SOMATOSTATIN AND INTESTINAL INFLAMMATION

SOMATOSTATINE EN INTESTINALE ONTSTEKING

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PROEFSCHRIFT

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Binnen een paar dagen was zijn jongensdroom om heroïeke daden tot zijn devies te maken vervlogen, en van Pater Emanuele had hij begrepen dat het zaak was warm te lopen voor Heroïeke Deviezen – en dat men zich, in plaats van een leven lang te strijden tegen een reus, beter een lang leven kon wijden aan het op vele manieren benoemen van een dwerg.

Umberto Eco, *Het eiland van de vorige dag*

Voor Jacqueline,

Thomas, Jorne en Stijn

Voor mijn ouders

CONTENTS

CHAPTER 1

INTRODUCTION TO SOMATOSTATIN IN INTESTINAL INFLAMMATION

Introduction

Since Dr Smethurst in 1859 was prosecuted for poisoning Mrs Banks, who apparently suffered from Crohn's disease[1], there have been many different theories about the nature of inflammatory bowel disease (IBD). The concept of idiopathic ulcerative colitis in the late 19th century[2] and its differentiation from idiopathic regional ileitis, later known as Crohn's disease[3], only appeared in the last hundred years. Much has still to be learned about the etiology and pathogenesis of these diseases. Before 1970, genetic factors or hypersensitivity to certain unknown micro-organisms or toxic agents were postulated[4], and these concepts have not as yet been refuted[5-8].

TABLE 1.1 Clinical presentation of acute IBD. Signs and symptoms depend on the bowel part involved. In Crohn's disease, lesions may be found in the entire digestive tract from mouth to anus. Ulcerative colitis only involves colonic segments.

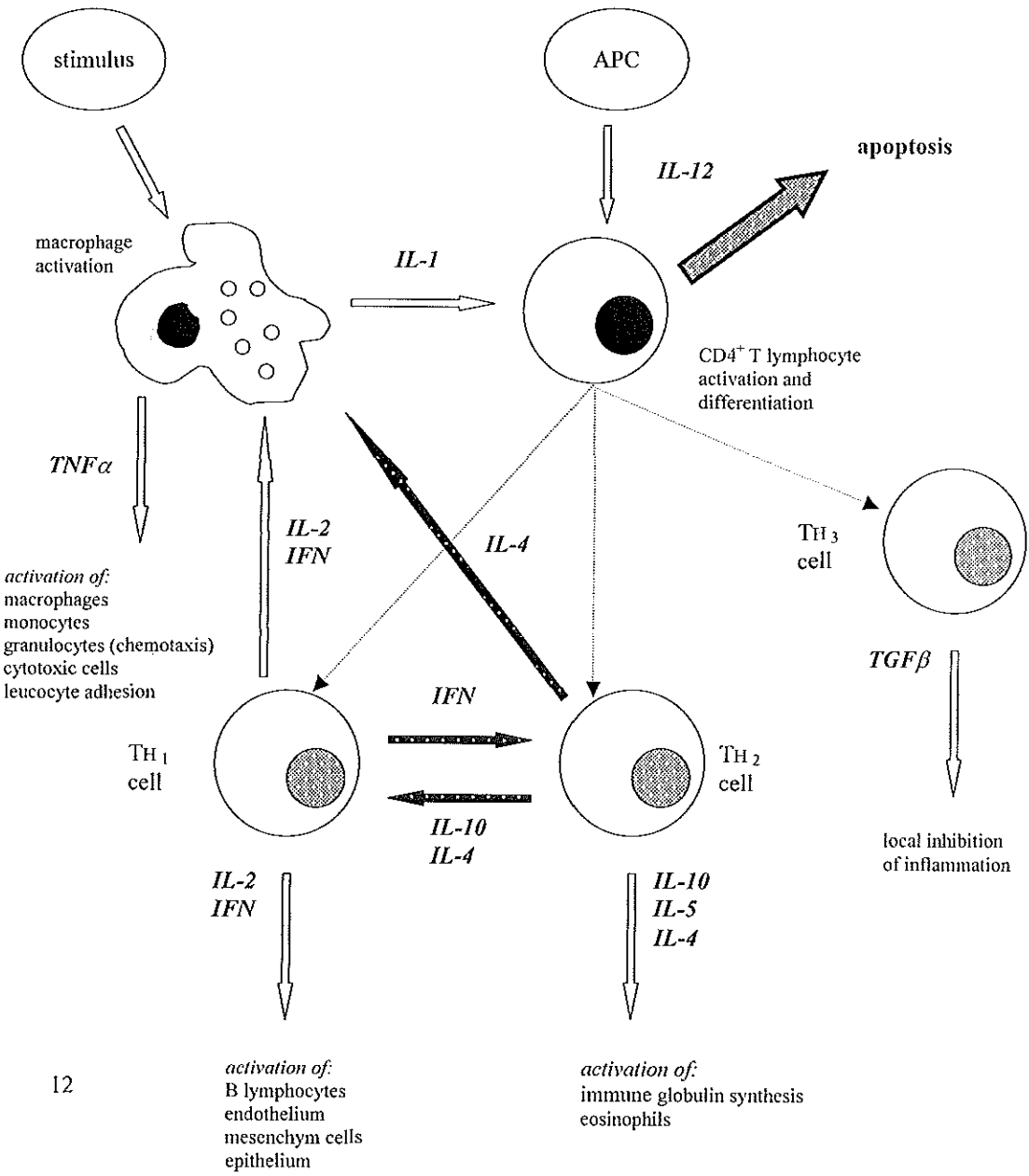
Crohn's disease	Ulcerative colitis
abdominal pain	diarrhoea
diarrhoea, usually non-bloody	rectal frequency
steatorrhoea	gross blood in stool
weight loss, anorexia, malaise	passage of mucopus
enteric fistula, abdominal mass	abdominal pain, rectal cramps
extra-intestinal symptoms:	extra-intestinal symptoms:
Fever	fever
arthralgia / arthritis	arthralgia / arthritis
erythema nodosum	pyoderma gangrenosum
pyoderma gangrenosum	iritis / uveitis
stomatitis aphthosa	
anal fissure, fistula or abscess	
iritis / uveitis	
chronic fatigue	
jaundice	

In the last two decades, research has concentrated on elements of the intestinal immune system in the pathogenesis of IBD.

Despite different clinical and histological manifestations, ulcerative colitis and Crohn's disease have many common features (TABLE 1.1). In both disorders intestinal damage appears to be immunologically mediated[9,10]. An inflammatory cascade is initiated by unknown stimuli which activate macrophages and intraepithelial T-lymphocytes (FIGURE 1.1)[11,12]. The immune reaction is augmented by recruitment of other inflammatory cells and production of cytokines and other mediators including eicosanoids, adhesion molecules and chemokines[13-18]. Due to an unknown disturbance in the immune response in IBD patients these inflammatory reactions do not subside, but result in sustained mucosal injury. A pro-inflammatory role has been attributed to the T-helper lymphocyte, as its depletion prevents IBD exacerbation[19]. A reduced suppressor T-cell proliferation in IBD mucosa may also contribute to this ongoing intestinal inflammation[20].

Although much is known about the clinical and epidemiological characteristics and the sequelae of intestinal inflammation and immune cascades, many pathogenic aspects of inflammatory bowel disease are still unidentified. As mentioned above, much attention has been paid to the role of leukocytes, cytokines and secondary inflammatory mediators. None of these factors seems to be of sole importance. Apart from activity of the primary immune cells, other constituents of the intestinal wall contribute to inflammation. Recent research has focussed on the interaction between the immune system and mucosal epithelial cells, vascular endothelium, intestinal smooth muscle and nerve cells[21,22]. Intestinal smooth muscle cells are capable of cytokine production[23], and play an important role in fibrosis and stricture formation, and in the disturbed gut motility, resulting in diarrhoea and abdominal cramps.

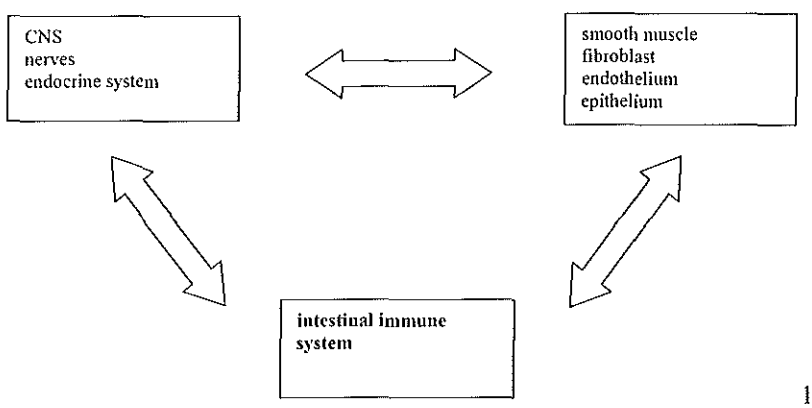
FIGURE 1.1 Schematic overview of immunologic events in mucosal inflammation in IBD: unknown stimuli activate intestinal mucosal macrophages, which produce several cytokines. Interleukin-1 (IL-1) stimulates T-helper (TH)-lymphocyte activity and differentiation into TH_1 and TH_2 cells. In addition, T-helper lymphocyte stimulation occurs through antigen presenting cells (APC), mediated by IL-12. Positive and negative feedback mechanisms from TH cell subsets regulate immunocyte activity and extent of inflammation. These autoregulatory circuits are disturbed in IBD, resulting in perpetuating inflammation. Balance between apoptosis and differentiation is important for feedback in the inflammatory cascade.
Modified after: Kirsner JB, Shorter RG (eds.) *Inflammatory bowel disease*. Williams & Wilkins, Baltimore, 1995.



The nervous system also has an impact on intestinal inflammation (FIGURE 1.2). Since the beginning of the 20th century, several reports on influence of central and peripheral nervous system on inflammatory processes have appeared[24]. The interaction of brain, neuroendocrine system (hypothalamus, pituitary gland, adrenals) and immune system is well known in several disorders like asthma and sepsis[25-27].

