin and its receptors play a role in the process of acute inflammation. No data are available on the role of the neurotensin receptor in inflammatory processes in humans. There are studies that have shown that the human peripheral lymphocytes express the neurotensin receptor and that human neutrophils react *in vitro* with locomotion and phagocytosis upon neurotensin stimulation [18,19]. We investigated the neurotensin binding in patients with a chronic inflammatory intestinal disease, inflammatory bowel disease (IBD). The mucosal increase in neurotensin receptor mRNA seen in rats [17] does not correspond with our findings in humans with chronic intestinal inflammation. This could be due to various factors, such as species-related differences, differences between acute and chronic inflammation and/or differences between mRNA and protein expression. In the smooth muscle of patients with IBD a decrease in neurotensin binding was found when compared to controls, although the difference was not statistically significant. However, correlation analysis showed that in colonic IBD muscle samples the degree of inflammation is inversely correlated with the number of binding sites. Depending on the degree of inflammation a decrease

was seen in the amount of neurotensin binding. It is known that neurotensin is a peptide involved in the regulation of intestinal motility. Therefore a decrease of binding sites for neurotensin could affect this motility, possibly resulting in a disturbed motility, as often seen in patients with IBD. When comparing inflamed with noninflamed areas of the intestine of patients with IBD, a significant decrease in neurotensin binding in patients with CD was seen in macroscopic inflamed ileum compared to noninflamed ileum. In the inflamed colon of patients with IBD the decrease in neurotensin binding compared to controls was rather small (82±16 vs. 93±25 fmol/g) and did not reach statistical significance. However, the decrease in the three CD samples was larger than in the UC samples (42±11 vs. 88±21 fmol/g) suggesting that changes in muscular neurotensin binding are more pronounced in CD than in UC patients. This is in agreement with the fact that the inflammatory process affects the smooth muscle in CD, but not in UC patients.

In summary, in control human intestinal mucosa neurotensin binding is detectable, however it is less than in the intestinal muscle. Within the intestine the expression of binding sites for neurotensin in the colon is significantly higher than in the ileum. In patients with IBD a small decrease was seen in neurotensin binding compared to controls sites. This was more pronounced in the inflamed samples than in the noninflamed IBD samples. There was an inverse correlation between the degree of inflammation and neurotensin binding. These findings may be related to the disturbed motility seen in patients with IBD. The present findings warrant further studies on the (patho)physiological significance of neurotensin receptors in both muscle and mucosa of the bowel.

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Identification of Neurotensin Binding Sites: Neurotensin receptor-1 and -3 are Expressed in the Human Gastrointestinal Tract

Submitted

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Abstract

Background: Neurotensin is involved in the regulation of gastrointestinal motility and inflammation. In patients with inflammatory bowel disease (IBD) the number of neurotensin binding sites decreases. Three receptors for neurotensin have been identified (neurotensin receptor-1, -2 and –3). Only limited data on the expression pattern of the different neurotensin receptors in the human intestine is available. **Aim:** This study examined which neurotensin receptors are present in the human gastrointestinal tract of control patients and patients with IBD. **Results:** Cold saturation studies showed no differences in the Kd's of neurotensin binding to the examined tissues. The overall mean Kd was 1.55±0.83 nM, which excludes the presence of the neurotensin receptor-2. Using the RT-PCR method, neurotensin receptor-3 was found in the muscle and mucosa and neurotensin receptor-1 only in the muscle; no neurotensin receptor-2 mRNA was detected in any of the examined tissues. A decrease in the mRNA levels of neurotensin receptor-1 and -3 was seen in inflamed and non-inflamed regions in the colon of IBD patients. Localization of the receptors was similar in controls and IBD patients: neurotensin receptor-1 was present in epithelium, smooth muscle, submucosal and myenteric plexuses, and neurotensin receptor-3 in the basal membrane and smooth muscle. **Conclusions:** No neurotensin receptor-2 expression was found in the human gastrointestinal tract. Human neurotensin binding to smooth muscle was due to the presence of both neurotensin receptor-1 and -3, whereas in mucosa the location of neurotensin receptor-1 and neurotensin receptor-3 was different. In IBD a decrease in the mRNA of the two neurotensin receptors was seen, but there was no difference in localization when compared to controls.

Introduction

Neurotensin, a neuropeptide in the gastrointestinal tract, is involved in the regulation of motility and inflammation [1-3]. Neurotensin exerts its effects by interacting with specific cell-surface receptors. So far, three receptors for neurotensin have been cloned: neurotensin receptor-1, -2 and -3. The first two receptors are, like most neuropeptide receptors, members of the family of seven transmembrane G-protein coupled receptors, whereas the third is structurally unrelated. Neurotensin receptor-3 belongs to a family of single transmembrane proteins with an extracellulair cysteine-rich domain and a furin cleavage site [4]. Neurotensin receptor-3 appears to be identical to sortilin, a sorting protein that binds to receptor-associated protein [4;5]. Neurotensin receptor-3 is mainly present in the Golgi apparatus, but a small amount can also be found on the plasma membrane. The membrane-bound neurotensin receptor-3 can be upregulated by neurotensin. Neurotensin binds with high affinity to neurotensin receptor-1 and -3 and with low affinity to neurotensin receptor-2. Upon binding of neurotensin, all three receptors are internalized. After internalization the neurotensin receptor-2 and -3 return to the plasma membrane whereas neurotensin receptor-1 is retained intracellularly [6;7]. The internalization of the neurotensin receptor-1 is important for the activation of the second messenger pathway Erk1/2 [8].

Recently, Martin *et al.* [9] reported heterodimer formation of neurotensin receptor-1 and -3 in HT29 cells. The complex was present on the plasma membrane and internalized after stimulation with neurotensin. Furthermore, they showed that intracellular signalling of the heterodimer was different from the signalling evoked by the neurotensin receptor-1 alone. This suggests that the neurotensin receptor-1 has a different function when expressed alone rather than when in complex with the neurotensin receptor-3. There is only limited data on the expression pattern of the different neurotensin receptor types in the human intestine. Most studies describe binding sites for neurotensin without further differentiation between the three types of receptors. In a previous study, a decrease in the number of neurotensin binding sites was shown in patients with inflammatory bowel disease (IBD) [10]. IBD is an inflammatory disease of the gastrointestinal tract that is characterized by disturbed intestinal and colonic motility, diarrhoea and weight

loss. Histologically the affected tissue shows ulcerations and infiltration of inflammatory cells [11]. The aim of the present study was to determine which neurotensin receptor types are present in the gastrointestinal tract of control and IBD patients. The affinity constant (Kd) of neurotensin binding was measured by cold saturation studies and mRNA levels of neurotensin receptor-1, -2 and -3 were studied with RT-PCR. Furthermore, immunohistochemistry provided information on the localisation of the different receptor types in the intestinal wall.

Materials and methods

Tissue sampling

Full thickness tissue samples were collected within 30 min of surgery at the Leiden University Medical Center, the Netherlands. Twenty patients with IBD (mean age 40 years; range 18-73 years) were included. Tissue was taken both from macroscopic inflamed and/or non-inflamed areas and both colonic and ileal tissue samples were taken. Tissue samples taken at least 10 cm away from the affected site from 8 patients with colonic neoplasms (mean age 63 years; range 49-74 years) were considered normal controls. The tissue was embedded in Tissue-Tek® O.C.T. compound and rapidly frozen on dry ice for the cold saturation studies, quick frozen in isopentane on dry ice for RT-PCR and embedded in paraffin for immunohistochemistry.

Cold saturation studies

To determine the affinity of the receptors for neurotensin in the human gastrointestinal tract, dose-inhibition curves were made using the autoradiographic method described in the previous chapter, but now using different concentrations (1 µM – 1 pM) of non-radioactive neurotensin (cold saturation studies). The dissociation constant, Kd, was determined using the non-linear least-squares curve-fitting program LIGAND [12]. The cold saturation studies where performed in a subset of 7 control and 11 IBD patients.

Quantitative RT-PCR

The expression of neurotensin receptor-1, -2 and -3 mRNA was determined with RT-PCR. Total RNA was isolated from mucosal and full thickness tissue samples from 8 control and 17 IBD patients by phenol-chloroform extraction of guanidine isothiocyanate lysates [13]. RNA of HT-29 cells was taken as positive control. 2 µg RNA was used to synthesize complementary desoxyribonucleic acid (cDNA) with Moloney Murine Leukemia virus reverse transcriptase (Invitrogen, La Jolla, CA, USA) and a random primer mix (Hoffmann-La Roche, Switzerland). The obtained cDNA was serially diluted from 1:4 to 1:1024. The diluted cDNA served as a template for the PCR using REDTaq™ DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA) and primers for neurotensin receptor-1, -2 or –3. The used amplification programs and primers have been described in table 1. Duplex PCR's were performed for neurotensin receptor-1 and –3. The amount of cDNA was standardized by the amount of β-actin mRNA in the different samples. PCR fragments were loaded on a 1.5% agarose gel and after electrophoresis, DNA was visualised with ethidium bromide under UV light and a digital picture was taken. The Intensity of the bands was measured in the digital picture with Scion (Washington D.C., USA) imaging software and plotted against the initial cDNA values on a log scale. Ratios of the integrated optical density per µg cDNA of a sample and the positive control were calculated.

Table 1. Primers Used to Detect Neurotensin Receptor mRNA

Primer		Sequence	Amplification program
Neurotensin	forward	CCGTCAAGGTCGTCATACAG	3 min 94°C; 2 min 56°C;
receptor-1	reverse	GATGGTGGAGCTGACGTAGAA	1 min 72°C - 35 cycli
Neurotensin	forward	GTCTCCTCAGCTTCATCGTAT	1 min 94°C; 1 min 58 °C;
receptor-2	reverse	TCCCCAAAGCCTGAAGCT	1 min 72°C - 35 cycli
Neurotensin	forward	AGAATGGTCGAGACTATG	3 min 94°C; 2 min 56°C;
receptor-3	reverse	AAGAGCTATTCCAAGAGGTCC	1 min 72°C - 35 cycli
β-actin	forward	GGGTCAGAAGGATTCCTATG	30 sec 94°C; 45 sec 56°C;
	reverse	GGTCTCAAACATGATCTGGG	1 min 72°C - 30 cycli

Immunohistochemistry

Immunolocalization of neurotensin receptors was assessed by an indirect peroxidase-labelled antibody method [14] with two polyclonal antibodies directed against neurotensin receptor subtype 1 and 3, respectively. Paraffin embedded tissue taken from 8 control patients and 19 IBD patients was cut into 4 µm sections and mounted on poly-L-lysine-coated glass slides. After deparaffinization and rehydration, antigen was retrieved by microwave heating the sections in 10 mM sodium citrate buffer (pH 6.0) for 4 min by 450 W. Thereafter, sections were immediately cooled in Tris-buffered saline (TBS) and subsequently rinsed in TBS with 0.001% saponin. After a wash step with TBS, slides were incubated for 20 min with 1.5% normal rabbit serum or normal goat serum so that neurotensin receptor-1 and -3, respectively, could block non-specific binding. Excess serum was drained off and sections were incubated overnight at 4ºC with goat anti-human neurotensin receptor-1 or rabbit anti-human neurotensin receptor-3 polyclonal antiserum (ITK Diagnostics B.V., Uithoorn, the Netherlands), appropriately diluted in TBS (1:100) containing 1.5% normal rabbit serum or normal goat serum. The sections were rinsed thoroughly in TBS and subsequently incubated with biotinylated rabbit anti-goat or goat anti-rabbit IgG (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% H_2O_2 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 sections, in which the primary antibody was replaced by TBS or that had been pre-incubated with the blocking peptide, were negative.

Statistics

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

Results

Identification of neurotensin binding sites

Figure 1 shows the inhibition curves for normal ileum and colon smooth muscle (values represent the mean of 3 (colon) or 5 (ileum) samples). The curves are also representative for IBD tissue. Analysis of the individual inhibition curves with LIGAND software revealed the presence of one high affinity binding site in the muscle of all but one sample. There were no significant differences in affinity between the different groups (table 2). The overall mean Kd value was 1.55±0.83 nM (n=18).

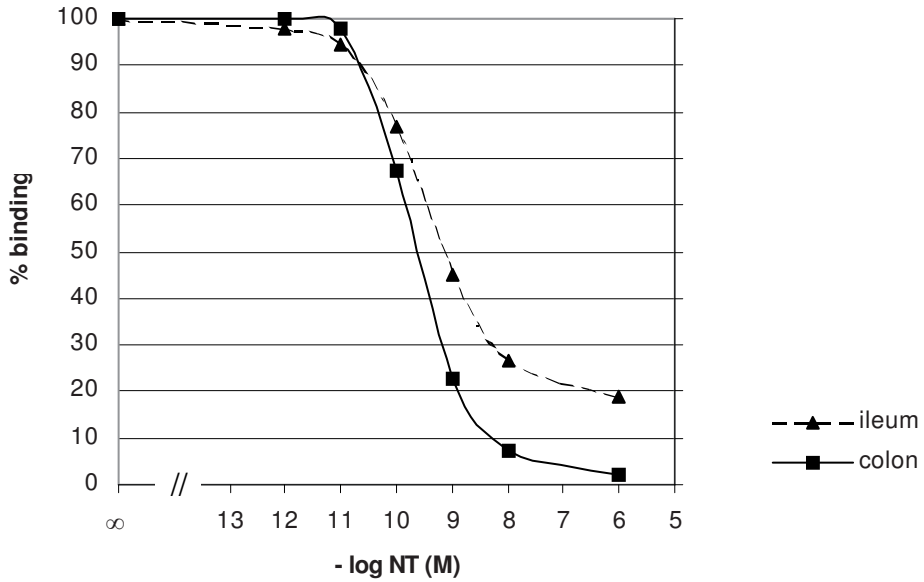

Figure 1. Neurotensin Inhibition Curves of Normal human Ileal and Colonic Smooth Muscle. Values are the mean of 3 (colon) or 5 (ileum) samples, NT=neurotensin.

Table 2. Dissociation Constants (Kd) of Neurotensin Binding Sites in Human Colon and Ileum

Muscle (nM)	controls	IBD	
		Quiescent	Active
Ileal	5.35±3.34 (4)	-	0.72±0.37 (2)
Colonic	0.47±0.11 (3)	0.18±0.01 (3)	0.52±0.28 (6)

Values represent mean±SEM (n) and have been calculated with LIGAND software based on the cold saturation method.

Neurotensin receptor-1

Neurotensin receptor-1 mRNA was present in detectable amounts in the full thickness intestinal samples. The quantity of neurotensin receptor-1 mRNA in control colon had a tendency to be higher than in control ileum and was significantly higher than in colon of IBD patients (see table 3). In the mucosa, neurotensin receptor-1 mRNA was only weakly detectable in a few samples (6 out of 20). Immunohistochemistry showed that in the colon moderate staining was present in the epithelium and the muscularis mucosa. In the submucosa the neurotensin receptor-1 was present in the submucosal plexus and endothelium. Both the longitudinal and circular muscles were heavily stained using the antibody against the neurotensin receptor-1. Also the myenteric plexus, lying between the two muscle layers, stained positive (figure 2 A-C). The staining of the human ileum was comparable with the staining of the colon, although it was less intense (figure 3 A-C). In IBD no difference in cellular localization of the receptor was seen.

Neurotensin receptor-2

No mRNA of the neurotensin receptor-2 was detected in muscle and mucosa of the intestine of control and IBD patients.

Table 3. Ratios of mRNA Expression in Human Intestine and in the HT-29 Cell Line, Corrected for β-actin

	neurotensin receptor-1		neurotensin receptor-3	
	mucosa	full thickness	mucosa	full thickness
Control colon	10.0±10.0 (3)	47±25 (3)	202±69 (3)	146±60 (3)
Control ileum	2.0± 2.0 (4)	7± 0 (2)	86±48 (4)	33± 9 (2)
Non-inflamed IBD colon	0.4± 0.4 (7)	15±15 (3)	90±33 (7)	68±17 (3)
Inflamed IBD colon	0.5± 0.5 (6)	4± 2 (5)	88±27 (6)	34±13 (5)

Values represent mean±SEM (n)

Neurotensin receptor-3

Clear expression of neurotensin receptor-3 was seen in all examined samples. The expression in the mucosal samples was higher than in the corresponding full thickness samples (mean difference 36; $p<0.05$ paired Student's t-test). For both full thickness and mucosal samples expression of neurotensin receptor-3 mRNA in control colon was higher than in colon of IBD patients ($p\leq0.05$). There was also a tendency for neurotensin receptor-3 mRNA to be higher in control colon than in control ileum, but due to the limited number of samples this was not statistically significant. The antibody against neurotensin receptor-3 gave a strong staining just underneath the epithelium (basal membrane) and several cells in the lamina propria (figure 2 A, D). Furthermore, the muscularis mucosa was positive. In the submucosa a strong staining of the stromal cells and only a moderate staining of the endothelium and plexuses (figure 2 B, E) was seen. Both the longitudinal and circular smooth muscles were heavily stained, but the myenteric plexus, found in between, was not stained for neurotensin receptor-3 (figure 2 C, F). Immuno-histological staining of human ileum was comparable with the human colon although less intense and with one major difference, namely, the basal membrane did not stain (figure 3). In IBD no difference in cellular localization of the receptors was seen.

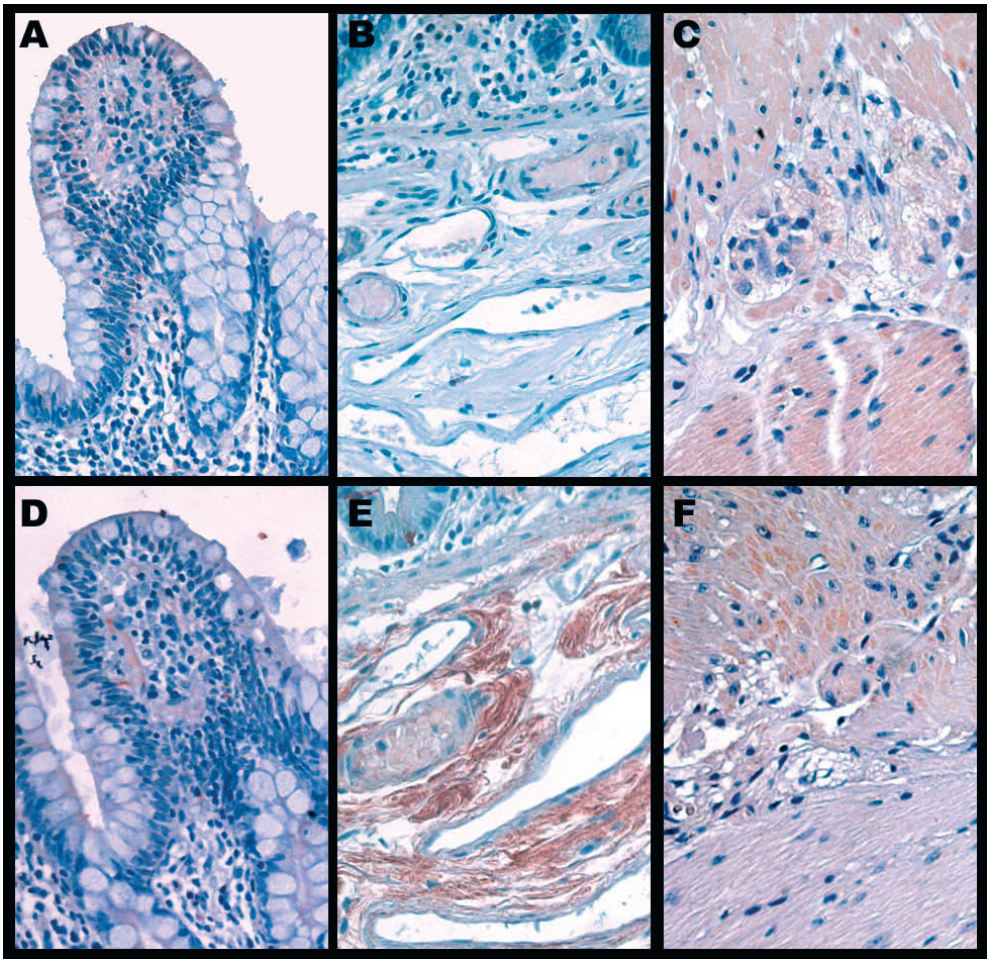

Figure 2. Immunohistochemical Localization of Neurotensin Receptor-1 (A-C) and -3 (D-F) in Colon of Control Patients, Using an Affinity-purified Polyclonal Antibody.

(A) Immunoreactivity for the neurotensin receptor-1 (brown/red) in the epithelial cells of the mucosa. (B) The submucosal plexus and endothelium stained positive for the neurotensin receptor-1. (C) Longitudinal and circular smooth muscle and the myenteric plexus stained positive for the neurotensin receptor-1. (D) Immunoreactivity for the neurotensin receptor-3 in the basal membrane of the mucosa. (E) The stromal cells stained strongly positive and the submucosal plexus and endothelium stained moderately positive for the neurotensin receptor-3. (F) Longitudinal and circular smooth muscle stained positive for the neurotensin receptor-3. Note the negative myenteric plexus.

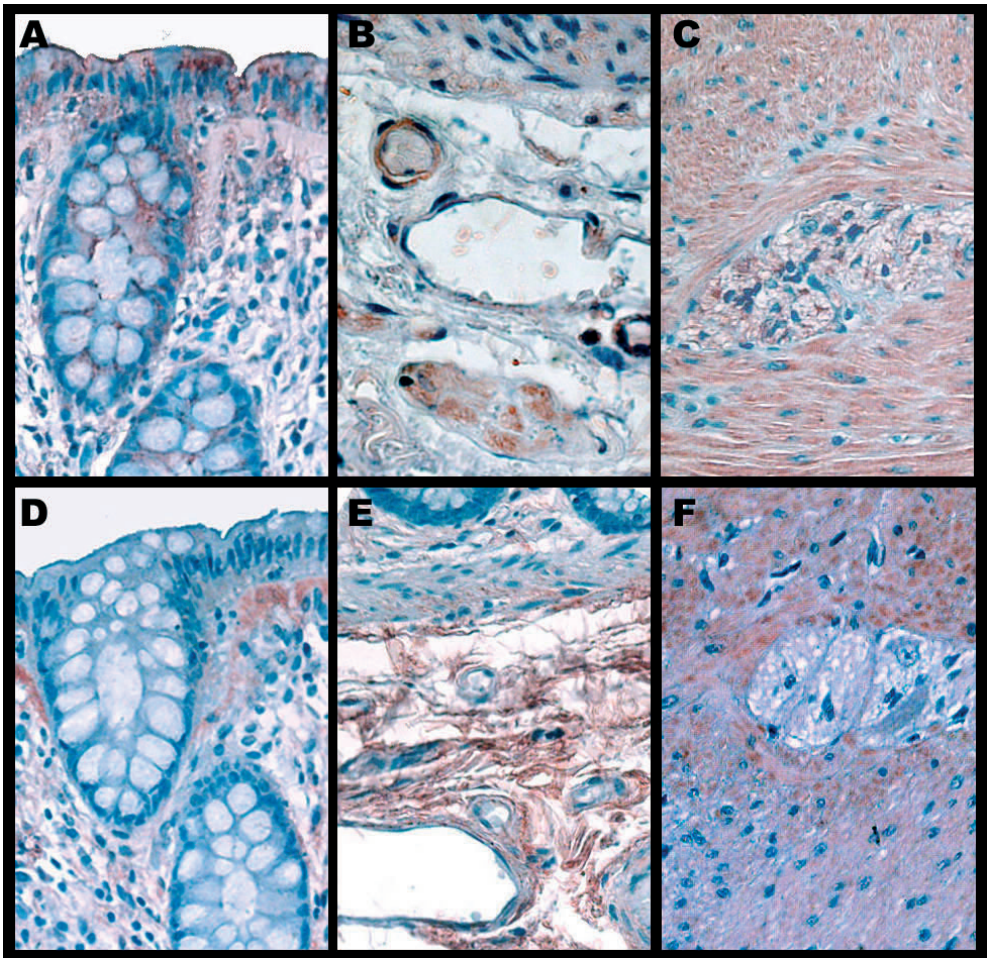

Figure 3. Immunohistochemical Localization of Neurotensin Receptor-1 (A-C) and -3 (D-F) in Ileum of Control Patients, Using an Affinity-purified Polyclonal Antibody.

(A) Immunoreactivity for the neurotensin receptor-1 (brown/red) in the epithelial cells of the mucosa. (B) The submucosal plexus and endothelium stained positive for the neurotensin receptor-1. (C) Longitudinal and circular smooth muscles and the myenteric plexus stained positive for the neurotensin receptor-1. (D) Immunoreactivity for the neurotensin receptor-3 in the epithelium. (E) The stromal cells stained strongly positive and the submucosal plexus and endothelium stained moderately positive for the neurotensin receptor-3. (F) Longitudinal and circular smooth muscles stained positive for the neurotensin receptor-3. Note the negative myenteric plexus.

Discussion

This article gives an overview of the neurotensin receptor types present in the human intestine in both control and IBD patient samples. Information about the receptor type and location is useful for the search for clinical applicable agonists and antagonist. The use of such drugs to act on other neuropeptide receptors has already been established. An example of such a drug is somatostatin. Cold saturation studies were used to determine the affinity of neurotensin for the neurotensin binding sites. In normal human colonic and ileal muscle one high affinity binding site was found with a Kd that was in the same range as that reported previously for the neurotensin receptor-1 and -3. This suggests that in normal human intestine the neurotensin receptor-2 is not expressed. There are no changes in affinity of neurotensin for its receptor in the intestine of patients with IBD as compared with patients without IBD. There was also no neurotensin receptor-2 detected with RT-PCR in the human intestine, confirming the absence of neurotensin receptor-2 in the human bowel. Previously, Schulz *et al*. described immunoreactivity of neurotensin receptor-2 in the human intestine adjacent to tumour specimens [15]. The discrepancy between their results and our results could be due to the fact that they took material immediately adjacent to the tumour and we took samples at least 10 cm from the affected areas. Both neurotensin receptor-1 and -3 mRNA were detected in human intestine, and both had a tendency to have more mRNA expression in colon than in ileum. Furthermore, there was a significant decrease in both mRNA types in the colon of IBD patients compared to controls. These results are in agreement with the results of storage phosphor autoradiography studies showing higher expression of neurotensin binding sites in control colon versus ileum and a decreased number of neurotensin binding sites in IBD [10]. The high mucosal mRNA content of neurotensin receptor-3 is contradictory to the low number of binding sites found by storage phosphor autoradiography. From the literature it is known that besides being present on the plasma membrane, neurotensin receptor-3 is also present in intracellular vesicles and that neurotensin receptor-3 is synthesized as a precursor which has to be activated by cleavage by furin [5;16]. This could be the case in the mucosa where there is a high amount of mRNA and probably precursor receptors that cannot bind

neurotensin. A high level of expression of neurotensin receptor-3 was found in the submucosa using immunohistochemistry. Furthermore, this technique demonstrated expression in the mucosa of the neurotensin receptor-1 and -3 types by different cells. This finding excludes the possibility of heterodimer formation as seen by Martin *et al.* [9] in HT-29 cells. In the smooth muscle, however, both receptors are expressed by the same cells, so theoretically heterodimers could be formed. This could be a mechanism by which neurotensin can exert different signalling functions in the mucosa and smooth muscle. Our data however cannot conclusively confirm that heterodimers are formed when both receptors are expressed. Furthermore, based on the immunohistochemical data, neurotensin receptor-3 is not present in the myenteric plexus, in contrast to neurotensin receptor-1.

In conclusion, in the human intestine, neurotensin receptor-2 was not expressed. Neurotensin receptor-1 and -3 were differently distributed in this tissue. In the smooth muscle, both receptors were present, but in the myenteric plexus only neurotensin receptor-1 could be demonstrated. In the mucosa neurotensin receptor-1 was found in epithelial cells and neurotensin receptor-3 in the basal membrane. No difference in distribution is seen between the intestine of IBD patients and controls, but the quantity of neurotensin receptors in IBD appeared to be lower.