The neuroimmune relationship has also been studied in several models of intestinal inflammation[25,28-30]. Most evidence for neuropeptide control of intestinal immunity evolves from indirect arguments, like the proximity of fine, neuropeptide-containing nervous filaments near the intestinal immune system[29,31]. Conflicting results emerge from in vitro studies. This is illustrated by intestinal wall substance P receptor density, which has been reported to be increased in IBD[32,33], whereas the number of substance P and vasoactive intestinal peptide containing nerves is decreased in colonic mucosa sections with active inflammation[34]. The level of substance P and calcitonin gene-related peptide in intestinal mucosa and muscle layers drops during the course of experimental colitis[35]. Pro- as well as anti-inflammatory actions of neuropeptides have been described[30]. In addition, intestinal inflammatory cells are capable of producing neuropeptides for local immunoregulatory activity[36].

FIGURE 1.2 Neuroinflammation. Relationship between nervous and endocrine system, immune system and effector cells. Communication (double arrow) through neurotransmittors, neuropeptides, hormones, cytokines, eicosanoids and other soluble mediators.



Somatostatin (SMS) is one of the commonest neuropeptides. Most of the body SMS content is stored in the digestive tract[37]. SMS exerts inhibitory actions on pancreas and intestinal secretion, gut motility and splanchnic bloodflow[38]. Moreover, SMS has several immune regulatory properties and may have a beneficial effect on intestinal inflammation[39,40]. Currently, this key inhibitory neuropeptide and its long-acting analogue octreotide are applied as therapeutic agents in several intestinal diseases, like variceal bleeding of the oesophagus, intractable diarrhoea and intestinal fistulae[41,42]. However, despite some optimistic auguries, their role in the management of inflammatory bowel disease still has to be defined[43,44].

Aim of the study

The research described in this thesis is aimed at examining the role of somatostatin (SMS) in several aspects of intestinal inflammatory processes and its possible therapeutic use. The literature on SMS involvement in intestinal inflammation is reviewed in chapter 2.

The main reason to start this series of studies was a report of beneficial effects of SMS on acute acetic acid-induced colitis in rats[39]. In view of the potential clinical advantages of SMS over the available drugs for patients with corticosteroid-resistant severe colitis, we decided to perform simultaneously a clinical therapeutic trial and some animal experiments, rather than to wait for the results of our experimental studies prior to clinical trials. There is an extensive experience with SMS and its analogue octreotide in clinical disorders, and these drugs have a remarkable lack of side effects. We studied the influence of SMS and its long-acting analogue octreotide on acute and non-acute inflammation in another model of murine colitis (dextran sulphate sodium-induced)[45], expecting

to find inhibition of mucosal inflammatory changes and diminished mucosal concentrations of proinflammatory cytokines (Ch. 3 and 4).

SMS is at present used in the management of diarrhoeal and motility disorders of different causes[46,47]. As disturbed motility occurs in intestinal inflammation, an in vitro study was performed on the reactivity of colon smooth muscles in murine dextran sulphate sodium-induced colitis. This study was aimed at detecting increased susceptibility of colon smooth muscle to prokinetic agents during inflammation and a possible inhibiting effect of SMS (Ch. 5).

Gut wall SMS receptor density has been reported to be increased in IBD[48]. We performed octreotide scintigraphy in active IBD in order to visualise these receptors in vivo. A new immunohistochemic technique was applied to intestinal specimens to localise these receptors in vitro (Ch. 6).

Based on the reported immune inhibitory effects of SMS and the beneficial effects on experimental intestinal inflammation[39,40], a clinical application for octreotide was sought in a multicenter trial in active ulcerative colitis (Ch. 7).

All results are discussed in the general discussion of the thesis and perspective for the relevance of this research is given (Ch. 8).

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CHAPTER 2

SOMATOSTATIN IN INFLAMMATORY BOWEL DISEASE

J.D.van Bergeijk, J.H.P.Wilson

In adapted form from Mediators of Inflammation 1997;6:303-9

Abstract

Various immunomodulating cells, interacting by molecular mediators control intestinal inflammation. Neuropeptides, released by enteric nerve cells and neuroendocrine mucosa cells, are able to affect several aspects of the general and intestinal immune system, with both pro- as well as anti-inflammatory activities. In inflammatory bowel disease (IBD) there is both morphological as well as experimental evidence for involvement of neuropeptides in the pathogenesis. Somatostatin is the main inhibitory peptide in inflammatory processes, and its possible role in IBD is discussed.

Introduction

Ulcerative colitis and Crohn's disease are both characterised by chronic, relapsing intestinal inflammation. The etiology of both forms of inflammatory bowel disease (IBD) is still unknown, despite intensive research in two main areas - abnormal regulation of the local immune response and exogenous factors, including infectious agents. The gastrointestinal immune system protects the organism against toxins, micro-organisms and dietary antigens within the gut lumen. It is generally assumed that luminal stimuli presented to intestinal mucosa activate intra-epithelial immunocytes resulting in release of mediators of inflammation. In IBD an inflammatory cascade is initiated and, due to insufficient or inappropriate immune reactions, intestinal damage results[1,2]. The reasons for inappropriate immune activation are unknown. Central roles are currently ascribed in this process to activated intestinal macrophages and T-lymphocytes[2] in combination with an imbalance between pro- and contra-inflammatory T-cells[3-5]. Treatment of IBD is aimed at reducing the production or action of mediators of inflammation (TABLE 2.1).

Delicate interactions of intestinal mucosal epithelium, smooth muscle cells, gut wall blood vessel endothelium, immunocytes and enteric nerve cells contribute to and regulate intestinal inflammatory changes. These processes are mediated by various chemical messengers, most of which are produced by lymphocytes, granulocytes and macrophages. Recent studies on etiopathogenesis and treatment in IBD have concentrated on these immunocyte products which include cytokines, eicosanoids and adhesion molecules. The contribution of other elements of the gastrointestinal immunological defence system to chronic intestinal inflammation is less well known. A potentially interesting area is the immunoregulatory role of the enteric nerves and neuroendocrine cells. Neuropeptides, like substance P (SP), somatostatin (SMS), vasoactive intestinal peptide (VIP) and calcitonin gene related peptide (CGRP), are the molecular

mediators of neuroregulation of the intestinal immune system, providing for interactions between nervous system and immunocytes. SMS is a key inhibiting factor of many biological processes. In this review the role of SMS as neuroimmune modulator in IBD will be highlighted, together with its possible future use in the treatment of IBD.

TABLE 2.1 Examples of drugs used in treatment of IBD.

aminosalicylates	• sulfasalazine • 5-aminosalicylic acid
corticosteroids	• old: prednisone / prednisolone • new: budesonide / beclomethasone
immunomodulatory agents	• azathioprine / 6-mercaptopurine • cyclosporine • FK506 • methotrexate
immunomodulatory agents, experimental use	• anti-tumour necrosis factorα • interleukin-10
common drugs under investigation	• antibiotics • heparin • transdermal nicotine • lidocaine

Neuroinflammation, neuropeptides and intestinal inflammation

The concept of neuroimmune interaction is in part derived from older clinical observation that inflammatory processes are influenced by emotional or physical stress. The immune system is subject to central nervous control and Pavlovian responses[6]. Although IBD patients do not have more emotional difficulties or

psychosocial stress compared to a normal population, disease activity and response to therapy are certainly influenced by the state of mental well-being[7]. The interaction between nervous system and the intestinal immune system is probably mediated by neuropeptides derived from enteric nerves and neuroendocrine cells[8-11].

Membrane-bound neuropeptide receptors are found on several immunocytes, including T-lymphocytes and monocytes[6,9,12-16]. Various neuropeptides affect intestinal lymphocyte function and several cells of the intestinal immune system also produce neuropeptides, suggesting a local immunoregulatory task[17-20]. Migration of immunocytes into the intestinal mucosa is affected by neuropeptides[21].

In addition there are several morphological arguments that suggest neuropeptide involvement in intestinal mucosal immunity. Intestinal mucosa contains SP, VIP and SMS immunoreactive nerve fibres and co-localisation is common[22,23]. Neuropeptides show a specific distribution along the gut. Transmural distribution can be different for different neuropeptides[24-27].

The reported studies of neuropeptide immunomodulation in the intestine need to be interpreted with some caution. Receptor binding studies often show unexpected concentration relationships and depend strongly on the local conditions[28-34]. Autoradiography is a semiquantitative approach and sampling error in taking mucosal biopsies or loss of neuropeptide containing cells by inflammatory damage, may account for some of the confusing results. Structural and species specific neuropeptide receptor heterogeneity may further complicate comparison of results[35-38]. Neuropeptides are known to exert different effects at different sites. For instance skin mast cells are susceptible to SMS, whereas SMS does not induce histamine release from intestinal mast cells[39].

Neuropeptides may be pro- or anti-inflammatory. Generally, substance P (SP) has a pro-inflammatory action. Macrophage interleukin-1 (IL-1) production and cytotoxicity is stimulated by SP[20,40] as is IL-1, tumour necrosis factor (TNF)-α and IL-6 release by human monocytes[31]. SP stimulates proliferation of and immunoglobulin synthesis by lymphocytes from spleen, mesenteric lymph nodes and Peyer's patches[28]. Moreover, in experimental infection with *Schistosoma mansoni* SP induces interferon (IFN)-γ production by granuloma macrophages[41]. However, a dose-dependent inhibition of immunoglobulin production of murine intestinal granuloma derived B-cells has been described[42]. Some of these pro-inflammatory effects of SP are opposed by SMS. SMS inhibits macrophage SP-induced IFNγ release[41]. SP-induced chemotaxis of neutrophils can be completely reversed by the SMS analogue octreotide[43].

In mucosal lymphocytes from resected human colon segments, DNA synthesis as measured by ^{3}H-thymidine uptake, is inhibited by low concentrations of SMS, VIP, SP and bombesin[44]. This inhibition might be principally achieved by T-cell suppression, as observed in lymphocytes that are stimulated by concanavalin A. The maximum inhibitory effects are obtained after 4 days of neuropeptide incubation[44]. VIP and SMS inhibit lymphocytic proliferation in a dose-dependent fashion[17,28,29,45]. The inhibitory effects of VIP and SMS on these lymphocytes are mediated by specific receptors, not by cytotoxicity[46-48].

VIP has both pro- and anti-inflammatory effects on intestinal T-cells and macrophages, inducing enhanced IL-5 release and inhibiting macrophage adherence[49,50]. T-cell cAMP increases on stimulation by VIP, but proliferation is inhibited[38,51]. However, reactivity of granuloma B-cells and macrophages is not affected[38,42].

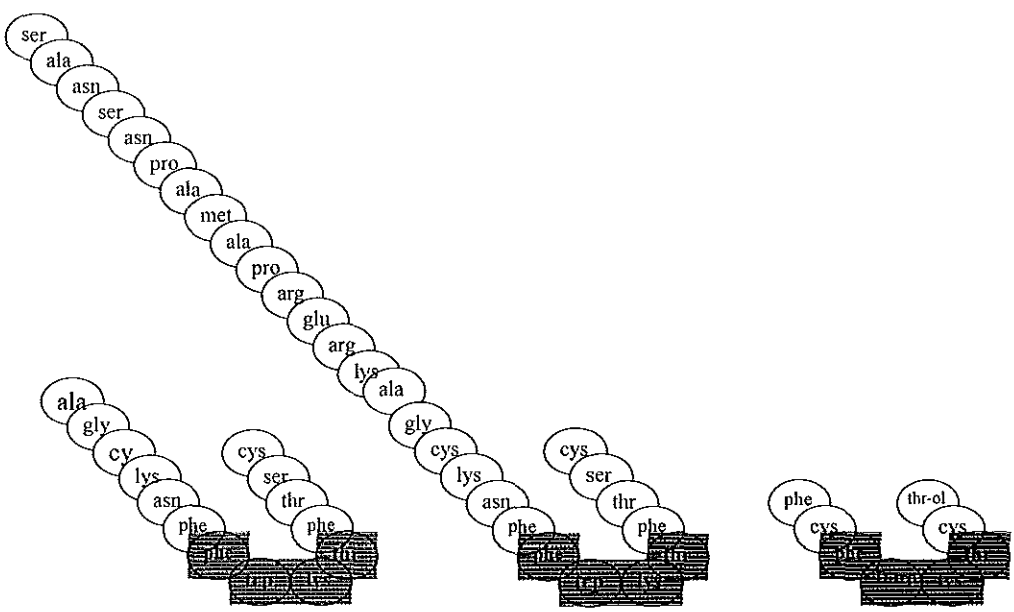
Conflicting results emerge from studies in IBD patients. SP content of inflamed colon is increased, but there is a substantial overlap with normal SP content[52,53].

SP receptor upgrading is observed in inflamed areas of IBD colon[54-57]. SP receptor expression in ulcerative colitis enteric nerves has been found to be normal[57] or increased[58]. For VIP the observations are even more confusing. Whereas VIP concentration in plasma of patients with active IBD is increased[59], colon VIP content is reported to be either decreased[53,54,60] or increased[55,61]. There is no clear relation between VIP content and disease activity.

Somatostatin

SMS was first extracted from ovine hypothalamus as an inhibitor of growth hormone secretion[62]. SMS is a peptide hormone. There are two biologically active forms, consisting of 14 or 28 amino acids respectively (FIGURE 2.1).

FIGURE 2.1 Schematic amino acid composition of somatostatin and octreotide. Shaded areas are essential for receptor binding.



Somatostatin-14 Somatostatin-28 Octreotide

Five types of SMS receptors have been discovered, each with specific binding characteristics for SMS subtypes and different SMS analogues[68]. SMS receptors are found in upper and lower parts of the normal digestive tract[66].

SMS is an inhibitor of several key functions in the body. SMS inhibits acid secretion, intestinal fluid absorption, intestinal and pancreas secretion. SMS has effects on splanchnic blood flow and gastric and intestinal motility[66]. SMS exerts its inhibitory effects by diminishing intracellular cAMP through G-protein activation (FIGURE 2.2). In addition, SMS impedes cellular influx of calcium. This impediment results from a direct effect on calcium channels and from increase of potassium conductance with subsequent cellular hyperpolarisation[64]. Circulating native SMS has a short half-life time. Long acting analogues like the decapeptide octreotide have been developed[69] and SMS analogues have been used to treat intractable diarrhoea, bleeding from oesophageal varices in portal hypertension, dumping syndrome and gastrointestinal fistulae[70-74]. Radioactive labelled SMS analogues serve as diagnostic tools in visualisation of gastrointestinal neuroendocrine tumours[75].

Immunomodulatory effects of somatostatin

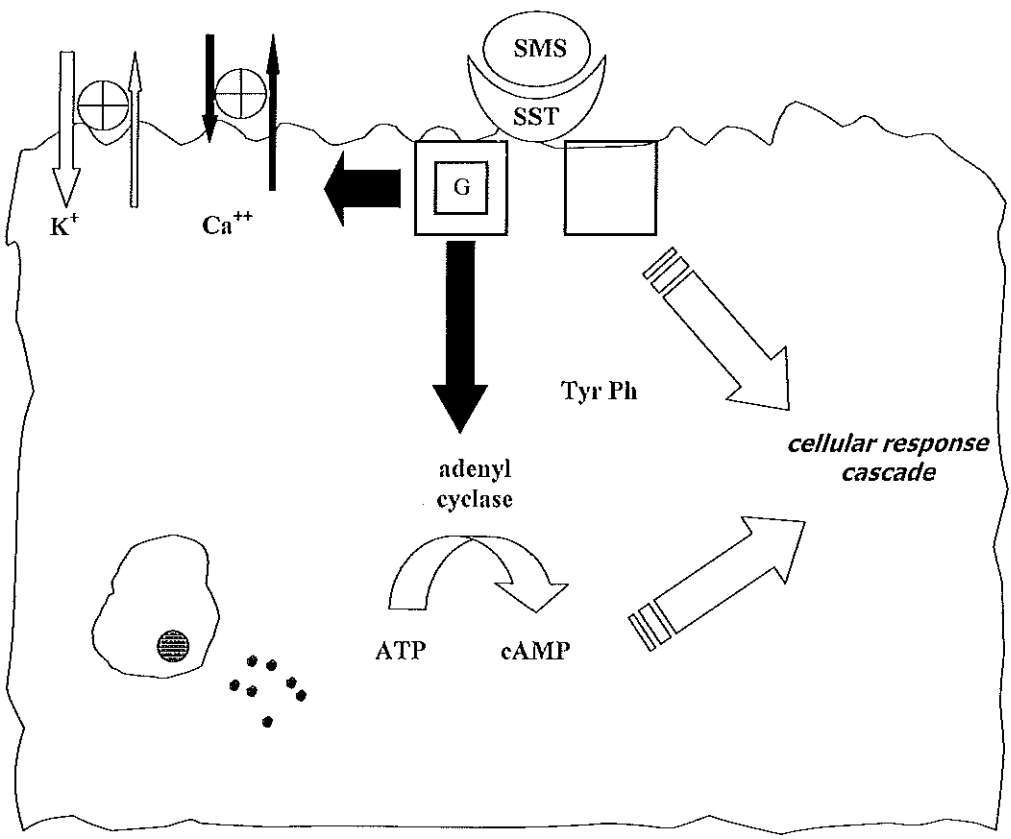
Studies on the immunomodulatory effects of SMS show several effects on T- and B-lymphocytes and macrophages. SMS receptors exist in spleen, liver, thymus and gastrointestinal lymphoid tissue, as well as on various immunocytes[12,15,76]. Immunomodulating actions of SMS were discovered as result of its antagonising effects of proliferation of rat lymphocytes, stimulated by hypothalamic extracts[77]. SMS inhibits responsiveness, immunoglobulin synthesis and proliferation of lymphocytes and granulocytes[15,45,46,78-80]. It reduces TNF release and cellular toxicity of stimulated rat peritoneal macrophages[30,81]. Inhibitory effects depend on local SMS concentration. Several *in vitro* studies show inhibition of

proliferation of lymphoid cells at low SMS concentrations and stimulation at high levels[46,77]. In an experimental model of intestinal inflammation with *Schistosoma mansoni*, SMS as well as its analogue octreotide decrease T-cell interferon production significantly[82].

Some studies report several immunostimulating effects by SMS. T-cell proliferation is seen, also at low SMS concentrations[83,84]. In rat peritoneal macrophages SMS stimulates cytotoxic reactivity, when given in low concentrations[81]. T-cell activation in a hybridoma T-lymphocyte cell line, measured by Il-2 release, is stimulated by SMS in a dose-dependent way[17]. However, IL-2 receptor expression is inhibited by SMS in human intestinal lymphocytes[47,48].

SMS controls inflammatory processes in *in vivo* experimental models. A reduction of inflammatory infiltrate and a diminished TNFα production occurs when SMS analogues are applied to animals in which carrageen-induced skin inflammation is established[85]. In this experiment intense SMS immunostaining was seen on leukocytes at peri-inflammatory sites. SMS reduces inflammation in experimental arthritis and ileal obstruction[86,87]. Similar beneficial effects on intestinal and colonic inflammation emerge from clinical observations in Crohn's disease and gold-induced enteritis[88,89]. SMS is able to reduce SP mediated inflammation induced by intestinal infection with *Trichinella spiralis*[90] and SP enhanced neutrophil chemotaxis[43].

FIGURE 2.2 Main cellular effects of somatostatin. Somatostatin receptors (SST) are cell membrane bound. After binding somatostatin (SMS) G-proteins (G) are activated, through which adenylcyclase is inhibited (dark arrow), and calcium influx into the cell is hampered. Potassium channels are opened, and potassium influx increases. Decreased activity of adenylcyclase leads to lower cyclic AMP (cAMP) levels, which also lowers intracellular calcium concentration. In smooth muscle cells relaxation is induced through this pathway. SMS also activates G-protein independent enzymes, like tyrosine phosphatase (Tyr Ph).
After: Law SF, Woulfe D, Reisine T. Somatostatin receptor activation of cellular effects. *Cellular Signalling* 1995;7:1-8.



Somatostatin in IBD

No systematic *in vivo* or *in vitro* studies on effects of SMS in IBD are available at present. Support for SMS induced immunomodulation in IBD is indirect and derived from morphological and biochemical analyses of intestinal tissue and blood specimens from IBD patients. From several studies a correlation between SMS activity and presence and intestinal inflammation emerges.

When measured in serum total body SMS release shows a circadian rhythm[91,92]. In active ulcerative colitis a higher 24-hour amplitude, higher average serum levels and a longer meal-stimulated peak level are observed[92,93]. As the serum concentration is only a faint mirror of the mucosal events, the impact of increased secretion of SMS is obscure. Same response patterns are seen in patients with duodenal ulcer or irritable bowel syndrome[93].