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Gastrin-releasing Peptide Receptor Expression is Decreased in Crohn's Disease but not in Ulcerative Colitis

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Abstract

Background: Gastrin releasing peptide (GRP) and neuromedin B are bombesin (BN)-like peptides involved in regulating motility and inflammation in the gastrointestinal tract, which may be useful in treating inflammatory bowel disease (IBD). Three bombesin-like peptide receptors have been reported, but no studies have investigated their localisation in normal and inflamed human intestine. **Aim:** To localize and characterize BN receptors in normal intestine and to see whether this is modified in IBD. **Methods**: Full thickness intestinal tissue samples were collected from 13 patients with Crohn's disease (CD), 11 with ulcerative colitis (UC), and 19 controls. BN receptor expression was characterized and quantified with storage phosphor autoradiography using BN, GRP, neuromedin B, and the synthetic analogue BN(6–14) as ligands. **Results:** Only BN receptor type 2 (high affinity for GRP) was present in intestinal tissue. Minimal BN binding was detected in the mucosa. In normal colonic smooth muscle, mean BN binding was 336 fmol/g tissue in longitudinal muscle, including the myenteric plexus, and 71 fmol/g in circular muscle. In CD, colonic smooth muscle BN binding was significantly decreased (longitudinal muscle, 106; circular muscle, 19 fmol/g), in contrast to UC (377 and 62 fmol/g, respectively). In CD, a small (not significant) decrease was seen in ileal muscle compared with controls (111 v 169 and 18 v 32 fmol/g tissue for longitudinal and circular muscle, respectively). **Conclusions:** In human intestine only GRP receptor expression is found; the highest expression is seen in longitudinal muscle and myenteric plexus of the colon. This expression is decreased in inflamed and non-inflamed colon of CD patients, but not in the colon of patients with UC.

Introduction

Bombesin (BN), a 14-amino-acid peptide was originally isolated from the skin of the amphibian *Bombina bombina* [1]. Gastrin-releasing peptide (GRP) and neuromedin B (NMB) belong to the BN-like peptide family in mammals [2]. In the gastrointestinal tract, GRP is found in neurons in the human intestine and stomach. The highest amounts of NMB-like immunoreactivity are found in nerves in the circular smooth muscle of the oesophagus and rectum. Peptides from the BN family exert a variety of central and peripheral functions. In the gastrointestinal tract they stimulate secretion from endocrine (gastrin, somatostatin) and exocrine cells (pancreas), exert direct effects on smooth muscle and they have mitogenic effects [3,4]. *In vitro* studies have shown that BN-like peptides also have immunoregulating functions: GRP is a potent chemoattractant of macrophages and lymphocytes [5] and is also able to enhance the phagocytic process in macrophages [6] and to stimulate cellular cytotoxicity and natural killer activity in human peripheral blood and lamina propria mononuclear cells [7-9]. In humans there are three receptors for the BN-like peptides, namely: the NMB receptor with a high affinity for NMB [10], the GRP receptor with a high affinity for GRP [11-13] and the bombesin receptor subtype 3 (BRS-3) [14,15] for which the natural ligand is not known yet. All three receptors belong to the family of seven transmembrane domains G-protein coupled receptors. In rats BN binding sites were localized to the circular muscle of the gastric fundus and antrum, the submucosal layer of the small intestine and the longitudinal and circular muscle and submucosal layers of the colon [16]. In humans, the NMB receptor is found in the mucularis mucosa of the oesophagus, while the GRP receptor is present in the pancreatic acini [17]. In addition to being abundant in the oesophagus and pancreas, BN recpetors are also present in ileal and colonic smooth muscle, but not in epithelial cells [18-20]. Until recently, little was known about the BRS-3 because of the lack of a ligand. Recently, a synthetic ligand, [D-Phe^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]-bombesin(6-14); (BN(6-14)), became available, which binds all three BN receptors, so that by using GRP and NMB as competitive ligands all three receptors can be distinguished [21,22]. In this way, BRS-3 has been demonstrated in human pancreatic islets [23]. However, no studies with BN(6-14) have been performed to characterize the BN receptor family

in the normal human gastrointestinal tract. Several studies have shown the ectopic expression of BN-R in human breast, prostate, lung and gastrointestinal carcinoma's [19,24-28] and therapeutic use of BN antagonist has been suggested. Furthermore, technetium BN analogues have already been used for diagnostic purpose in clinical studies [29,30]. In addition, BN and its receptors might be of importance in inflammatory conditions. Inflammatory bowel disease (IBD) is an inflammatory disease of the gastrointestinal tract. Crohn's disease (CD) and ulcerative colitis (UC) are two different forms of IBD: CD is affecting the full thickness of the wall of colon or the small intestine, whereas UC primarily affects the mucosa of the colon. Disturbed intestinal and colonic motility, diarrhoea and weight loss clinically characterize both diseases. Histologically, the affected tissue shows ulcerations and the infiltration of inflammatory cells [31].Because the effects of BN are established only after binding with its receptor, it is important to know the BN receptor status of patients with IBD. It is known that the expression pattern of receptors for other neuropeptides, such as substance P, is altered in IBD [32]. In addition, Mantyh *et al*. detected binding sites for BN in IBD [33].The aim of our present study was to localize and quantify all three receptors for BN in the human intestinal tract and to study whether this expression is modified in the inflamed and non-inflamed intestine of patients with IBD.

Material and methods

Tissue samples

Colonic or ileal tissue samples were collected at the Leiden University Medical Centre, the Netherlands. Thirteen patients with CD and 11 with UC were included in our study and tissue was taken from both the inflamed and non-inflamed areas, as indicated by the pathologist. As normal controls, tissue was taken at least 10 cm from the affected site of 19 patients with a non-inflammatory related disease, mainly colonic tumours. Table 1 summarizes the patients' characteristics. After surgical resection, full thickness tissue samples were immediately embedded in

Tissue-Tek® O.C.T. compound and frozen on dry ice. Tissue was stored at -80°C until use.

Table 1 Patients characteristics

group	n	Age range in years (mean)	sex		tissue location		inflammation		no anti-infl. drugs
			M	F	ileum	colon	yes	no	
Controls	19	34-74 (55)	10	9	8	12	-	20	19
Crohn's disease	13	18-73 (37)	3	10	9	8	10	7	2
Ulcerative colitis	11	19-72 (40)	4	7	-	13	8	5	2

Storage phosphor autoradiography

Storage phosphor autoradiography was used to identify the presence of receptors. The technique gives information on the localisation, quantity and the binding characteristics of the receptors. 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 according to the protocol described previously [34]. Slides were incubated with 75 pM ^{125}I-[D-Tyr^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]-bombesin(6-14) for cold saturation studies or ^{125}I-[Tyr^{4}]-BN for quantification studies (Perkin Elmer Life Science, Boston, Massachusetts, USA). The first ligand has a high affinity for all three human receptors and the second binds preferentially with the GRP receptor. BN(6-14) (Polypeptide Laboratories GmbH, Wolfenbuttel, Germany), BN, GRP and NMB (Bachem AG, Bubendorf, Switzerland) were used as non-radioactive ligands to detect non-specific binding. Dose inhibition curves were made using different concentrations (1 µM – 1 pM) of the non-radioactive ligands (cold saturation studies) to determine which receptor types were present. The dissociation constant, Kd, was determined using the non-linear least squares curve fitting program LIGAND [35] to obtain information about the characteristics of the receptor. Binding was quantified with a storage phosphor screen and ImageQuant® software (Molecular Dynamics, Sunnyvale, CA, USA). Slides with 10 µl drops of different concentrations of radiolabeled ligand were used for

standardization. Rat brain sections were used as a positive control. Binding is expressed as fmol/g tissue. Serial sections were stained with haematoxylin and eosin to distinguish the different layers in the intestine.

Data analysis

Data were expressed as mean (SEM). An unpaired Student's t-test was used for statistical assessment of differences between means. Values of $p < 0.05$ were considered significant.

Results

Localization of BN binding sites

In the colon and ileum of control patients BN binding was seen in the longitudinal smooth muscle layer and the myenteric plexus. Binding to the circular smooth muscle layer was weaker. A low binding signal was found in the mucosa of the colon, but not in the ileum. A similar distribution pattern was seen in the colon and ileum of patients with IBD, but the intensity in the colonic longitudinal and circular smooth muscle of patients with CD was lower than that seen in controls and patients with UC. Figure 1 provides a representative picture of the binding pattern for a normal colon and the inflamed colon of a patient with CD.

Identification of binding sites

Cold saturation inhibition curves using the universal ligand ^{125}I-[D-Tyr^{6}, β-Ala^{11}, Phe^{13}, Nle^{14}]-bombesin(6-14) were performed to identify the BN receptor types present in the intestine of control subjects and patients with IBD. Figure 2 shows an inhibition curve of BN receptors in a control colon; this curve is also representative of the curves seen in the ileum and in patients with IBD. This inhibition curve shows that the binding sites have a high affinity for GRP and BN(6-14), but a lower affinity for NMB, which is characteristic of the GRP receptor. No receptors with a high affinity for NMB or only a high affinity for BN(6-14) (BRS-3) were detected in human intestinal tract. The Kd for the affinity of BN binding was calculated using

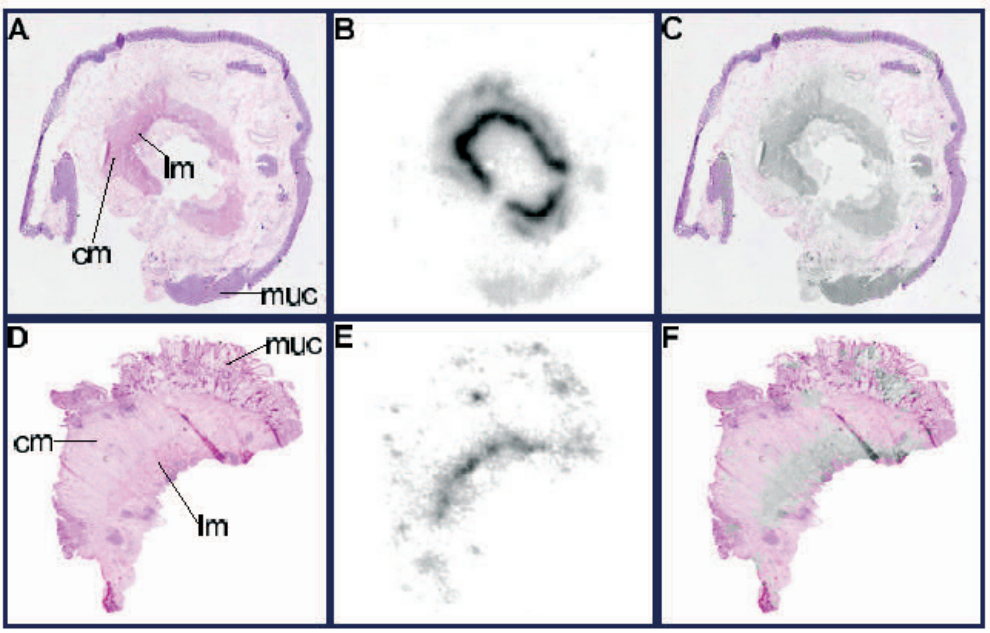

Figure 1 ^{125}I-BN Binding to (A-C) Control and (D-F) Inflamed Crohn's Disease Colonic Tissue. A, D: Haematoxylin and eosin staining. B, E: Specific binding of ^{125}I-BN on the serial sections; therefore the non-specific binding image is subtracted from the total binding image. The intensity of the greyscale equals the amount of binding sites. C, F: The precise location of BN binding is shown by merging the binding image with the haematoxylin and eosin stained image, which gives a qualitative result; BN, bombesin; muc, mucosa; cm, circular muscle; lm, longitudinal muscle

LIGAND software. For some tissue a two site model was possible, but this was never significantly better than the one site model, and the Kd_2 was always extremely high (range 235-15236 nM). The mean Kd_1 for BN was 2,54 nM.

Quantification of bombesin binding sites in control patients

Binding detected with storage phosphor autoradiography was quantified using ImageQuant® software. In colonic smooth muscle of control patients, the mean (SEM) BN binding was 183 (43) fmol/g tissue, but a pronounced difference was seen between the two muscle layers. Mean (SEM) BN binding in the longitudinal muscle, including the myenteric plexus, was 336 (66) fmol/g tissue, whereas binding in circular muscle was only 71 (20) fmol/g tissue ($p<0.01$). Significantly lower binding was found in the ileum of control patients compared with the colon.

The difference in binding between the longitudinal and circular muscle seen in the colon was also seen in the ileum: mean (SEM) binding was 169 (54) fmol/g and 32 (11) fmol/g (p=0.02), respectively. In the mucosa, only the colon showed a weak binding (mean, 10; SEM, 4 fmol/g tissue).

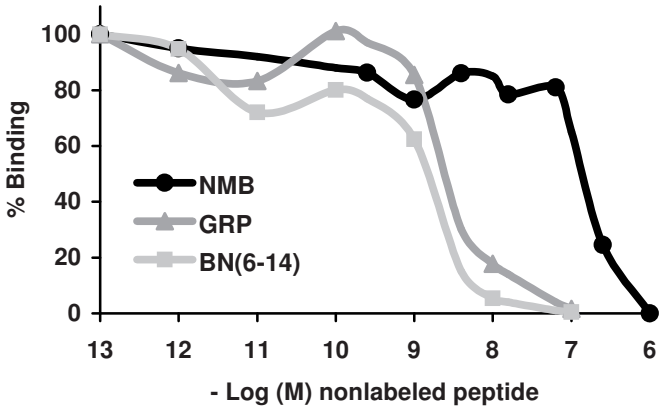

Figure 2. Competitive Inhibition of ^{125}I-BN(6-14) Binding to Human Control Colon Tissue was incubated with 75 pM ^{125}I-BN(6-14) and the indicated concentration of NMB (●), GRP (▲) and BN(6-14) (■).