SMS containing cells and submucosal ganglion cells in surgical specimen from IBD patients can be visualised by immunohistochemical staining and quantified by counting the SMS containing cells per mm. Mucosal SMS content can be assessed by radioimmunoassay of homogenised biopsy specimen. In normal colon mucosa, SMS containing endocrine cells show the highest density in the distal parts. In active IBD this distinct difference disappears. Studies prior to the discovery of SMS showed a decrease of neuroendocrine enterochromaffin cells in diseased rectum of ulcerative colitis patients[94,95]. In ulcerative colitis there is an actual decrease in SMS containing cells, especially in the distal part of the colon[96-99]. Although these changes may be secondary to inflammatory damage to mucosal SMS containing cells, this is refuted by the fact that other mucosal neuropeptides like SP show increased levels in these cases[98]. SMS containing submucosal ganglion cells are evenly distributed along the colon in normals and IBD patients, but the number of these cells is decreased in IBD[97]. In colon epithelial cell cultures from patients with active ulcerative colitis, decreased SMS

generation is observed. This loss of SMS production correlates with disease activity[100].

In Crohn's disease the loss of SMS containing colonic cells is less evident. A tendency towards decrease of SMS positive cells with increasing disease activity has been reported[97]. No difference of SMS content is seen in mucosa or normal ileum and terminal ileitis[98]. Mucosal biopsies from inflamed jejunum in Crohn's disease show normal levels of SMS, but soluble SMS is increased[101]. This may reflect instability of SMS storage granules due to inflammation.

Several arguments for SMS involvement in mucosal inflammation emerge from scintigraphic studies. An increased density of SMS receptors is found in areas of granulomatous inflammation like tuberculosis, sarcoidosis and Wegener's granulomatosus[102-104]. From SMS receptor measurements in granulomas of murine intestinal *Schistosoma mansoni* infestation emerge the same results[82].

High-affinity SMS receptors are found in normal jejunum, ileum and colon[105]. Apart from presence in colon mucosa and nerve plexus, SMS receptors are found in the germinal centres of colonic lymph follicles. SMS receptors are seen in gut associated lymphatic tissue, like palatine tonsils, Peyer's patches, vermicular appendix and isolated lymphatic follicles in the colon[106] and SMS is isolated from nervous tissue in Peyer's patches[107]. The precise function of SMS in this gut-associated lymphoid tissue has not yet been settled. High receptor density is seen in the luminal parts of secondary lymph follicles, but they are absent from the corona of B-lymphoid cells. Lymphoid aggregates without a germinal centre do not show receptor activity[106]. Interaction of SMS and lymphocytes from these germinal centres is reasonable. As these receptors have high affinity for SMS, a specific immunomodulatory role of SMS is anticipated. Other indications for a direct influence of SMS on intestinal immunocytes can be derived from electron microscopic studies of the gut wall. SMS containing enteric nerve fibres are

present in a very high density near intestinal lymph follicles, coming close to follicular lymphocytes[107].

Inhibitory effects of SMS on vascular cell proliferation have been described[108]. Expression of high affinity SMS receptors is seen in intramural veins of inflamed intestinal mucosa in IBD or peri-inflammatory veins in rheumatoid arthritis[109,110]. No difference is seen in SMS receptor content of normal and inflamed tissue in mucosa, nerve plexus or lymphatic follicles. Precise cellular localisation of the SMS receptors is not possible from autoradiography, due to insufficient resolution of this visual technique. Histologically these receptor positive veins are normal, but the surrounding tissue often is infiltrated by leukocytes and receptor positivity is correlated with IBD activity[109]. However, expression of SMS receptors in vessel walls could be a non-specific response to inflammation as this phenomenon is also seen in peritumoral tissues in resected SMS receptor negative colon adenocarcinoma and other malignancies[111,112].

In animal experimental models of inflammatory bowel disease there are several suggestions of a role of SMS in the inflammatory mucosal responses. In murine experimental colitis SMS prevents mucosal damage effectively, especially when given before the introduction of the toxin[113]. Parallel to decrease of mucosal lesions a decrease of inflammatory mediators such as leukotriene B4 and platelet activating factor is seen.

Interleukin-2 receptor expression and DNA synthesis in intestinal lamina propria lymphocytes (LPL) is reduced by SMS[44,47,48]. Proliferation of Peyer's patch derived lymphocytes is inhibited by SMS. Inhibitory effects of SMS on lymphocytic proliferation are more pronounced in intestinal derived T-cells than in spleen lymphocytes[114]. Affinity of lamina propria lymphocytes for SMS-binding was found to be 1000 times higher than peripheral blood lymphocytes in one study[48].

However, conflicting results emerge from a study in which effects on human peripheral blood lymphocytes and intestinal LPL were compared. The effects of SMS on intestinal LPL were minimal. Although both lymphoid cells expressed high affinity SMS receptors, intestinal lymphoid proliferation was poorly inhibited by SMS. Immunoglobulin synthesis was affected in a dose-related way in both peripheral and intestinal lymphocytes[48]. The fact that these results run counter to earlier reported data, may be due to species differences and the lack of SMS receptor subtyping.

Intestinal granulomas induced by *Schistosoma mansoni* are smaller in presence of SMS. Their output of IFNγ and immunoglobulin is diminished by SMS. As these same granulomas are also capable of producing SMS, this suggests a local immunoregulatory role for SMS[19,82,115]. This is supported by the interesting observation that intra-epithelial lymphoid cells can stimulate isolated intestinal epithelium to produce SMS[116].

Conclusion

From morphological studies it is clear that neuropeptides are probably involved in the control of the intestinal immune system. Immune stimulatory and inhibitory effects emerge from various *in vitro* studies and from sparse *in vivo* experiments. SMS has been shown to inhibit immunological processes at various sites following different stimuli. As SMS receptors show a high density in the gastrointestinal associated lymphoid tissue and in inflammatory granulomas in murine *Schistosoma mansoni* infestation, it seems likely that SMS and its receptors play a role in immunological events of the digestive tract. Mucosal SMS content is reduced in active IBD. The beneficial effects of SMS and SMS analogues on experimental inflammation of skin, joints and intestine and the few

reports on open studies in patients with IBD suggest that SMS and SMS analogues could possibly be beneficial in IBD and should stimulate further clinical studies.

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CHAPTER 3

SOMATOSTATIN PREVENTS MUCOSAL INFLAMMATION IN ACUTE DEXTRAN SULPHATE SODIUM-INDUCED COLITIS

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Submitted

Abstract

Somatostatin (SMS) is a potent immune inhibitory neuropeptide, able to reduce acetic acid-induced acute colon damage in rats. We studied the proposed anti-inflammatory effect of somatostatin in acute dextran sulphate sodium (DSS)-induced colitis. This model is characterised by neutrophil infiltration of colon mucosal and submucosal regions.

Osmotic pumps containing saline or somatostatin were subcutaneously inserted in 4 groups of BALB/c mice. Somatostatin was released at 120 μg per day (6 μg/g mouse/day). At day 5 of the study two groups (one saline, one somatostatin) were exposed to DSS 10% (w/v) in their drinking water. Two days afterwards all mice were sacrificed and small bowel, colon, thymus and spleen were removed for cytokine analysis. Colon and ileum specimens were examined microscopically.

DSS induced a mild, superficial colitis. Somatostatin effectively reduced intestinal neutrophil infiltration, without changing macroscopic disease activity. Interleukin (IL)-6 levels were decreased in DSS treated animals, irrespective of somatostatin administration. A role of macrophages in this colitis model is suggested. Other cytokines (IL-1β, IL-2, IL-10, interferon γ) in colon, thymus and spleen specimens were equal in all four groups, indicating that intestinal or peripheral blood lymphocytes are not involved in the early phase of DSS-induced colitis, nor in the anti-inflammatory effect of somatostatin.

We conclude that somatostatin prevents DSS-induced colitis effectively, by an as yet undefined mechanism.

Introduction

Several neuropeptides are morphologically and functionally related to the intestinal immune system. Whereas most neuropeptides have pro-inflammatory properties, somatostatin (somatotropin release inhibiting factor, SMS) exerts mainly immune inhibitory effects[1]. SMS reduces intestinal lymphocyte immunoglobulin synthesis and proliferation, and also peritoneal macrophage activity[2-6]. Carrageenin-induced inflammation in rats is attenuated by SMS, irrespective of local or systemic administration[7]. In human disease beneficial effects of SMS in rheumatoid arthritis and in thymoma have been reported[8,9]. The anti-inflammatory role of SMS is disputed by failing to improve *E.coli* lipopolysaccharide-induced inflammation in mice[10]. Octreotide is a long-acting, synthetic SMS analogue. When given prior to induction of pancreatitis octreotide improves mortality, yet without changing the inflammatory response histologically[11]. Moreover, immune inhibitory effects may aggravate inflammation as T cell activity in response to parasite infestation is restrained by SMS[12]. The SMS analogue octreotide prevents acute mucosal damage by acetic acid, that was accompanied by a reduction of mucosal SMS content[13]. Acetic acid induces colitis by epithelial damage. Dextran sulphate sodium (DSS)-induced colitis in rodents is a very reproducible experimental model of colitis that has several similarities with human inflammatory bowel disease. DSS changes intestinal macrophage activity as well as intestinal microflora[14,15]. When administered repetitively, DSS causes a chronic colitis, which is not seen in acetic acid-induced colitis. Although T-lymphocytes are activated during DSS exposure, colitis may be induced in T cell depleted animals[16]. In our colitis model we investigated the potential beneficial effect of SMS, continuously released from a subcutaneous pump in mice. We expected a reduction of mucosal leukocyte infiltration and intestinal proinflammatory cytokines.

Methods

All studies were approved by the Erasmus University Ethical Committee for Animal Experiments. Osmotic pumps (Alzet, Germany, type 1007D) were implanted during ether anaesthesia in the nuchal subcutis in four groups of 8 female young adult BALB/c mice. Pumps were filled with either saline (groups A and B) or SMS-14 (groups C and D). SMS-14 (Somatofalk®) was obtained from Tramedico, Weesp, the Netherlands. The pumps released SMS in a continuous daily dose of 120 μg. At day five after pump implantation groups B and D were exposed to DSS 10 % (w/v, MW > 500 kDa, Pharmacia, Sweden) in drinking water, to which they had free access. After obtaining a macroscopic disease activity index (see below) at day seven all animals were killed by cervical dislocation after an overnight fast. The small bowel, colon, thymus and spleen were removed immediately. After careful cleansing, the colon was divided into three equal parts. From each part and from the terminal ileum specimens were fixated in formaldehyde, processed and embedded in paraffin wax for histological evaluation. Sections were stained with a standard haematoxylin and eosin staining. After measuring tissue specimen weights the rest of the colon, approximately 2 cm of terminal ileum, thymus and spleen were placed in a standard Krebs' buffer (pH 7.4) containing in mmol/L: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2 \cdot 2H_2O$, 1.2 $MgSO_4 \cdot 7H_2O$, 25 $NaHCO_3$, 1.2 KH_2PO_4 and 8.2 D-glucose. After homogenising (Ultra-Turrax, Polytron, Switzerland) specimens were centrifuged during 10 min. at 2800 x g, 4 °C. The supernatants were kept at -20 °C until analysis. Cytokine ELISA's were performed using commercial antibody pairs (Biosource, Belgium) for specific mouse interleukin (IL)-1β, IL-2, IL-6, IL-10, and interferon (IFN)-γ. ELISA's were performed according to the manufacturer's instructions.

All experiments and analyses were performed in a blinded manner. Cytokine values are given in pg per mg wet tissue (mean ± standard error).

Macroscopic evaluation included body weight, stool consistency, rectal bleeding, appearance of hair, liveliness, colon colour and distension, and serosal thickening (maximum total score 10 points). Colon and ileum inflammation was scored histologically according to Okayasu: 0 = no inflammation; 1 = focal neutrophil infiltration; 2 = diffuse infiltration, crypt abscesses, goblet depletion; 3 = ulceration (15). Histology scores of proximal, middle and distal colon segments were added and the average of all scores was taken as the final histology score. Statistical analysis was performed by an unpaired Student's t-test for cytokine results and histological scores were compared using a Wilcoxon rank sum test for independent samples (statistical significance: $p \leq 0.05$).

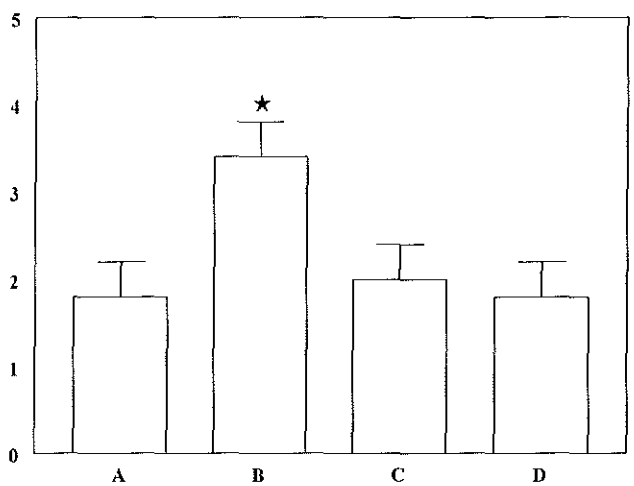

FIGURE 3.1 Histology according to Okayasu criteria (see text and ref. 15), mean ± SEM.
A = control; B = DSS; C = SMS; D = SMS / DSS.
B statistically different from A, C and D (* $p < 0.01$)

Results

Macroscopy/histology

All mice were sacrificed at day 7 between 9 and 10.00 am. Macroscopic scores were significantly higher ($p < 0.001$) in DSS treated groups receiving saline (2.5 ± 0.4) or SMS (2.5 ± 0.4) compared to controls (0.1 ± 0) and SMS alone (0.1 ± 0). Group B, not receiving SMS, had developed a mild colitis, involving mucosal and submucosal layers (score 3.4 ± 0.4) significantly different from controls (1.8 ± 0.4) and SMS treated groups (2.0 ± 0.3 and 1.8 ± 0.4 respectively). SMS treated groups did not differ from controls (FIGURE 3.1). All ileal specimens were normal (score = 0).

Colon

IL-6 levels were significantly lower in DSS-receiving groups irrespective of SMS administration (FIGURE 3.2). Other cytokine values are depicted in TABLE 3.1. IL-1β levels in SMS/DSS treated animals were lower than in all other groups, though statistical significance was not reached. Also IL-2 levels in DSS and SMS/DSS groups were lower than controls (NS). IL-10 concentration in DSS and SMS/DSS receiving mice was lower than controls (NS). IFN-γ was barely detectable in all colon specimens.

TABLE 3.1 Cytokines in colon homogenates; only statistical significant difference in IL-6 levels. Mean ± standard error (pg/mg tissue)

	control	DSS	SMS	SMS / DSS
IL-1β	120 ± 38	107 ± 49	180 ± 60	60 ± 23
IL-2	110 ± 19	53 ± 28	138 ± 11	83 ± 21
IL-10	165 ± 41	93 ± 17	153 ± 43	117 ± 27
IL-6	315 ± 68	112 ± 24*	357 ± 90	90 ± 23*
IFNγ	4 ± 2	3 ± 2	6 ± 3	7 ± 3

* $p < 0.05$ compared to control

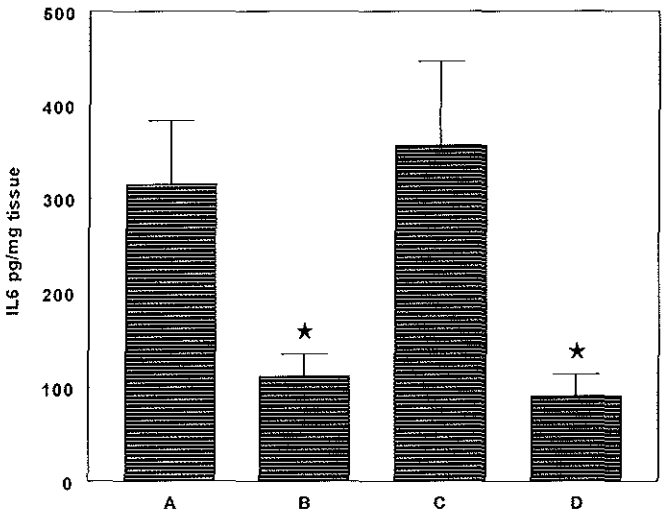

FIGURE 3.2 Interleukin-6 (IL-6) in pg per mg colon tissue (see table 1), mean ± SEM. A = control; B = DSS; C = SMS; D = SMS / DSS. Levels equal in B and D, but in both groups statistically different from A and C (* $p < 0.05$).

Thymus and spleen

Although IL-1β, IL-2, IL-6 and IL-10 levels in SMS treated animals were higher than in the other groups, no statistical significance was reached (TABLE 3.2). IFNγ was hardly detectable (data not shown). No statistical significant differences in cytokine levels were found in spleen tissue (TABLE 3.3). SMS groups tended to lower IL-2 levels (NS). IFNγ was not detectable.

TABLE 3.2 Cytokines in total thymus homogenates; no statistical differences
Mean ± standard error (pg/mg tissue). ND = not detectable.

	control	DSS	SMS	SMS / DSS
IL-1β	13 ± 2	20 ± 5	31 ± 13	23 ± 4
IL-2	10 ± 2	17 ± 2	32 ± 20	15 ± 3
IL-6	55 ± 11	58 ± 19	178 ± 126	59 ± 21
IL-10	25 ± 5	29 ± 8	55 ± 27	37 ± 9
IFNγ	ND	ND	ND	ND

TABLE 3.3 Cytokines in total spleen homogenates; no statistical differences
Mean ± standard error (pg/mg tissue). ND = not detectable.

	control	DSS	SMS	SMS / DSS
IL-1β	6 ± 1	8 ± 2	5 ± 2	9 ± 3
IL-2	4 ± 1	5 ± 2	3 ± 1	5 ± 1
IL-6	10 ± 4	5 ± 2	8 ± 1	5 ± 3
IL-10	8 ± 3	5 ± 2	6 ± 1	6 ± 2
IFNγ	ND	ND	ND	ND

Discussion

High molecular DSS is able to induce an acute, superficial colitis in mice within two days of exposure. DSS induces neutrophil infiltration of mucosal and submucosal areas. In this early phase of inflammation, DSS disables mucosal macrophage phagocytose function after cellular uptake by these cells[15,17]. This enables intraluminal anaerobes to invade the intestinal mucosa and induce colitis[14]. As inflammation lasts after discontinuing DSS, other mechanisms must also be involved. Direct toxicity to intestinal epithelial cells and interference with leukocyte adhesion is suggested[18]. Although DSS influences activity of T- and B-lymphocytes *in vitro*[19-21], lymphocytes do not seem essential for the induction of DSS colitis[22,23]. On the other hand, T lymphocyte derived cytokines are found in mouse colon mucosa by immunohistochemic techniques in chronic DSS-induced colitis[24]. In our study the mucosal IL-6 levels were lower in DSS treated animals. As IL-6 is a mainly macrophage derived cytokine, this drop of IL-6 content may reflect diminished macrophage activity. Olson et al. found unchanged plasma IL-6 concentrations in mice after two days of DSS exposure, but mucosal IL-6 content was not measured[25].

Marked amounts of colon neutrophils were found in DSS-induced colitis. SMS prevented this neutrophil infiltration effectively. The influence of SMS on intestinal leukocytes may be more distinct in the early phase of their activation[4].

SMS did not influence macroscopic disease activity, as the macroscopy score in both DSS receiving groups did not differ. In this stage SMS did not reduce diarrhoea or bloodloss. In this early DSS-induced inflammatory state, although statistically different from controls, animals did not have severe disease (mean macroscopy score 2.5 on a 10 point scale). In acetic acid induced colitis however, octreotide pre-treatment diminished diarrhoea, parallel to a reduction of mucosal damage[13]. In our study SMS prevented inflammation when administered 5 days before exposure to DSS. This is in line with the results of the study of Eliakim et al., in which a marked inhibition of acetic acid-induced inflammation occurred in rats that were pre-treated with octreotide[13].