Quantification of bombesin binding sites in IBD patients

Binding to both muscle layers was greatly decreased in the colon of patients with CD. In the circular muscle, mean (SEM) BN binding was 19 (4) fmol/g tissue, and in the longitudinal muscle (including the myenteric plexus) binding was 106 (30) fmol/mg tissue; binding at both these sites was significantly lower than that seen in controls (figure 3). In patients with UC, such a decrease was not seen. Binding in both muscle layers was comparable with controls (mean (SEM) 62 (10) and 377 (58) fmol/g tissue; figure 3). In the ileal muscle of patients with CD there was also a small, although not significant, decrease compared with controls (mean (SEM) 111 (20) vs. 169 (54) fmol/g tissue and 18 (4) vs. 32 (10) fmol/g tissue, for longitudinal and circular muscle, respectively). Within the CD and UC patient groups no

differences were seen between inflamed and non-inflamed regions and there was no association with the use of anti-inflammatory drugs. Furthermore, no difference was detected between male and female patients in all three groups.

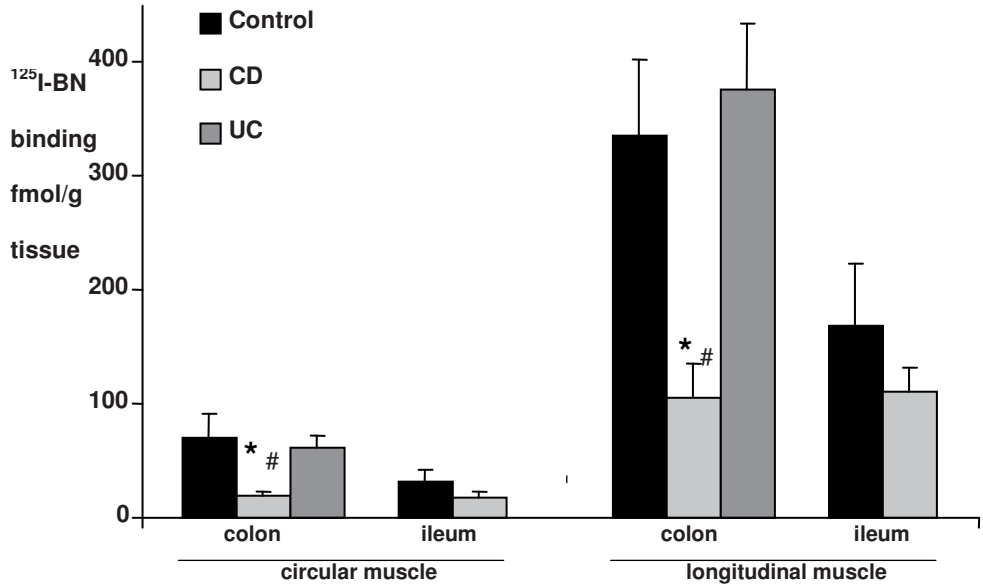

Figure 3 Quantity of ^{125}I-BN Binding to Smooth Muscle of Human Colon and Ileum as Detected with Autoradiography. *Left*: BN binding to circular smooth muscle of controls, patients with CD and patients with UC. The first three bars represent colonic tissue and the last two bars ileum (12, seven, 15, eight and nine samples, respectively). *Right*: BN binding to longitudinal muscle, including the myenteric plexus, of controls, patients with CD and patients with UC. The first three bars represent colonic tissue and the last two bars ileum(12, seven, 15, eight and eight samples, respectively). Values are means (SEM). *$p < 0.05$ compared with controls; #$p \leq 0.01$ compared with UC BN, bombesin; CD, Crohn's disease; UC ulcerative colitis.

Discussion

In our present study, we studied the quantity and localization of receptors from the BN family in the mucosa and smooth muscle of the human distal intestinal tract. Both normal control patients and patients with IBD were studied. Patients with mainly colonic neoplasms were used as normal controls. Previous studies [24,27]

have shown that colon cancers can aberrantly express the GRP receptor, but that normal epithelium surrounding the cancer does not.

The cold saturation inhibition studies showed that there were no binding sites with a high affinity for NMB, indicating that there are no detectable amounts of the NMB receptor present in the human colon and ileum. In addition, no BRS-3 were detected in the human colon and ileum; when these receptors are present a binding curve with a high affinity for BN(6-14) and a low affinity for GRP and NMB should been seen. From these inhibition studies it can be concluded that only the GRP-R is present in the human intestine. The highest concentrations of the GRP receptor were present in the myenteric

plexus and longitudinal smooth muscle. This finding is in agreement with a study of Rettenbacher and Reubi [36]. Low numbers of binding sites were present in the mucosa of the colon only. Other studies have shown that the epithelium does not express the GRP receptor [19,37]. The mucosal binding sites found in our study are probably found on neurons in the lamina propria or the muscularis mucosa, although the resolution of autoradiography was not high enough to confirm this. Immunohistochemical studies are needed to obtain information on this matter, but to date no antibodies for the GRP receptor are available.

The receptor types present and their localization were not altered in patients with IBD, although there was a pronounced decrease in the number of binding sites in the smooth muscle of patients with CD compared with the normal control patients. This decrease was not seen in patients wit UC. The use of anti-inflammatory drugs did not seem to affect GRP receptor expression. CD and UC are both chronic inflammatory conditions of the intestine, but in patients with CD this is a transmural inflammation, whereas in those with UC only the mucosal layer is affected. The change in receptor expression in the smooth muscle of patients with CD but not in those with UC is in agreement with the involvement of the disease in the muscle. Previously, Mantyh and colleagues [38] found no changes in BN binding sites in IBD patients compared with controls; this discrepancy could be the result of the different methods used for quantification. Quantification with a storage phosphor screen, as used in our study, is more sensitive and has a higher dynamic range than quantification with film, as used by Mantyh *et al.* [39].

Rat studies have shown that BN treatment attenuated TNBS (2.3.6-trinitrobenzenesulfonic acid) induced colonic damage and stimulated histopathologically apparent mucosal proliferation in rats [40]. Earlier, Chu *et al.* showed that BN improved survival in a lethal model of methotrexate induced enterocolitis in rats, possibly by maintaining gut mucosal structure [41]. In the mucosa of patients with IBD very few binding sites for BN were seen. This suggests that if the administation of BN has any effect it will be seen in the human mucosa, acting via indirect mechanisms such as the release of other trophic agents --- for example, neurotensin and peptide YY.

At present, the clinical relevance of our findings is unknown. BN is known to stimulate contraction of the colonic muscles. The lower presence of BN receptors in the intestinal muscles of patients with CD may protect the patient against enhanced motility. Alternatively, the decrease in BN receptors may delay intestinal healing because rat studies have shown that the administration of BN protects against TNBS induced intestinal inflammation. Our study does not allow us to draw conclusions about the potential beneficial effects of BN administration or blocking of the BN receptors in CD. Diagnostically, the difference between BN receptors in the colon of CD and UC may be useful for the differentiation between the two diseases --- for example, by *in vivo* receptor scintigraphy.

In conclusion, in human colon and ileum the only BN receptor found is the GRP receptor. The highest expression is seen in longitudinal muscle and the myenteric plexus of the colon. This expression was decreased in inflamed and non-inflamed colon of patients with CD, but not in patients with UC, when compared with normal colon from controls.

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Motilin Receptor Expression in Smooth Muscle, Myenteric Plexus and Mucosa of Human Inflamed and Noninflamed Intestine

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Abstract

Background: Besides regulation of upper gastrointestinal motility, motilin seems to play a role in the inflammatory response. Motilin receptor expression in human intestine has not been studied thoroughly. This study aimed to describe the intestinal distribution of motilin receptors in inflammatory bowel disease (IBD) and control patients. **Methods:** Quantitative autoradiography, immunohistochemistry and reverse-transcriptase polymerase chain reaction (RT-PCR) were used to detect motilin receptors in tissue of 25 IBD patients (13 Crohn's disease (CD), 12 ulcerative colitis (UC)) and 19 patients with a neoplasm (controls). **Results:** Median muscular motilin binding was 3 and 8 fmol/g tissue in colon and ileum, respectively. In the gastroduodenal region the median was higher (93 fmol/g). In UC colonic muscular motilin binding was significantly increased compared to controls (7 versus 3 fmol/g, $P \leq 0.05$). Expression in CD was similar to controls. Besides the binding found in the muscular compartment, motilin binding was also found in the mucosa, which was even higher than in the muscle (3 versus 11 and 8 versus 27 fmol/g for colon and ileum ($P \leq 0.06$), respectively). RT-PCR and immunohistochemistry confirmed mucosal motilin receptor expression. The mucosal motilin receptors were located in the epithelial cells. In the muscular compartment receptors were strongly present in the myenteric plexus and weakly in the smooth muscle cells. In IBD tissue the expression pattern was not different. **Conclusions:** The motilin receptor is expressed in human colonic and ileal smooth muscle. Further, motilin receptor expression was also shown in the mucosa. Muscular binding in UC patients is increased but no different expression pattern was found.

Introduction

Motilin, a 22 amino-acid biological active peptide, is released by endocrine cells of the upper gastrointestinal mucosa. The 1- to 2-hour interval increases in plasma motilin levels are synchronized with phase III myoelectric contraction of the stomach and intestine [1] and it is now well accepted that motilin is involved in initiating the interdigestive motor complex. Studies with intravenous infusion of motilin in humans showed that motilin induces increased antrum contraction frequency [2,3]. Motilin receptor location studies, to obtain more information about possible functions of motilin are mainly performed in animals. These studies showed some interspecies differences. In the rabbit, the most frequently studied animal, receptors for motilin are found in the antrum, small and large intestine with exception of the cecum. Most receptors were found in colon followed by antrum from where an aborally decreasing gradient was seen over the small intestine [4-7]. Studies in antrum and colon to test if the motilin receptor expression is mostly located on nerve or muscle cells are contradictory [4,5,8-10]. In the small intestine receptors are only found in the smooth muscle fraction. In cat and guinea pig, in contrast with the results in rabbit, no receptors are found in the colon but antrum and small intestine do express binding sites [11-13] and motilin receptors are clearly present in the myenteric plexus of guinea pig ileum [14]. Only a few articles described studies on motilin binding sites in humans. The highest frequency was found in nerve fractions of antrum, but also binding sites in duodenum and colon were detected while there were no receptors demonstrated in ileum and jejunum [15-17]. Due to the species differences it is important that more studies are performed on motilin receptor expression in human intestine. A few years ago an orphan human G-protein receptor (GPR38-A) was identified as a motilin receptor [18] and more techniques became available to detect this motilin receptor. GPR38-A mRNA was detected in enteric neurons of human ileum and colon [18].

Many gastrointestinal hormones also act as a neuropeptide and the presence of the motilin receptor in nerve fractions and in enteric neurons indicates that this could also be the case for motilin. From other neuropeptides in the gastrointestinal tract it is known that they have a function in the regulation of the inflammatory process. For example substance P has been proven to be an attractant for

macrophages and its receptor expression is increased in the inflamed intestine [19]. In rabbit there have been some preliminary studies to the role of motilin in the inflammatory process. It was shown that in a TNBS colitis model the motilin receptor expression in smooth muscle was decreased and that there was less contractility in response to motilin administration [20,21]. They also showed that interleukin-11 could reverse these effects. The aims of our study were to describe the distribution of the motilin receptor in the human lower gastrointestinal tract (ileum and colon), and to see if in tissue of patients with inflammatory bowel disease (IBD) motilin receptor expression is changed as compared with normal controls. Here 3 different techniques where used i.e. quantitative autoradiography, immunohistochemistry and reverse-transcriptase polymerase chain reaction (RT-PCR).

Material and methods

Tissue samples

Colonic or ileal tissue samples were collected at the Leiden University Medical Centre, the Netherlands. Twenty-five IBD patients (13 Crohn's disease (CD), 12 ulcerative colitis (UC); mean age 39), were included in our study and tissue was taken from both inflamed as noninflamed areas (table 1). Eighty-two percent of the IBD patients used anti-inflammatory drugs; the type of drugs is specified in table 2. As controls, macroscopically normal intestine was taken at least 10 cm from the affected site of 19 patients (mean age 58) with colonic neoplasm's. None of the control patients used anti-inflammatory drugs. Mucosa was snap frozen in isopentane on dry ice; full thickness tissue samples were embedded in Tissue-Tek® O.C.T. compound and frozen on dry ice. A further 2 antrum samples and 3 duodenal samples were collected as positive controls. Tissue was stored at -80°C until use.

Table 1. Patients Characteristics

Group	n	age in years	sex		tissue location		inflam-mation	
			M	F	ileum	colon	yes	no
Controls	19	34-74 (mean 58)	10	9	8	12	-	20
Crohn's disease	13	18-73 (mean 37)	3	10	9	8	10	7
Ulcerative colitis	12	19-72 (mean 39)	4	8	-	14	9	5

Table 2. Type of Anti-inflammatory Drugs Used by the Patients

Group	Controls	Crohn's disease	Ulcerative colitis
none	19	1	1
Corticosteroids	0	2	2
Corticosteroids + 5- aminosalicylicacid	0	6	5
Corticosteroids + 5- aminosalicylicacid + immunosuppressors	0	2	2
Corticosteroids + immunosuppressors	0	0	1
5-aminosalicylic acid	0	1	0
Immunosuppressors	0	0	1

MPO-assay

The extent of neutrophil infiltration was quantified by measuring myeloperoxidase activity (MPO) to confirm the macroscopic classification of inflammation and to grade the inflammation. Tissue was homogenized and 25 µl of the homogenate was used in an assay described by Krawisz *et al.* [22] to detect MPO activity. The reaction kinetics was followed for 30 minutes and a sample of human polymorphonuclear neutrophils was used for standardization. MPO activity is expressed in arbitrary units.

Peptide labelling

Motilin (Bachem AG, Switzerland) was iodinated using the chloramine T oxidation method. Iodinated peptide was separated from unincorporated iodide by chromatography on a Sephadex-G50 column with Tris-HCL elution buffer (50 mM,

pH 8.0) [23]. The specific activity of ^{125}I-motilin was estimated to be ≈2000 Ci/mmol.