The mode of action of mucosa protection by SMS to DSS is still speculative. A direct inhibition of SMS on neutrophil influx may be anticipated, but such an effect is not supported by other studies. Octreotide did not influence endotoxin-induced impairment of leukocyte migration[10], and high doses SMS may even stimulate leukocyte movement *in vitro*[26,27]. An inhibition of lymphocyte activity as stated before[2-5] is not likely. IL-1β, IL-2, IL-10 and IFNγ were similar in all study groups, suggesting that lymphocytes be not involved in this phase of DSS-induced colitis as reported by others[22]. Influence from peripheral lymphocytes is not very feasible, as DSS did not affect the cytokine levels of spleen and thymus homogenates. SMS may hamper bacterial invasion of the mucosa in the early phase of DSS-exposure. However, in a study of bacterial translocation in mice, octreotide reduced the beneficial effects of a high fibre diet on the bacterial migration[28].

Colon macrophages phagocytose DSS, which disturbs their function in the early phase of DSS-induced colitis[14,15,17], probably reflected by the low colon IL-6 levels we found. As a marked reduction of macrophage phagocytotic response by SMS is reported[6], this may result in diminished DSS uptake by macrophages. This is not supported by the low IL-6 levels in SMS/DSS treated animals,

suggesting that DSS still has a chance to affect macrophage activity. However, we did not study the chemoattractive activity of macrophages, which may be diminished by SMS[29].

In both acetic acid- and DSS-induced colitis the enteric nervous system is involved in the protection against these toxins[30,31]. Impaired neuron function in these models promotes mucosal neutrophil influx. DSS induces nerve cell hyperplasia in the distal colon, with increase of pro-inflammatory neuropeptides like substance P and neuropeptide Y[32]. As SMS is a well-known substance P antagonist and inhibits several immunological effects of this proinflammatory neuropeptide[33,34], it seems likely that this may diminish DSS-induced colon damage.

In conclusion, in our animal model, SMS prevented DSS-induced colitis effectively. Characteristically, it affected DSS-induced neutrophil infiltration in colon mucosa and submucosa. This anti-inflammatory effect of SMS still has to be settled. Lymphocyte inhibition seems not involved. Probably SMS inhibits macrophage chemoattractive activity or SMS interferes with proinflammatory neuropeptides or protects the enteric nerve system.

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CHAPTER 4

SOMATOSTATIN DOES NOT ATTENUATE INTESTINAL INJURY IN DEXTRAN SULPHATE SODIUM-INDUCED SUBACUTE COLITIS

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Abstract

Acute colitis induced by acetic acid in rats is attenuated by the long-acting somatostatin (SMS) analogue octreotide. We studied the potential beneficial effect of SMS on non-acute experimental colitis. BALB/c mice received either saline, SMS-14 (36 or 120 μg daily) or octreotide (3 μg daily) subcutaneously delivered by implant osmotic pumps. A non-acute colitis was induced by administration of dextran sulphate sodium (DSS) 10 % in drinking water during 7 days. DSS evoked a mild, superficial pancolitis, most characterised by mucosal ulceration and submucosal influx of neutrophils. Neither SMS-14 nor octreotide reduced the mucosal inflammatory score or macroscopical disease activity, although the suppressed intestinal levels of interleukin-1β (IL-1β), IL-6 and IL-10 during DSS were further reduced by both SMS and octreotide. A slight increase of neutrophil influx was seen during SMS administration in animals not exposed to DSS. In conclusion, SMS or its long-acting analogue did not reduce intestinal inflammation in non-acute DSS induced colitis. However, based on the cytokine profile observed, SMS-14 and octreotide may further reduce the intestinal macrophage and TH_2 lymphocyte activity, which has been suppressed by DSS.

Introduction

Neuropeptides play key roles in intestinal inflammation[1]. One of the most active inhibitory neuropeptides is somatostatin (SMS), which has dose dependent inhibitory effects on lymphocyte proliferation and cytokine production, immunoglobulin synthesis and macrophage function[2]. It stimulates the development and immunologic activity of intestinal granulomas in murine *Schistosomiasis mansoni* infection[3,4]. Mucosal depletion of SMS is observed in inflammatory bowel disease[5-8]. In contrast, blood SMS levels in active IBD are elevated, suggesting a defensive role in intestinal mucosal injury[9,10]. Octreotide, a long-acting SMS analogue, is able to reduce epithelial damage in an animal model of acute experimental colitis[11]. No data are available on the effects of somatostatin or its analogues on chronic colitis.

When laboratory animals are exposed to dextran sulphate sodium (DSS) in their drinking water, a mild colitis develops. Although one of the characteristic events evoked by DSS is an enhanced T-cell response[12], colitis is induced irrespective of the presence or absence of lymphocytes[13,14]. DSS-induced colitis is a highly reproducible model of intestinal inflammation, mimicking certain aspects of human inflammatory bowel disease[15]. In the acute phase it is characterized by a predominantly left-sided, acute colitis, which is not contiguous[16,17]. By one week of DSS exposure, a mild subacute colitis develops, which responds to several immune-modulating agents[18-20]. Certainly pro-inflammatory neuropeptides (substance P, neuropeptide Y) are involved in DSS-induced colitis[21]. The relevance of inhibitory neuropeptides like SMS in this model has not been studied.

We aimed to determine the role of SMS in DSS-induced subacute intestinal inflammation in mice, focussing on intestinal leukocyte infiltration and cytokine content. Pro- as well as anti-inflammatory cytokines were examined in this study.

Based on the described immune inhibitory effects, we anticipated a beneficial effect of SMS administration on intestinal mucosal damage induced by DSS.

Methods

Experimental protocol

All experiments were approved by the Laboratory Animal Ethics Committee of the Erasmus University. Forty young female BALB/c mice (20 to 22 g) were randomly allocated to 8 groups (A to H). All mice had osmotic pumps (Alzet, Germany) inserted subcutaneously in the upper region of the back under ether anaesthesia. Osmotic pumps were filled with saline, SMS-14 or octreotide. Groups A and B received normal saline. The pumps in groups C, D, E and F were filled with SMS acetate, containing SMS-14 (Somatofalk®, Tramedico, Weesp, the Netherlands). These pumps continuously released SMS-14 subcutaneously during 7 days (groups C and D 36 μg, and groups E and F 120 μg daily). Groups G and H received octreotide 3 μg daily (Sandostatine®, Novartis, Basle, Switzerland). Immediately after insertion of the pumps animals in groups B, D, F and H were exposed to DSS 10% in their drinking water during 7 days. Control groups were allowed to drink normal water. All animals had free access to drinking water and normal food. After to an overnight fast, animals were killed by cervical dislocation 7 days after implantation of the osmotic pumps. All operations and sacrificing took place between 9 and 11 am.

Macroscopy

Severity of inflammation was documented macroscopically, based on changes of bodyweight and general appearance (hair, liveliness), stools and rectal bleeding, and color, distension and serosal appearance of the colon directly after opening of the abdomen. A maximum macroscopic score of 10 points was allocated, according to the following: as adolescent mice gain at least 5% bodyweight per

week, change of bodyweight of more than 5% was scored 0, 0-5% gain = 1, loss of bodyweight = 2. Hair was normal (0) or dull (1); mice were lively (0) or apathetic (1). Stools were normal (0), semiliquid (1) or liquid (2). Rectal bleeding was given 1 point. Colon colour was normal (0) or red (1); distention was absent (0) or remarkable (1). The serosal aspect was normal (0) or thickened (1).

Intestinal specimens

Immediately after sacrificing, the colon and small bowel were removed. Faecal contents were carefully extruded. Fragments of terminal ileum, and proximal, middle and distal colon were cut to a standardized length alongside a measuring rod. Specimens for histology were placed immediately in formaldehyde. For cytokine analysis fragments were kept in Krebs buffer after weighing.

Histological examination

Histological sections from ileum, and proximal, middle and distal parts of the colon were stained (haematoxylin and eosin) and scored to extent of inflammation (0=none, 1=mild, 2=moderate, 3=severe), damage (0=none, 1=superficial, 2=involving m.mucosae, 3=transmural), and regeneration (3=none, 2=focal, 1=multifocal, 0=complete)[13]. Assessments were made by a blinded committee. Scores from proximal, middle and distal colon parts were added to obtain a total histology score per animal.

Cytokine analysis

Tissue specimens were fragmented during 10 seconds in Krebs buffer (Ultra-Turrax, Polyton, Switzerland) and centrifuged (10.000xg, 10 min., 4°C). The supernatant was stored at –80°C for cytokine assay. Levels of interleukin-1 beta (IL-1β), IL-6, IL-10 and interferon gamma (IFNγ) were measured by ELISA kits for assay of mouse cytokines (Biosource, Belgium). Levels are expressed as cytokine per tissue weight (pg/mg).

Analysis

Descriptive analysis was performed concerning macroscopic changes. Cytokine values were expressed as mean ± standard error. Results were analysed using a Wilcoxon two-sided rank sum test for small samples (macroscopy/microscopy) or an unpaired t-test (cytokine levels). P-values under 0.05 were considered statistically significant.

Results

Macroscopy

All DSS treated animals developed colitis and revealed a significantly higher macroscopy score than controls (6.2 ± 1.2 versus 0.4 ± 0.2, $p < 0.01$). Macroscopy scores in groups C, E and G ('non-inflamed') did not differ from controls (0.8 ± 0.6, 0.8 ± 0.4, and 0 ± 0, NS compared to A). Neither SMS nor octreotide affected macroscopy scores significantly in the colitis groups D, F and H (4.8 ± 1.6, 5.8 ± 0.9 and 7.0 ± 1.4, NS compared to B).

Histology

No inflammation was seen in the ileum. A mild pancolitis was induced by DSS. Mucosal infiltration of polymorphonuclear neutrophils (PMN) was the most remarkable finding. Histology scores from proximal and distal colon parts in animals exposed to DSS did not differ. Scores in groups B (7.2 ± 0.5), D (8.8 ± 0.8), F (6.8 ± 0.6) and H (8.6 ± 1.2) were significantly higher than group A (3.0 ± 0.3, $p \leq 0.05$). Scores from group E (3.6 ± 1.7) and G (4.2 ± 0.8) were higher than controls, but these differences were not statistically different. In group C inflammatory score was high (5.2 ± 0.8), merely due to PMN infiltration. Mucosal damage was not obviously present in group C as the total microscopy score minus the score for PMN infiltration was 1.6 ± 0.5 points for group C, compared to 0.8 ± 0.2 points for controls ($p > 0.05$).

Cytokines

IFNγ was barely detectable in most samples (≤ 0,4 pg/mg). Statistical analysis was not possible, due to the abnormal distribution of data. IL-1β levels in groups B, C, D, F, G and H (15.5 ± 3.3, 19.3 ± 3.5, 19.2 ± 7.3, 5.9 ± 1.7, 10.6 ± 1.8 and 10.1 ± 5.4 pg/mg) were significantly lower than in group A (27.4 ± 5.3 pg/mg, $p \leq 0.05$). Mucosal IL-1β concentration in group E (27.5 ± 7.9 pg/mg) was not different from A (FIGURE 4.1a). In F the IL-1β levels were significantly lower than in B ($p < 0.001$). IL-6 levels in groups B, D, F, G and H (21.3 ± 5.1, 21.4 ± 5.2, 14.1 ± 4.1, 27.4 ± 2.8 and 12.1 ± 5.9 pg/mg) were lower than in group A (54.5 ± 11.2 pg/mg, $p < 0.01$, FIGURE 4.1b). IL-6 levels in C (45.6 ± 5.5) and E (51.7 ± 6.8 pg/mg) did not differ from A ($p > 0.4$). D, F, G and H did not significantly differ from B ($p = 0.2$). IL-10 concentration (FIGURE 4.1c) in group B (9.7 ± 3.8 pg/mg) was lower than in A (20.9 ± 6.2 pg/mg), but statistical significance was not reached ($p = 0.17$). IL-10 levels in D, F and H (6.7 ± 1.7, 6.3 ± 2.4 and 6.1 ± 0.7 pg/mg) were lower than in controls ($p < 0.05$). In groups C (16.2 ± 3.8 pg/mg), E (22.8 ± 6.9 pg/mg) and G (14,9 ± 4.9 pg/mg) IL-10 levels did not significantly differ from controls.

Discussion

In our study DSS induced a mild, superficial colitis in BALB/c mice, both left and right sided, rarely involving layers beyond the muscularis mucosae. The most characterising finding was mucosal infiltration by neutrophils (PMN). Neither SMS nor octreotide attenuated DSS induced mucosal inflammation. Animals given low doses of SMS and octreotide on normal drinking water showed a marked mucosal infiltration by PMN, without mucosal damage. In these groups mucosal IL-1β content was reduced, perhaps reflecting a negative feedback on IL-1β by increased PMN influx. This increased PMN influx was

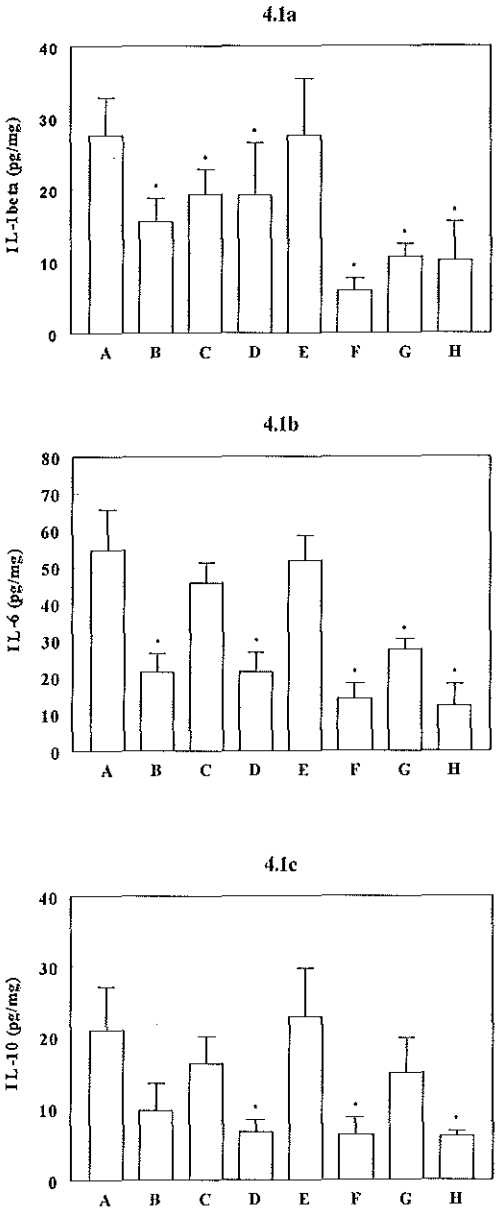

FIGURE 4.1 Cytokine levels in colon mucosa in pg/mg tissue weight. A = saline; B = saline/DSS; C = SMS 36 µg; D = SMS 36 µg/DSS; E = SMS 120 µg; F = SMS 120 µg/DSS; G = OCT 3 µg; H = OCT 3 µg/DSS (*p>0.05 vs A).

limited to the colon, as all ileal specimens were normal. As neutrophil migration into the intestinal mucosa is the prominent phenomenon in DSS-induced colitis, the meaning of this infiltration without mucosal damage is not clear. *In vitro* leukocyte migration is stimulated by SMS by an as yet undefined mechanism[22,23].

Several beneficial effects of SMS or SMS analogues on intestinal inflammation of various aetiologies have been demonstrated[24-27]. In an acute model of experimental colitis induced by acetic acid octreotide in high dose attenuated mucosal inflammation and local cytokine and eicosanoid production[11]. The most distinct effect was seen when octreotide was administered the day before induction of acute colitis. As no follow-up studies on rate of mucosal regeneration were performed, no data are known on the possible beneficial effects of octreotide in the post-toxic period of acetic acid. The differences in results with acetic acid-induced colitis and our findings might be due to the use of different models or different species or both. Acetic acid induced colitis is an acute toxic model in which non-specific inflammation prevails[15]. DSS induced murine colitis is a model of acute as well as non-acute colitis[13,16,17]. This model has been found suitable for studying involvement of mediators of mucosal inflammation and evaluation of antiinflammatory treatment[18,20,28]. DSS most likely acts as a direct mucosal toxin, as inflammation occurs irrespective of the presence of lymphocytes or other mucosal immunocytes[13,14,29]. However, in the non-acute DSS induced colitis intestinal macrophages, neutrophils and lymphocytes are involved[13,19,30]. Corticosteroids, cyclosporin A or anti-neutrophil serum attenuate DSS-evoked inflammatory changes[18-20].

In chronic DSS induced colitis an enhanced T_{H_1} response is observed, which is accompanied by increased intestinal IFNγ content[31]. However, we were not able to detect IFNγ in our intestinal specimen, perhaps due to the low concentrations observed after 7 days of DSS exposure[31]. IL-4 levels are elevated and IL-5 concentrations drop after 14 days of DSS exposure[32], indicating differential

effects on TH_2 activity. We found low mucosal IL-10 levels in DSS induced colitis compared to controls, what may be a direct or indirect effect of DSS on TH_2 lymphocytes. SMS and octreotide did not abolish this effect, but even further reduced IL-10 concentrations. We observed a striking decrease of IL-1β and IL-6, after 7 days of DSS administration. This reduction was enhanced by SMS 120 μg and octreotide. In the DSS model of experimental colitis, reduction of IL-1β and IL-6 suggests a reduced activity of mucosal macrophages as well as TH_2 lymphocytes. The reduction of pro-inflammatory cytokines may reflect a phase of mucosal regeneration in this stage of subacute colitis after one week of DSS exposure. The study period was probably too short to detect beneficial effects of SMS on the rate of post-exposure healing. Moreover, SMS has been reported to delay intestinal mucosal regeneration[33]. The dual effect of different doses SMS may reflect different local mucosal concentrations and subsequent receptor activation. This effect is not influenced by the short half time of this instable compound, considering similar results obtained by octreotide.

In conclusion, in mice SMS or octreotide administered subcutaneously during one week of oral DSS exposure did not attenuate colon mucosal inflammation. Low levels of mucosal IL-1β following DSS administration, were further decreased during high doses SMS. During administration of SMS a marked influx of PMN in colonic but not ileal mucosa was observed, also in animals not receiving DSS. These animals showed lower concentrations of mucosal IL-1β than control animals. The meaning of this promotive role of SMS on neutrophil migration still has to be settled. SMS and octreotide amplified the DSS-induced decline of intestinal levels of IL1β, IL6 and IL10, suggesting further inhibition of macrophages and TH_2 lymphocytes.

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CHAPTER 5

RESPONSE TO CARBACHOL AND SALBUTAMOL IN DEXTRAN SULPHATE SODIUM-INDUCED COLITIS IS MODULATED BY SOMATOSTATIN

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Submitted

Abstract

The effects of somatostatin (SMS) and its analogue octreotide (OCT) were studied on contractile activity of mouse colonic smooth muscle in experimental colitis, induced by addition of dextran sulphate sodium (DSS) 10 % (w/v) to the drinking water.

BALB/c mice received subcutaneous osmotic pumps delivering either normal saline, somatostatin 6 μg or octreotide 0.15 μg per g body weight per day. DSS 10% (w/v) was given to half the mice for 7 days immediately after implantation of the osmotic pumps. Mice were killed at day 8 and parts of the transverse colon were used to study isotonic contraction in an organ bath.

DSS 10% induced a mild, superficial colitis, leaving the muscular layer uninvolved. SMS or OCT did not affect the degree of inflammation.

Concentration-response curves were obtained by adding carbachol (CARB) in increasing concentrations (10^{-8} – 10^{-3} M). After maximal contraction with 10^{-3} M CARB, increasing concentrations of salbutamol (SALB) were administered (10^{-8} – 10^{-3} M). From both log dose-response curves a half-maximum concentration was calculated ($EC50_{CARB}$ and $EC50_{SALB}$).

Both $EC50_{CARB}$ and $EC50_{SALB}$ increased significantly in DSS-induced colitis, suggesting a decreased sensitivity to both agents. After SMS and OCT treatment $EC50_{CARB}$ returned to the control value, whereas $EC50_{SALB}$ was further increased. Maximum SALB-induced smooth muscle relaxation was decreased after DSS exposure.

Introduction

Intestinal inflammation is accompanied by altered gut smooth muscle function and motility, manifested clinically as diarrhoea and a decreased reservoir function. Diarrhoea may be due to an increase of the number of colonic giant migration contractions, rather than increased colon contractility[1]. Inflammation decreases maximum smooth muscle cell contractility and force after stimulation with muscarinic agonists, without affecting the half-maximum dose (EC50)[2-4]. Less is known about the influence of intestinal inflammation on smooth muscle relaxation. Adrenergic stimuli are one of the mediators of colon smooth muscle relaxation[5-7], with different effects in circular (both α- and β-adrenergic) and longitudinal (β-adrenergic) muscle cells[7].