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. Binding of ^{125}I-motilin to tissue sections was carried out by a modification of Sakai et al's protocol [5], which was optimized in our laboratory. In brief, slides were air dried for 30 min and preincubated in 50 mM Tris-HCl (pH 8.0) containing 0.05% PMSF and 0.4% BSA for 20 min. For total binding, slides were incubated with 50 mM Tris-HCl, 10 mM $MgCl_2$, 0.05% PMSF, 0.4% BSA and 600 pM ^{125}I-motilin at pH 8.0 for 180 min. Alternate serial sections were incubated with addition of 1µM nonradioactive motilin to determine nonspecific binding. After incubation sections were washed five times for 5 min with 50 mM Tris-HCl pH 8.0 containing 0.05% PMSF and 0.4% BSA at 4°C. Washed slides were rapidly dried with a stream of cold air. Slides were placed in a storage phosphor cassette for 40 hours at room temperature. The latent image stored in the storage phosphor screen was visualized by laser scanning of the screen in PhosphorImager® (Molecular Dynamics, Sunnyvale, CA, USA). The data of the digitized image were processed with ImageQuant® software (Molecular Dynamics), the nonspecific binding image was subtracted from the total binding image to create the specific binding image. Slides with 10 µl drops of different concentrations of radiolabeled ligand were used for standardization. Binding is expressed as fmol/g tissue. Serial sections were stained with hematoxylin/eosin to distinguish between the smooth muscle and mucosa.

Immunohistochemistry

Immunolocalization of motilin receptors was assessed by an indirect peroxidase-labeled antibody method [24] with a polyclonal antibody directed against motilin receptor GPR38-A in a subset of 8 control patients and 19 IBD (11 CD, 8 UC) patients. In brief, cryostat tissue sections (7 µm) were cut at -20°C and mounted on poly-L-lysine coated glass slides. After 50 min of air drying tissue was fixed in acetone (-20°C) for 10 min and then air dried for another 30 min. Next the tissue

was rinsed in Tris buffered saline (TBS, 0.05 M, pH 7.5) and treated for 1 min with 0.25% periodic acid in TBS to block endogenous peroxidase. After 2 wash steps with TBS the slides were incubated for 20 minutes with 1.5% normal rabbit serum (NRS) to block nonspecific binding. Excess serum was drained off and sections were incubated for 2 hours at room temperature with goat antihuman GPR38-A polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), appropriately diluted (1:640) in TBS containing 1.5% NRS. The sections were rinsed thoroughly in TBS, and subsequently incubated with biotinylated rabbit anti-goat Ig (Dako, Glostrup, Denmark; 1:200 in TBS) and peroxidase-labeled streptavidin (Dako; 1:100 in TBS) for 30 minutes 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% H_2O_2 for 10 minutes, resulting in a red staining. Finally, sections were lightly counterstained in Mayer's hematoxylin and mounted in Aquamount™ (BDH, Germany). To asses the specificity of the staining, control experiments where performed on serial sections in which the primary antibody was replaced by TBS or preabsorbed with the blocking peptide.

RT-PCR

The expression of GPR38 mRNA was determined with a semiquantitative RT-PCR; β-actin mRNA expression was used for standardization of the amount of used cDNA. Total RNA from 8 control and 17 IBD (9 CD, 8 UC) mucosal tissue samples was isolated by phenol chloroform extraction of guanidinium isothiocynate lysates [25]. RNA of TE671 cells was used as positive control. With M-MLV reverse transcriptase (Invitrogen, La Jolla, CA, USA) and a random primer mix (Hoffmann-La Roche, Switzerland) cDNA was synthesized from 2 µg RNA. The obtained cDNA was serially diluted from 1:4 to 1:1024. The diluted cDNA served as a template for the PCR using REDTaq™ DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA) and primers for GPR38 (forward, CACGTTGGCAGAATCATTTAC; reverse, TCCCATCGTCTTCACGTTAG) or β-actin (forward, GGGTCAGAAG-GATTCCTATG; reverse, GGTCTCAAACATGATCTGGG) (Sigma-Genosys, UK). Following amplification programs were used 1 minute 95°C; 1 minute 53°C; 1 minute 72°C 36 cycles and 30 seconds 94°C; 45 seconds 56°C; 1 minute 72°C 30

cycles for GPR38 and β-actin respectively. PCR fragments were loaded on a 1.5% agarose gel and after electrophoresis DNA was visualized with ethidium bromide under UV light and a digital picture was made. Intensity of the bands was measured with Scion (Washington D.C., USA) imaging software and plotted against starting amount of cDNA on log scale. Ratios of the integrated optical density per µg cDNA between samples and positive control were calculated.

Statistics

Data is expressed as median values with confidence intervals. For statistical assessment of differences between groups, Wilcoxon signed ranks test and a Mann-Whitney U-test were used for paired and unpaired data respectively. Values of $P < 0.05$ were considered significant.

Results

Motilin receptors in normal human gastrointestinal tract

Quantification of motilin receptor expression

With autoradiography the median motilin binding found in the colonic and ileal smooth muscle was 3 and 8 fmol/g tissue, respectively (table 3). This is lower ($P < 0.05$) than the amount of binding sites present in the human gastroduodenal region (93 fmol/g tissue; range 90-167) which was used as positive control. Besides binding in the muscular compartment, specific motilin binding was found in intestinal mucosa (table 3). In colon and ileum the motilin receptor expression in the mucosa was higher than in the smooth muscle (colon: 11 vs. 3 fmol/g, $p \leq 0.06$; ileum: 27 vs. 8 fmol/g, $P \leq 0.05$) (table 3), while in the gastroduodenal region mucosal binding tended to be lower than the binding found in smooth muscle (44 vs. 93 fmol/g). Figure 1 shows representative pictures of the location and intensity of motilin binding in normal human colon and ileum.

Table 3. Quantity of ^{125}I-motilin Binding (fmol/g tissue) in Control Human Intestine as Measured by Autoradiography

	mucosa			muscle		
	median	CI	n	median	CI	n
Gastroduodenal region	44	3-100	3	93	8-225	3
Ileum	27	9-48	8	8* ⊾	4-14	8
Colon	11	4-36	11	3*	2-7	12

* P<0.05 versus antrum and duodenum, ⊾ P<0.05 vs. paired mucosa, CI = 95% confidence interval, n = number of tested tissue samples

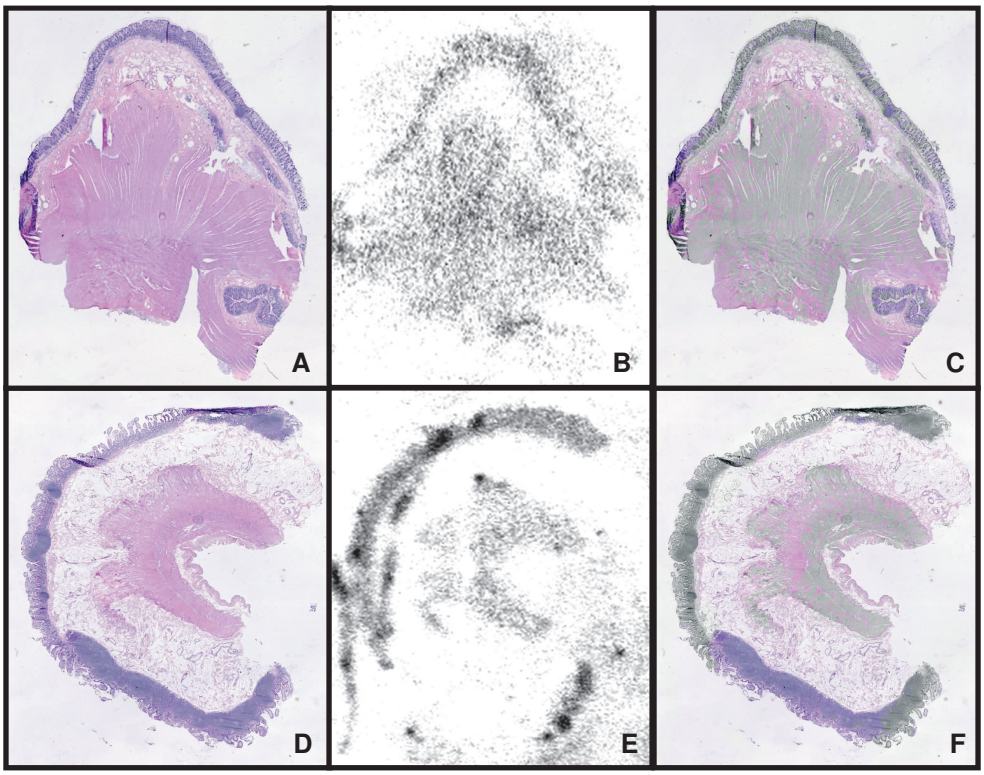

Figure 1 ^{125}I-motilin Binding to Control Colon (A-C) and Ileum (D-F).

A, D: The eosin/haematoxylin staining. B, E: Specific binding of ^{125}I-motilin on the serial sections, the intensity of the greyscale equals the amount of binding sites. C, F: The precise location of motilin-binding is shown by merging the binding image with the eosin/haematoxylin staining, which gives a qualitative result.

Localization of motilin receptor GPR38-A

The result of motilin binding in the mucosa was confirmed by immunohistochemistry with an antibody against the GPR38-A receptor. In addition to the manufacturer's assurance of specificity of the antibody for the human GPR28-A receptor, control experiments in which the antibody was preabsorbed with the blocking peptide confirmed the specificity of the antibody. The staining in smooth muscle and myenteric plexus were completely blocked and the pronounced staining in the mucosa was reduced by half. Control sections in which the primary antibody was replaced by TBS where all negative. It was shown that the motilin receptor was expressed in the epithelial cells of the mucosa and there was a difference between colon and ileum in the fact that in the ileum the top of the villi were negative for the motilin receptor and the crypts stained positive while in colon all epithelial cells stained positive (figure 2). In mucosa of antrum also all epithelial cells stained positive while in duodenum a comparable gradient was seen as in ileum. The smooth muscle of all parts of the intestine stained weakly positive, with staining in the antrum being strongest and in ileum weakest. Within the smooth muscle the expression of the motilin receptor was clearly positive in the myenteric plexus in all parts of the gastrointestinal tract and this staining was stronger than that in the surrounding muscle cells (figure 3).

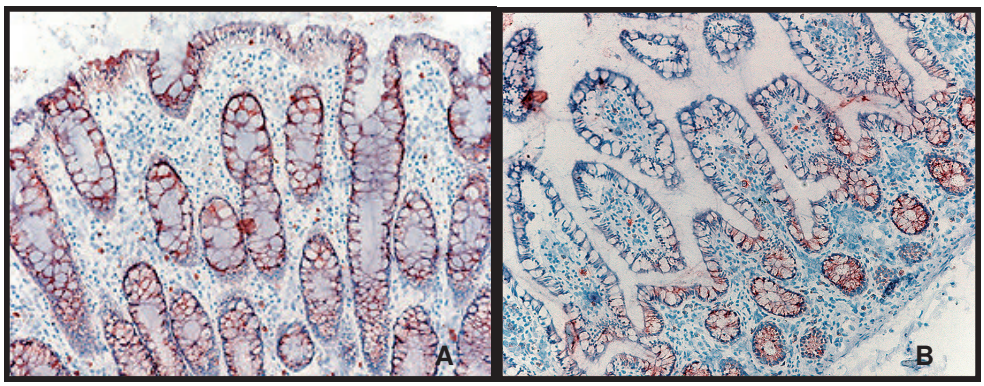

Figure 2. Immunohistochemical Staining (brown/red) for GPR38-A in Control Colonic (A) and Ileal (B) Mucosa. Note that the epithelial expression in ileum is only present in the crypts.

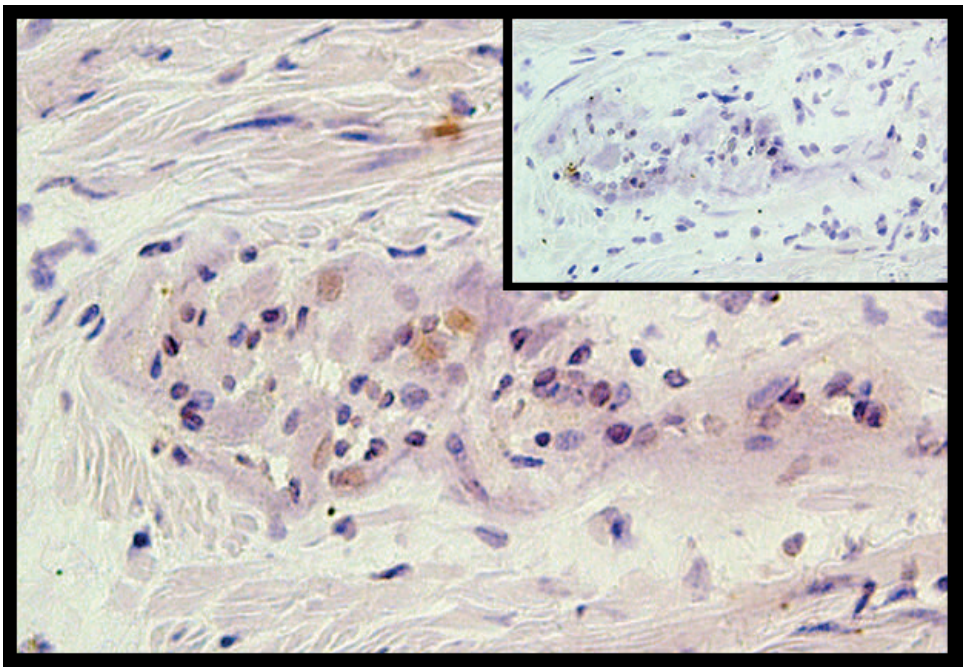

Figure 3. Immunohistochemical Staining (brown/red) for GPR38-A in Control Colonic Myenteric Plexus. The corresponding control where the primary antibody was preabsorbed with the blocking peptide is inserted.

GPR38 mRNA expression

RT-PCR of RNA extracted from the mucosal intestinal tissue samples showed that mRNA of the motilin receptor GPR38 was in detectable levels present in the mucosa of antrum, duodenum, ileum, and colon (figure 4).

Motilin receptor expression in IBD

There was a 20-year age difference between controls and IBD patients. However, we found no correlation between age and motilin receptor expression in the control group, indicating that age does not influence the motilin receptor expression. The MPO assay showed that the classification in macroscopically inflamed and non-inflamed areas correlated well with the degree of neutrophil infiltration ($r = 0.42$, $P=0.009$). MPO values in the inflamed IBD group were significantly higher than

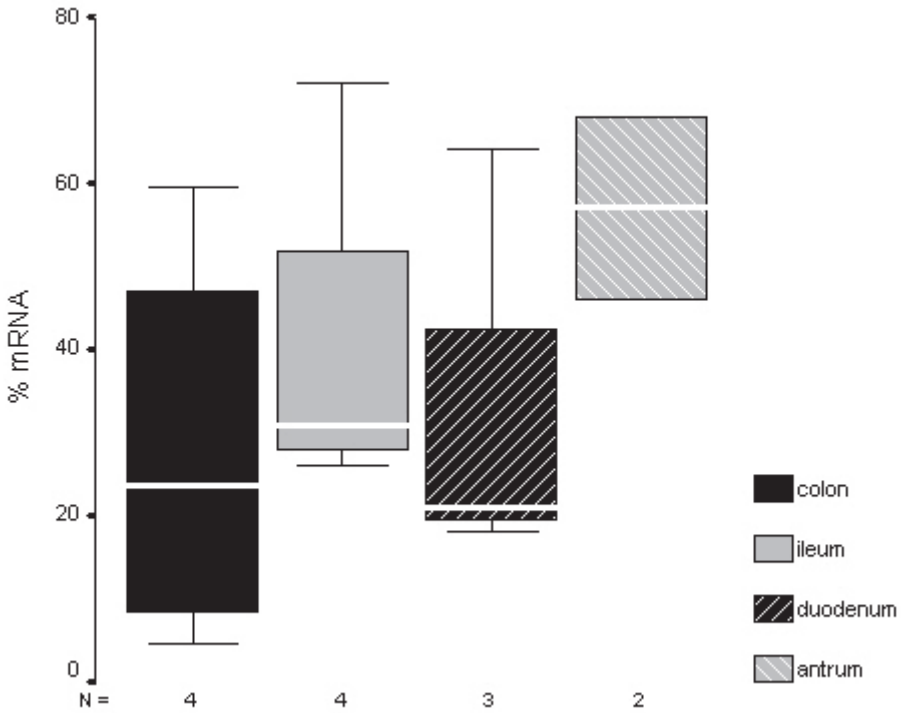

Figure 4. GPR38 mRNA Expression in Control Human Intestinal Mucosa. Data is expressed as a ratio of the amount of mRNA expression in mucosa and TE671 cell line. Data are corrected for β-actin expression. The white line represents the median value and the box 50% of the values.

those in the non-inflamed IBD group (10.0±1.1 versus 5.6±1.8 U/mg tissue; $P<0.05$). Neutrophil infiltration in control tissue was comparable to the non-inflamed IBD group (5.8±0.7 versus 5.6±1.8 U/mg tissue). In UC colonic muscular motilin binding was higher versus controls and lower versus adjacent mucosa (7 versus 3, 7 versus 19 fmol/g, $P\leq0.05$). There was no correlation with the presence/intensity or absence of inflammation. There were no differences in the amount of motilin binding in CD patients compared to controls. In both, the motilin binding was lower in colonic and ileal smooth muscle than adjacent mucosa (colon: 4 versus 9, ileum: 9 versus 23 fmol/g, $P\leq0.02$), with the binding in ileal smooth muscle being significantly higher ($P\leq0.01$) than that in colonic smooth muscle (figure 5). In patients with IBD immunohistochemistry showed that there was no difference in the

localization of the motilin receptor when compared with the control patients. Also RT-PCR showed no differences in GPR38 mRNA expression in mucosal tissue In samples of CD, UC or control patients (figure 6 and 7).

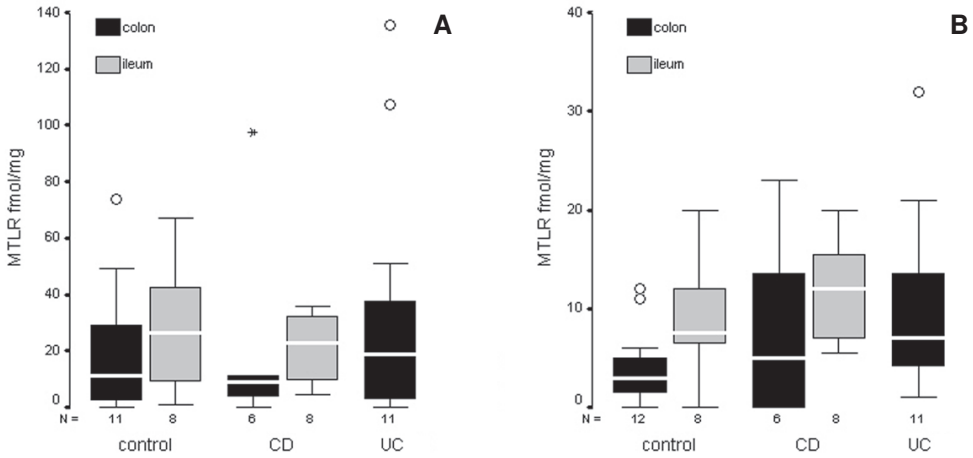

Figure 5. Quantity of ^{125}I-motilin Binding (fmol/g tissue) in Mucosa (A) and Smooth Muscle (B) of Colon and Ileum of IBD Patients and Controls as Measured by Autoradiography. The white line represents the median value and the box 50% of the values.

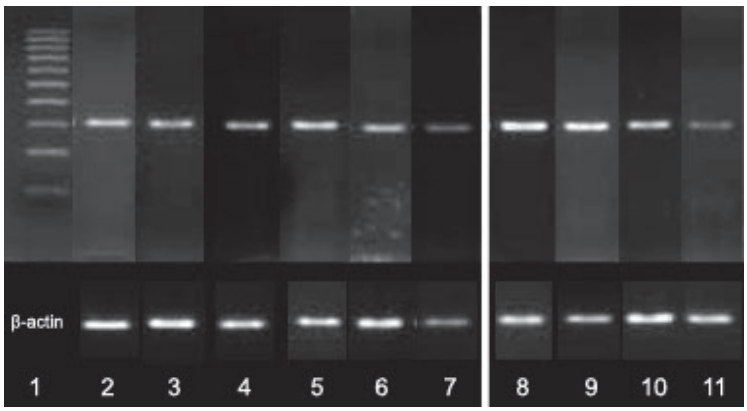

Figure 6. GPR38 mRNA Expression in Colonic and Ileal Mucosa of IBD Patients and Controls. The first lane shows a marker, lane 2 until 7 colonic mucosal tissue and lane 8 till 11 ileal mucosa tissue from controls (2,3,8,9) Crohn's disease patients (4,5,10,11) and ulcerative colitis patients (6,7) respectively. The upper band is GPR38 mRNA (294 kb) and underneath the corresponding β-actin band is inserted.

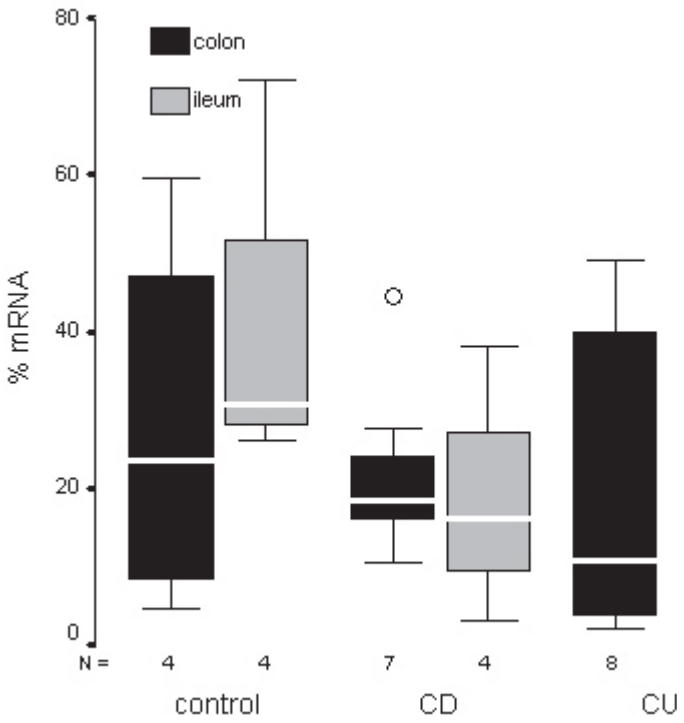

Figure 7. GPR38 mRNA expression in colonic and ileal mucosa of IBD patients and controls. Values are a ratio of the amount of mRNA expression in TE671 cell line. Data are corrected for β-actin expression. The white line represents the median value and the box 50% of the values.

Discussion

Motilin is a peptide that is involved in the intestinal motility but the distribution of the receptor through which motilin exerts its effect is not well know for humans. This study describes the motilin receptor expression in humans as measured by 3 different techniques. The most commonly known function of motilin is the regulation of upper gastrointestinal motility, and indeed most binding sites for motilin are found in the smooth muscle compartment of antrum and duodenum. But this is not the only place were motilin receptors are expressed; we showed that in a lesser extent motilin binding sites are present in colonic and ileal muscular compartment. Earlier, Feighner *et al*. already showed mRNA expression in enteric neurons of colon and ileum [18]. We now show that beside mRNA also the receptor itself with autoradiography and immunohistochemistry. The presence of motilin receptors in colon and ileum smooth muscle and myenteric plexus indicates that motilin beside

its regulatory function in the upper gastrointestinal tract can also be involved in the regulation of lower gastrointestinal motility. Motilin agonists are now used therapeutically to accelerate gastric emptying but the finding of receptors in the lower gastrointestinal tract gives the possibility to study if these compounds are also useful in diseases as slow transit obstipation where the colonic motility is disturbed.

Besides its presence in the smooth muscle the motilin receptor was also expressed in the human intestinal mucosa. Most studies that examined the location of the motilin receptor in humans discarded the mucosa from the smooth muscle. We found that the amount of receptors in the mucosa varies along the gastrointestinal tract. Highest concentrations were seen in duodenum and antrum and lowest in colon. It is not known yet what the function of motilin receptors is in this intestinal compartment. In ileal and duodenal mucosa it was seen that only the crypts stained positive while the top of the villi were negative, while in colon and antrum all epithelial cells stained positive. It could be that motilin plays a role in epithelial secretion and in other epithelial functions. The fact that beside muscular motilin receptor expression the receptor was also in relatively high quantities present in the mucosa of the intestinal tract suggests that motilin receptors might have some other functions apart from its role in the regulation of motility.

Beside motilin receptor expression in normal human intestine we also studied the expression in the intestine of IBD patients. In recent years motilin has been more acknowledged as a neuropeptide and studies in the past have shown that the expression of some neuropeptide receptors are changed in IBD [26]. In experimental colitis studies in rabbits the contractility in response to motilin is decreased due to a decrease in motilin receptor density [20,27], but no studies were done in human intestine. Furthermore, location studies in normal intestine have already shown that there are pronounced interspecies differences. It is therefore important to obtain information about the motilin receptor expression and distribution in human inflamed intestine. This study shows a small increase of motilin receptors in the colonic smooth muscle of UC patients, but no major changes in the motilin receptor expression pattern were seen. There was no relation with the grade of inflammation. This result is in contrast with the above

mentioned study in rabbits, emphasizing the difference between rabbits and humans in case of neuropeptide receptor expression and the importance of such studies in humans.

Overall we can conclude that beside its role in the regulation of the upper gastrointestinal motility, motilin may have a role in the motility regulation of the lower intestinal tract since receptors for motilin are also expressed at that location. Besides the expression of motilin receptors in the smooth muscle, the receptors are also expressed in the mucosa as shown by 3 different techniques. Further studies have to be done to obtain more information about the precise role of the motilin receptors in the mucosa.

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Summarizing Discussion

Neuropeptides

Neuropeptides are signalling peptides that are produced by neural, endocrine and/or immune cells. They exert their effects by binding to G protein-coupled receptors on the target cells where they act as neurotransmitters, paracrine regulators or systemic hormones. These neuropeptides are involved in different human neoplastic and non-neoplastic diseases. For instance, the receptors for somatostatin, gastrin-releasing peptide and substance P are overexpressed in neuroendocrine tumours [1], prostatic carcinomas [2] and rheumatoid arthritis [3] respectively. With the use of radiolabelled peptides these receptors can be applied to in vivo scintigraphy or peptide radiotherapy. These and other neuropeptides, with their receptors, have been suggested to be involved in the pathology of inflammatory bowel disease (IBD), a chronic inflammatory disease of the gastrointestinal tract that is characterized by changes in motility, pain and diarrhoea. Based on clinical-histological and macroscopic features IBD is divided into two entities: Crohn's disease and ulcerative colitis. **Chapter One** of this thesis gives an overview of the role of neuropeptides in the inflammatory process in general and in IBD in particular. The molecules are secreted by nerves of the enteric nervous system and endocrine cells in the mucosa which are both in close proximity to inflammatory cells in the mucosa. These immune cells have receptors for several neuropeptides on their surfaces and a subgroup of these immune cells is itself capable of secreting neuropeptides. Further, the neuropeptides are involved in the regulation of the motility of the intestine and in chloride secretion. The involvement of neuropeptides in the inflammatory response, motility and chloride secretion, three processes that are impaired in IBD, suggests a role for these neuropeptides in the pathology of IBD. Furthermore, there may be a therapeutic and/or diagnostic application for these neuropeptides or their antagonists in IBD. However, before interventions can be made, it is important to obtain more knowledge about the status of the receptors of these neuropeptides in tissue of IBD patients.

Chapter two gives an outline of the various studies performed in thesis.

Techniques

In recent years molecular techniques have become increasingly important in receptor research, but it should be kept in mind that mRNA levels do not necessarily correlate with the number of receptors expressed in the examined tissue. An example of this discrepancy between mRNA levels and receptor expression is shown in **chapter three**. Although autoradiographic binding studies did show an increase of the substance P receptor expression in mucosa of IBD patients, there was no increase in the amount of mRNA in the same tissue. Although molecular techniques also have specific advantages (sensitive, fast, information of the different receptor types), they do not provide information about receptor affinity, specificity and selectivity. Therefore, and for reasons of quantification of receptor protein, radioligand binding studies are still necessary. Even better is a combination of molecular and binding techniques, with additional immunohistochemistry for information about the precise location of the receptors. This will give a complete picture of the neuropeptide receptor status in the tested tissue.

Substance P

Substance P belongs to the family of mammalian tachykinins and is present in the enteric nervous system, where it is an important mediator of neurogenic inflammation. However, the peptide also exerts other pro-inflammatory functions [4]. In **chapter three** the expression of the substance P receptor was studied in patients with IBD using the three above-mentioned techniques. Other studies have already shown the results of one or two techniques, but this is the first time that three complementary techniques describe the receptor status in the same tissue. Autoradiography showed that the number of substance P binding sites in the mucosa of IBD patients was higher (55 compared to 18 fmol/g in control patients) but that the amount of mRNA was not increased. Immunohistochemistry showed that the increased substance P binding to the mucosa of IBD patients is not due to expression of the receptor at new locations, but rather to an increase in the number

of receptors at the normal locations. Further, our data suggest that this increase in substance P receptor expression level is due to an internal pool of receptors or early synthesis of new receptors as the mRNA content is not increased in the inflamed intestine of IBD patients. As a consequence of the increased substance P receptor expression an increased inflammatory activity will probably be seen in response to substance P. Therefore NK-1 receptor antagonist are expected to decrease the substance P mediated immune cell activity, ion secretion, intestinal permeability and colonic motility. Highly selective antagonists for the NK-1 receptor are currently in use in rheumatoid arthritis, depression and anxiety, but the potential beneficial effects of these antagonists in IBD first have to be shown by controlled clinical studies.

Neurotensin

Another neuropeptide receptor studied in this thesis is the receptor for neurotensin, a 13 amino-acid peptide located in the entire gastrointestinal tract, with the highest concentration in the ileum [5]. Neurotensin is involved in several processes in the gastrointestinal tract such as chloride secretion [6], motility [7] and inflammation [8]. Neurotensin acts via three different receptors with the neurotensin receptor-1 being the most important in the gastrointestinal tract [9]. We studied the receptor status of this neuropeptide in IBD and control tissue with the three techniques described above. In **chapter four** the results of storage phosphor autoradiography are given and in **chapter five** a differentiation is made between the various receptor types for neurotensin and the additional PCR and immunohistochemistry data are presented. Autoradiography showed neurotensin binding to colonic and ileal smooth muscle with binding in the colon being higher than in the ileum. Also the mucosa showed neurotensin binding, but the expression level was very low. Further, we found that in the smooth muscle of inflamed tissue of patients with IBD fewer receptors were expressed compared to the non-inflamed regions. In **chapter five** cold saturation studies showed that this expression was due to neurotensin receptor-1 and -3. No neurotensin receptor-2 was found. RT-PCR showed mRNA of the neurotensin receptor-1 and -3 being present in the human intestine. The

mRNA levels of neurotensin receptor-1 and -3 were lower in the inflamed smooth muscle of IBD patients, as was the neurotensin binding described in **chapter four**. Immunohistochemistry showed no changes in the location of the receptors. The decreased binding sites for neurotensin in IBD suggest that the receptor normally has a beneficial effect on inflammatory status of the intestine. This is confirmed by a mice DSS model where administration of a neurotensin receptor-1 antagonist worsened the inflammatory response and injection of neurotensin had a healing effect [10].

Gastrin releasing peptide

The third group of neuropeptide of interest to us was the family of bombesin-like peptides and their receptors. This peptide family has several functions in the gastrointestinal tract, such as the stimulation of endocrine and exocrine secretion, the involvement in smooth muscle contraction, and the regulation of immunological processes [11-13]. Gastrin-releasing peptide and neuromedin B are two members of this family that are present in humans [14]. There are three receptors described for the bombesin-like peptides [15-17] and in **chapter six** we showed that in the human intestine only the gastrin-releasing peptide receptor is expressed. This receptor was most prominently present in the longitudinal muscle (including the myenteric plexus) of the colon and to a lesser degree also in the circular smooth muscle of colon and ileum. However, in the mucosa the gastrin-releasing peptide receptor was only found in low quantities in the colon, but not in the ileum. In patients with Crohn's disease the expression of the gastrin-releasing peptide receptor was less in the colonic smooth muscle. In patients having ulcerative colitis this was not the case. The difference in gastrin-releasing peptide receptor expression between patients with Crohn's disease and ulcerative colitis makes this receptor a possible target to distinguish between the two diseases. Further the decrease of the receptors in Crohn's disease may have a protective effect in these patients on the enhanced motility.

Motilin

In **chapter seven** we described studies on the expression of the motilin receptor. Motilin is a gastrointestinal peptide mainly known for its function in phase III of the migrating motor complex [18]. But motilin also has a functional role in the motility of the lower gastrointestinal tract [19-21]. By using autoradiography we showed that motilin binding sites are present in the smooth muscle of colon and ileum but that their numbers are less than in the upper gastrointestinal tract. Besides the muscular motilin binding, we showed that in the colon and ileum the motilin receptor is also expressed in the mucosa. The precise function of motilin in the mucosa is not yet known, but the confirmation of the presence of the receptor by three different techniques supports the idea that it is present in the mucosa. In patients with IBD we found that the level of expression of the motilin receptor in the mucosa was similar to that in the controls. In the colonic smooth muscle a slight increase in motilin binding was seen in ulcerative colitis patients compared to controls but this was not the case in patients with Crohn's disease. Overall there were no major differences in motilin receptor expression between IBD patients and controls, suggesting that motilin does not play an important role in the inflammatory process in IBD.

Interactions

In summary, this thesis describes the expression of the receptors of the neuropeptides substance P, neurotensin, gastrin-releasing peptide and motilin in control and IBD human tissue. In the normal intestine, receptors for all four peptides are present in the colonic mucosa with the receptors for substance P and motilin in the highest density. In ileal mucosa the gastrin-releasing peptide receptor is not present, while neurotensin binding to ileum is found but less than to colon. In contrast, the expression level of substance P and motilin receptors is higher in ileal mucosa than in colon. In smooth muscle tissue, expression of all four receptors is found. The expression of the motilin receptor, however, is low, with expression in the ileum being a little higher than in colon. For the other three receptors the

binding to the ileum is lower than to the colon. Literature has shown that there are usually interactions between the various peptides. The secretion of one neuropeptide can be stimulated by another neuropeptide, cytokine, and/or hormone. For example, the contractions seen in dog studies in response to bombesin are mediated by substance P [22]. Other studies have shown that bombesin is also able to increase the release of neurotensin and motilin [23,24]. In our study the ileal neurotensin muscular binding was correlated with the amount of bombesin binding in longitudinal and circular smooth muscle ($r = 0.77$ and $r = 0.68$, respectively), supporting the in the literature found connection between bombesin and neurotensin. Further we found that there was an inverse correlation between the binding of substance P to colonic mucosa and neurotensin to colonic smooth muscle ($r = - 0.53$). Thus, the increase in mucosal substance P receptors seen in IBD goes together with the decrease of neurotensin receptors in smooth muscle of IBD. It is known that the proinflammatory cytokines IL-1β, IL-12, IL-18 and TNFα can increase expression of NK-1 receptors via a mechanism involving the transcription nuclear factor kβ (NF-kβ). In the promoter region of the human NK-1 receptor binding sites for NF-kβ are present [25,26]. Further the substance P – NK-1 receptor interaction stimulates COX-2 gene expression. In mice models of experimental colitis it was shown that the COX-2 expression was elevated and that this was normalized by administration of a specific NK-1 receptor antagonist [27]. Also the expression of the neurotensin receptors is linked with NF-kβ and COX-2 expression [10,28]. This same secondary messenger pathway can explain the correlation between the expression of both receptors. However, since neurotensin, substance P and their receptors are localized in the central nervous system as well as along the length of the entire gastrointestinal tract these neuropeptides are able to activate many different cells and pathways and is it difficult to completely elucidate their connection. Beside the knowledge of the expression of these neuropeptide receptors also a better understanding of the cellular and molecular mechanisms involved in neuropeptide signaling pathways are necessary before treatment options can be determined.

Conclusions

In IBD patients, there is an increase in substance P receptors in the mucosa and a decrease in neurotensin and gastrin-releasing peptide receptors in the smooth muscle. The clinical relevance of the increase in substance P receptors and decrease in neurotensin and gastrin-releasing peptide receptors was not studied in this thesis. Additional studies will have to be performed to find out if this different level of expression is due to the inflamed status of the intestine or if it contributes to the inflammation process in the intestine. Overall this thesis contributes to a better understanding of neuropeptide receptor expression in control and IBD human tissue. This could lead to the discovery of new functions for these neuropeptides and it may give new possibilities for diagnostic and therapeutic applications in patients with IBD.

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Samenvatting

Neuropeptiden

Neuropeptiden zijn signaaleiwitten die geproduceerd worden door zenuw, endocriene en/of immuuncellen. Door te binden aan G-eiwit gekoppelde receptoren kunnen zij hun werking uitoefenen. Ze kunnen werken als een neurocriene (in het zenuwstelsel), paracriene (in locale cellen) of hormonale (via het bloed elders in het lichaam) stof. De neuropeptiden zijn naast hun reguliere functies in het menselijk lichaam ook betrokken bij diverse ziektebeelden. Zo zijn bijvoorbeeld het aantal receptoren voor somatostatine, bombesine en substance P verhoogd in respectievelijk neuro-endocriene tumoren [1], prostaat kanker [2] en reumatoïde artritis [3]. Door het gebruik van radioactief gelabelde eiwitten kunnen deze receptoren en daarmee de ziekte zichtbaar gemaakt worden (in vivo scintigrafie) of ze kunnen gebruikt worden voor lokale radiotherapie.

Van deze en andere neuropeptiden en hun receptoren wordt gedacht dat ze ook een rol spelen in de pathologie van inflammatoir darmlijden (IBD), een chronische ontstekingsziekte van het maag-, darmkanaal. Het ziektebeeld van IBD wordt gekarakteriseerd door veranderingen in darmbewegingen (motiliteit), pijn en diarree. Alle drie de functies worden onder andere gereguleerd door neuropeptiden. Op basis van klinische, histologische en macroscopische kenmerken kan IBD in twee ziekten worden onderverdeeld, namelijk de ziekte van Crohn en colitis ulcerosa.

In het **eerste hoofdstuk** van dit proefschrift is een overzicht gegeven van de rol die neuropeptiden in het algemeen en in IBD in het bijzonder, spelen bij het ontstekingsproces. De eiwitten kunnen worden uitgescheiden door de zenuwen van het enterische zenuwstelsel (het zenuwstelsel dat in de darm gelegen is) en door endocriene cellen in de mucosa van het maag- darmkanaal. Zowel de zenuwen als de endocriene cellen zijn dicht gelokaliseerd bij de ontstekingscellen die zich in de darmmucosa bevinden. Op het celoppervlak van deze ontstekingscellen bevinden zich receptoren voor neuropeptiden, hierdoor kunnen de ontstekingscellen door de neuropeptiden geactiveerd kunnen worden. Sommige van deze ontstekingscellen kunnen vervolgens zelf ook weer neuropeptiden uitscheiden. Daarnaast zijn neuropeptiden ook betrokken bij de regulatie van de motiliteit van het maag- darmkanaal en bij chloride secretie (van belang bij het

ontstaan van diarree). De betrokkenheid van de neuropeptiden bij deze drie processen die alle drie verstoord zijn in IBD patiënten, leidt tot de hypothese dat neuropeptiden een rol spelen in de pathologie van IBD. Een betere kennis over mogelijke wijzigingen in het aantal of type receptoren voor de neuropeptiden in patiënten met IBD zou kunnen leiden tot de ontwikkeling van nieuwe diagnostische of therapeutische mogelijkheden. Het is dus van belang dat de kennis over de expressie van deze receptoren bij patiënten met IBD toeneemt.
In het **tweede hoofdstuk** wordt een beschrijving gegeven van de verschillende onderzoeken die zijn uitgevoerd in het kader van dit proefschrift.

Methoden

Voor het onderzoek beschreven in dit proefschrift is een drietal verschillende technieken gebruikt om de expressie van neuropeptide receptoren te bestuderen. In de afgelopen jaren zijn moleculaire technieken (DNA en RNA niveau), waaronder reverse-transcriptase polymerase chain reaction (RT-PCR) steeds belangrijker geworden in het receptor onderzoek. Een nadeel van deze techniek blijft echter dat de hoeveelheid mRNA in een cel niet noodzakelijkerwijs gecorreleerd is met het aantal receptoren dat tot expressie wordt gebracht. Een voorbeeld hiervan wordt gezien in hoofdstuk drie: terwijl autoradiografische bindingsstudies een toename van het aantal substance P receptoren in de mucosa van IBD patiënten liet zien, was er geen toename in de hoeveelheid mRNA in dat weefsel. Dit neemt echter niet weg dat moleculaire technieken hun eigen voordelen hebben, ze zijn erg gevoelig, snel en er kan gemakkelijk onderscheid gemaakt worden tussen de diverse types receptoren voor een neuropeptide. Echter voor informatie over de affiniteit, specificiteit en selectiviteit van een receptor zijn radioligand bindingsstudies (autoradiografie) noodzakelijk. Hierbij wordt radioactief gemerkt neuropeptide bij het weefsel gevoegd zodat het aan de mogelijk aanwezige receptoren voor dit neuropeptide kan binden. Eenmaal gebonden kan het radioactief gemerkte neuropeptide zichtbaar gemaakt worden en kan de hoeveelheid worden geteld. De derde toegepaste techniek is immunohistochemie, hiermee kan met behulp van specifieke antilichamen onder de microscoop

bekeken worden wat de exacte locatie (welk celtype) van de receptoren in het weefsel is. Door de drie hierboven beschreven technieken te combineren is het mogelijk om een totaalbeeld van de neuropeptide receptor status in het onderzochte weefsel te krijgen.

Substance P

Substance P behoort tot de tachykinine familie, voor deze groep neuropeptiden zijn een drietal receptoren beschreven [4]. Substance P is onder andere aanwezig in het enterische zenuwstelsel en is daar een belangrijke mediator van de neurogene ontsteking, daarnaast heeft het ook nog een aantal andere ontstekings-bevorderende functies [5]. In **hoofdstuk drie** wordt de expressie van de substance P receptor (NK-1R) bij patiënten met IBD bestudeerd met behulp van de hierboven genoemde technieken. Andere onderzoeken hebben al wel eens de resultaten laten zien van één of twee van de beschreven technieken maar dit is voor het eerst dat de drie elkaar aanvullende technieken samen worden gebruikt in hetzelfde weefsel. Met de autoradiografie hebben wij laten zien dat de hoeveelheid substance P die aan de mucosa van IBD patiënten bindt hoger is dan de hoeveelheid in het controle weefsel (55 versus 18 fmol/g). De RT-PCR toonde echter aan dat de hoeveelheid NK-1R mRNA niet was toegenomen. Immunohistochemie liet zien dat de toegenomen binding van substance P in de mucosa het gevolg is van een toegenomen hoeveelheid binding op de bekende locaties en niet doordat de receptor ook op nieuwe plaatsen tot expressie wordt gebracht. Verder kan uit deze gegevens geconcludeerd worden dat de toename van de gemeten substance P receptoren afkomstig is van een interne voorraad van receptoren omdat de hoeveelheid mRNA, de bron voor nieuwe eiwitten, gelijk is gebleven in de ontstoken darm van IBD patiënten. Het verhoogde aantal substance P receptoren in IBD patiënten kan leiden tot een sterkere ontstekingsreactie zodra er substance P vrij komt. Dit leidt dan ook tot de hypothese dat NK-1 antagonisten een verlaging van de door substance P gemedieerde ontstekingsprocessen (geactiveerde immuuncellen, ion-secretie, verhoogde darmpermeabiliteit en motiliteit) tot gevolg heeft. Selectieve

antagonisten voor de NK-1 receptor worden nu al toegepast bij ziekten als reumatoïde artritis, depressie en angsten. Of deze antagonisten ook een positieve invloed zullen hebben bij IBD patiënten zal in gecontroleerde klinische studies bestudeerd moeten worden.

Neurotensine

Een andere neuropeptide receptor die beschreven wordt in dit proefschrift is de neurotensine receptor. Neurotensine is een eiwit van 13 aminozuren dat in het gehele maag- darmkanaal voorkomt maar met name in het ileum [6]. Neurotensine is betrokken bij de regulatie van diverse processen in de darm waaronder chloride secretie [7], beweging [8] en ontsteking [9]. Neurotensine kan zijn effect uitoefenen door te binden aan drie verschillende receptoren, maar de neurotensine receptor-1 is de belangrijkste in het maag- darmkanaal [10]. In de onderzoeken, die beschreven worden in dit proefschrift, is het voorkomen van de neurotensine receptoren bestudeerd in de darm van IBD- en controle patiënten. In **hoofdstuk vier** worden de resultaten van de autoradiografie gegeven en in hoofdstuk vijf wordt een onderscheid gemaakt tussen de drie verschillende typen neurotensine receptoren met behulp van de technieken RT-PCR en immunohistochemie. De autoradiografie liet neurotensine binding zien in de spier van de dikke darm (colon) en in iets mindere mate ook in de spier van het laatste deel van de dunne darm (ileum). Tevens werd in de mucosa neurotensine binding gedetecteerd maar deze hoeveelheid was zeer klein. Tenslotte werd nog gevonden dat in de ontstoken spier van IBD patiënten minder neurotensine receptoren tot expressie komen dan in de niet ontstoken delen. In **hoofdstuk vijf** werd vervolgens met behulp van verdringingsproeven aangetoond dat de in hoofdstuk vier gedetecteerde binding het gevolg is van de aanwezigheid van de neurotensine receptor-1 en -3. Er is geen neurotensine receptor-2 gevonden. Met RT-PCR werd het mRNA van de neurotensine receptor-1 en -3 aangetoond in de humane darm. De hoeveelheid mRNA in de ontstoken darm van IBD patiënten was lager dan in de niet ontstoken darm, wat in overeenstemming is met de afgenomen neurotensine binding in de ontstoken darm van IBD patiënten zoals beschreven in hoofdstuk vier.

Immunohistochemie liet geen verandering zien in de lokalisatie van de neurotensine receptoren in het weefsel van IBD patiënten in vergelijking met de controles. De afname van het aantal bindingsplaatsen voor neurotensine in het ontstoken weefsel van IBD patiënten suggereert dat de receptor normaal een beschermend effect heeft op de darm. Dit wordt bevestigd door een muizenmodel waarin de toediening van een neurotensine receptor-1 antagonist leidde tot een verslechtering van de geïnitieerde ontsteking en waar de toediening van neurotensine juist een helend effect had [11].

Gastrine releasing peptide

Het derde neuropeptide waarvan de receptor expressie bestudeerd werd maakt deel uit van de bombesine-familie. Deze familie oefent diverse functies uit in het maag- darmkanaal waaronder de stimulatie van endocriene en exocriene secretie, spiercontracties en in de regulatie van het immuunsysteem [12-14]. Gastrine-releasing peptide en neuromedin B zijn twee leden van deze familie die bij de mens voorkomen [15]. Er zijn drie receptoren voor deze groep van neuropeptiden beschreven [16-18] en in **hoofdstuk zes** laten we zien dat in de humane darm alleen de gastrine-releasing peptide receptor tot expressie komt. Deze receptor komt met name voor in de longitudinale spier en myenterische plexus van het colon en in mindere mate ook in de circulaire spier van colon en ileum. In de mucosa werd alleen in het colon een zeer lage hoeveelheid gastrine-releasing peptide receptor aangetroffen en niet in het ileum. Bij IBD patiënten werd een verschil gezien tussen patiënten met de ziekte van Crohn, bij wie het aantal receptoren in de spier van het colon is afgenomen, en patiënten met colitis ulcerosa, waar dit ongewijzigd was. Door dit verschil zou de gastrine-releasing peptide receptor eventueel gebruikt kunnen worden om beide ziektebeelden van elkaar te onderscheiden. De afname van gastrine-releasing peptide receptoren die gezien wordt in patiënten met de ziekte van Crohn kan een beschermingsmechanisme zijn van deze groep tegen de verstoorde motiliteit gezien in IBD.

Motiline

In **hoofdstuk zeven** is het onderzoek naar de expressie van de motiline receptoren beschreven. Motiline is een maag- darm eiwit dat met name bekend is door zijn rol in fase III van het migrerende motor complex [19]. Maar motiline heeft ook een rol in de motiliteit van de rest van het maag- darmkanaal [20-22]. Met behulp van autoradiografie hebben we in dit proefschrift aangetoond dat er motiline bindingsplaatsen aanwezig zijn in de spier van het colon en ileum, alhoewel de hoeveelheid lager is dan in het eerste deel van het maag- darmkanaal. Naast de binding in de spier hebben we ook laten zien dat de receptor voorkomt in de mucosa. De exacte functie van motiline receptoren in de mucosa is nog niet bekend, maar daar ze met drie verschillende technieken zijn aangetoond staat hun aanwezigheid niet meer ter discussie. Bij patiënten met IBD is de mucosale motiline receptor expressie onveranderd. In de spier van het colon werd een geringe toename gezien van de motiline binding bij patiënten met colitis ulcerosa maar niet bij patiënten met de ziekte van Crohn. Maar echt grote veranderingen in de motiline receptor expressie in IBD patiënten in vergelijking met controles werden er niet gezien, hetgeen suggereert dat motiline waarschijnlijk geen heel belangrijke rol speelt in het ontstekingsproces van IBD.

Interacties

Samengevat, dit proefschrift beschrijft de expressie van de receptoren van de neuropeptiden substance P, neurotensine, gastrine-releasing peptide en motiline in weefsel van controle en IBD patiënten. In de mucosa van controle colon worden de receptoren van alle vier de neuropeptiden tot expressie gebracht, met de receptoren van substance P en motiline in de hoogste concentraties. In de mucosa van het ileum wordt de gastrine-releasing peptide receptor niet tot expressie gebracht, de neurotensine receptor is wel detecteerbaar maar de hoeveelheid is minder dan in het colon. Dit is in tegenstelling tot de receptoren van substance P en motiline want die worden juist in de mucosa van het ileum meer tot expressie gebracht dan in het colon. In de spier komen ook de receptoren van alle vier de

neuropeptiden voor, maar de hoeveelheid motiline receptoren is laag (ileum iets hoger dan het colon). Van de receptoren voor de andere drie neuropeptiden is de binding in het ileum lager dan de binding die in het colon gezien wordt.
Uit de literatuur is bekend dat er vaak interacties optreden tussen diverse eiwitten. De secretie van het ene neuropeptide kan gestimuleerd worden door een ander neuropeptide, cytokine of hormoon. Bijvoorbeeld, in honden worden darm-contracties gezien na stimulatie met bombesine, het werkinginsmechanisme van bombesine blijkt echter via substance P te lopen [23]. Andere studies hebben laten zien dat bombesine ook in staat is om de secretie van neurotensine en motiline te verhogen [24,25]. In onze studie was de neurotensine binding in het ileum gecorreleerd met de hoeveelheid bombesine binding in de longitudinale en circulaire spier (r= 0.77 en r = 0.68), dit ondersteunt de in de literatuur beschreven connectie tussen bombesine en neurotensine. Verder vonden wij ook dat in het colon er een negatief verband is tussen de binding van substance P in de mucosa en neurotensine in de spier (r = -0.53). Oftewel de toename van substance P receptoren in de mucosa van de IBD patiënt gaat gepaard met een afname van de neurotensine receptoren in de spier van diezelfde patiënt. Het is bekend dat de ontstekingsbevorderende cytokines IL-1β, IL-12, IL-18 en TNFα het aantal NK-1 receptoren kunnen laten toenemen via een mechanisme waarbij ook de transcriptie factor NF-kβ betrokken is [26,27]. Daarnaast stimuleert de interactie van substance P met zijn receptor de expressie van COX-2. In een ontstekingsmodel in muizen heeft men laten zien dat de verhoging van COX-2 gezien bij ontstekingen te niet kon worden gedaan door de toediening van een specifieke NK-1 receptor antagonist [28]. Ook de expressie van neurotensine receptoren is gekoppeld met de expressie van de signaaleiwitten NF-kβ en COX-2 [11,29]. Deze gezamenlijke regulatie mechanismen kunnen een verklaring zijn voor de correlatie tussen de expressie van beide receptoren. Maar de grote diversiteit aan plaatsen waar beide receptoren in de mens voorkomen alsmede het grote aantal cellen waarop zij invloed kunnen uitoefenen maakt het ingewikkeld om een dergelijke interactie te beschrijven. Er is dan ook meer kennis van dergelijke mechanismen nodig voordat nieuwe therapieën onderzocht kunnen worden.

Conclusies

Bij patiënten met IBD is er sprake van een toegenomen substance P receptor expressie in de mucosa en een afname in neurotensine en gastrine-releasing peptide (alleen bij Crohn patiënten) receptoren in de spier. De klinische relevantie van deze toename in substance P receptoren en afname in neurotensine en gastrine-releasing peptide receptoren is niet bestudeerd in dit proefschrift. Aanvullende studies zullen uitgevoerd moeten worden om te bekijken of deze veranderingen het gevolg zijn van het ontstekingsproces in de darm van IBD patiënten of dat de veranderde expressie juist het ontstekingsproces in de darm verergert. Echter dit proefschrift beoogt een betere kennis van neuropeptide receptor expressie in darmweefsel van IBD- en controle patiënten. Deze kennis kan gebruikt worden bij het zoeken naar mogelijkheden voor het gebruik van deze neuropeptiden in nieuwe diagnostische testen en in de therapie van patiënten met IBD.

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Nawoord

Het ligt er dan eindelijk: **mijn proefschrift!** Gedurende het traject heb ik mij wel eens afgevraagd of ik het ooit nog af zou krijgen, maar iedere keer vond ik weer de tijd en motivatie om door te gaan. En nu kan ik met trots zeggen dat het gelukt is.

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Pascale ter Beek

Curriculum Vitae

De auteur van dit proefschrift werd op 13 augustus 1974 geboren in Almelo. Zij behaalde in 1992 haar VWO diploma aan het Pius X College te Almelo. In datzelfde jaar begon zij met de opleiding Gezondheidswetenschappen aan de Universiteit Maastricht. Tijdens haar studie heeft zij 10 maanden stage gelopen bij de vakgroep moleculaire celbiologie en genetica van de Universiteit Maastricht alwaar ze onder begeleiding van Dr. J.L.V. Broers onderzoek deed naar de expressie van A- en B-type lamines tijdens proliferatie en differentiatie in de HL-60 cellijn. Eind 1997 werd het doctoraal diploma met als afstudeerrichting Biologische Gezondheidskunde behaald. In september 1998 is zij als assistent in opleiding begonnen bij de vakgroep Maag-, darm- leverziekten van het Leids Universitair Medisch Centrum. Onder verantwoordelijkheid van Prof. dr. C.B.H.W. Lamers en Dr. ir. I. Biemond heeft zij gedurende 5 jaar onderzoek gedaan naar de neuropeptide receptor expressie bij patiënten met inflammatoir darmlijden, hetgeen geresulteerd heeft in een aantal publicaties en de totstandkoming van dit proefschrift. Aansluitend heeft zij ruim een jaar onderzoek gedaan naar de ontwikkeling van een muizenmodel voor myasthenia gravis bij de vakgroep Neurofysiologie van het Leids Universitair Medisch Centrum onder leiding van Dr. P.C. Molenaar. Tegenwoordig is zij als pharmacovigilance officer werkzaam bij sanofi-aventis.