Enteric nerve function and the intestinal content of neuropeptides are changed in inflammatory bowel disease[8,9]. Neuropeptides like substance P (SP), vasoactive intestinal polypeptide (VIP) and somatostatin (somatotropin release inhibiting factor, SMS) are important mediators in the nervous control of intestinal motility and mucosal inflammation[10]. In general, SP and VIP are thought to promote smooth muscle cell contractility[10]. SMS is an important inhibitory neuropeptide. The effects of SMS on muscle activity are complex. In healthy subjects it diminishes colon transit time by an as yet unknown mechanism[11,12]. Interference of SMS with agonists and antagonists affecting contraction has been shown in *in vitro* studies. Preincubation of smooth muscle cells with SMS attenuates carbachol (CARB)-induced contraction[13]. In bronchial smooth muscle cells SMS reduces the β-adrenergic-induced relaxation[14]. In the rat colon a dose-dependent contractile effect of SMS and its analogues is observed, with a marked desensitisation if the drug is re-administered after five minutes[15]. However, there are species differences. In dog colon muscular strips SMS inhibits spontaneous contraction[16] and in guinea pig colon a slight contractile effect is seen in the

circular muscles and an inhibition of carbachol-induced contraction in both circular and longitudinal muscular cells[13].

SMS and its analogues have anti-inflammatory effects[17] and beneficial effects on bowel movements and diarrhoea in active Crohn's disease have been reported[18,19]. However, it is not clear whether the beneficial effects are due to modulation of the gut immune system or direct inhibition of smooth muscle activity.

Dextran sulphate sodium (DSS)-induced colitis is an animal inflammatory model characterised by a mild, superficial colonic inflammation, leaving the muscular layers intact[20]. In earlier studies in this colitis model in mice we found a marked loss of maximal contraction on carbachol stimulation and a diminished nitroprusside-induced relaxation in the inflamed colon, especially in the transverse part[21]. We studied the effects of subcutaneous administration of SMS and its long-acting analogue octreotide on *in vitro* transverse colon smooth muscle contraction and relaxation in mice with dextran sulphate sodium (DSS)-induced colitis. Octreotide has a plasma half-life of 20 minutes; SMS has a very short plasma half-life after single subcutaneous administration (about 1 minute)[22]. We gave both drugs through subcutaneous osmotic pumps, making a continuous delivery possible.

Methods

The University Experimental Animal Ethic Committee approved all experiments. The experiments were all performed in a blinded fashion.
Six groups of female young adult BALB/c mice (20 – 22 g) were kept under standardised laboratory conditions. In all mice osmotic pumps (model 1007 D, 0.5 μL per hour, Alzet, Germany) were implanted subcutaneously in the

suprascapular region. Pumps were filled with either normal saline, or SMS (SMS-14, Somatofalk®, Tramedico, Weesp, The Netherlands) or the long acting SMS analogue octreotide (OCT, Sandostatin®, Novartis, Basle, Switzerland). They continuously released saline, 6 μg SMS or 0.15 μg OCT per g body weight per day (dose difference based on different half-lives). Thereafter, mice were randomly allocated to either normal drinking water or drinking water containing 10 % (weight/volume) dextran sulphate sodium (MW > 500000, Pharmacia, Sweden). All mice receiving DSS 10% developed a mild superficial colitis after one week, only involving the colon mucosa and submucosa. After an overnight fast at day 8 all mice were killed by cervical dislocation. The colon was removed, pericolonic fat was detached manually and faecal contents were gently extruded. Colon specimens were kept in formaldehyde. Glass mounted sections were stained (haematoxylin and eosin) for microscopic evaluation. Histological grading concerned neutrophil influx (0-3 points), damage (0-3) and regeneration (0-3)[20,23]. The transverse colon was cut out and immediately placed in standard Krebs' buffer (pH 7.4), containing in mmol/L: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2 \cdot 2H_2O$, 1.2 $MgSO_4 \cdot 7H_2O$, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 8.3 glucose.

Longitudinal transverse colon specimens (approx. 10 mm length) were mounted entirely in a double jacket organ bath in carbonated (5% CO_2 in O_2) Krebs' buffer with a constant temperature of 37° C. One end of the colon specimen was attached to a stationary clamp. Contractile responses were measured isotonically under a 0.5 g load and recorded via a non-elastic wire by a Penny & Giles transducer and a rectilinear ink-writing plotter.

Carbachol (Sigma Chemicals) in increasing concentrations from 10^{-8} to 10^{-3} M was cumulatively added to the bath fluid and muscle contraction was measured. Equilibration of contractile response was allowed during at least 1 minute before a new dose of CARB was administered. We found a maximal muscle contraction at carbachol (CARB) concentration of 10^{-3} M. As in a pilot study precontraction by KCl had induced muscular inertia (data not shown) we defined maximal

contraction (100 %) at CARB concentration of 10^{-3} M and related all responses (contraction and relaxation) to this maximum. After equilibration at maximal contraction, relaxation was obtained by adding salbutamol (SALB) (generous gift of Glaxo Wellcome, UK) in increasing concentrations from 10^{-8} to 10^{-3} M, always after relaxation had reached a steady state.

For both drugs log-dose-response curves were plotted (GRAPHPAD-PRISM™). From these we derived the measure of contraction or relaxation as compared to a standard contraction induced by CARB 10^{-3} M (100 %). For both CARB and SALB the half-maximum dose ($EC50_{CARB}$, $EC50_{SALB}$) was calculated by use of the GRAPHPAD-PRISM™ software. Results are given in mean and 95% confidence intervals. Statistical analysis was performed by a t-test for colon muscle response and by a Wilcoxon rank-sum test for histological scores. A p-value lower than 0.05 was considered statistically significant.

Results

Histology

All DSS treated animals developed a mild but significant colitis. Histology scores from DSS treated animals were significantly higher than controls. Neither SMS nor OCT attenuated this microscopic score (TABLE 5.1). Mucosa and submucosa showed infiltrates of predominantly neutrophils in DSS treated animals. Muscle layers were not infiltrated or visibly damaged by inflammatory cells.

Contraction

Maximum contraction was derived at CARB concentrations of 10^{-3} mM (FIGURE 5.1). $EC50_{CARB}$ results are listed in TABLE 5.2. In controls (mean 1.1 x 10^{-6} mmol/L) $EC50_{CARB}$ values were significantly lower than in the DSS-saline group (2.3 x 10^{-6} mmol/L) ($p = 0.005$). SMS enhanced $EC50_{CARB}$ values (2.2 x

10^{-6} mmol/L) compared to controls (p = 0.03), whereas $EC50_{CARB}$ in the OCT group did not significantly differ from controls (1.4 x 10^{-6} mmol/, p = 0.5). $EC50_{CARB}$ values in DSS treated animals were not significantly different from controls in both SMS (0.9 x 10^{-6} mmol/L) and OCT (1.1 x 10^{-6} mmol/L) groups (p = 0.5), and significantly lower than in DSS/saline (resp. p = 1.0 x 10^{-6} and 7.2 x 10^{-6}).

TABLE 5.1 Histology score (mean ± SEM).

	control	DSS
saline	3.0 ± 0.3	7.2 ± 0.5*
SMS 6 µg	3.6 ± 1.7	6.8 ± 0.6*
OCT 0.15 µg	4.2 ± 0.8	8.6 ± 1.2*

*p < 0.05 compared to control

Relaxation

FIGURE 5.2 depicts colon smooth muscle relaxation after maximum contraction at 10^{-3} mM CARB. Maximum relaxation was seen at SALB $10^{-3.5}$ mM. DSS reduced maximum relaxation significantly compared to controls (p < 0.01). In terms of percentage, SMS and OCT even showed lower relaxation compared to controls and DSS treated mice (p < 0.01). TABLE 5.3 shows $EC50_{SALB}$ results. SMS (5.6 x 10^{-6} mmol/L) showed significantly higher values compared to controls (4.7 x 10^{-6} mmol/L, p = 0.02), but OCT (4.5 x 10^{-6} mmol/L) did not (p = 0.5). DSS (12.6 x 10^{-6} mmol/L) and DSS receiving groups that were treated by SMS (27.1 x 10^{-6} mmol/L) or OCT (12.6 x 10^{-6} mmol/L) showed significantly higher $EC50_{SALB}$ values than controls with p values resp. 8 x 10^{-6}, 8 x 10^{-9} and 1 x 10^{-12}. SMS even showed higher $EC50_{SALB}$ than DSS (p = 0.0005).

TABLE 5.2 EC50 (x 10^{-6} M) of carbachol (mean and 95 % confidence intervals)

	control	DSS
saline	1.1 (0.4-0.8)	2.3 (1.7-2.9)*
SMS 6 μg	2.2 (1.2-2.3)*	0.9 (0.7-1.2)**
OCT 0.15 μg	1.4 (0.9-1.9)	1.1 (0.8-1.3)**

$p < 0.05$ compared to control/saline
** $p < 0.001$ compared to DSS/saline

control = control group
saline = receiving normal saline
DSS = dextran sulphate sodium
SMS 6 μg = treated with somatostatin 6 μg/g body weight daily
OCT 0.15 μg = treated with octreotide 0.15 μg/g body weight daily

Table 5.3 EC50 (x 10^{-6} M) of salbutamol (mean and 95 % confidence intervals). For legend see table 1.

	control	DSS
saline	4.7 (4.1-5.2)	12.6 (9.0-16.3)**
SMS 6 μg	5.6 (5.0-6.2)*	27.1 (19.1-35.0)**°
OCT 0.15 μg	4.5 (3.8-5.3)	12.6 (11.3-13.9)**

* $p < 0.05$ compared to control/saline
** $p < 0.0001$ compared to control/saline

° $p < 0.001$ compared to DSS/saline

Discussion

Many of our concepts of colon motility control are still hypothetical. It is probable that circular colon muscle plays a key role in propulsion of luminal contents and the taeniae-like configuration of the longitudinal muscle cell modulates colonic reservoir function[24]. The relation between altered muscle cell

function and disturbed motility in intestinal inflammation is largely uncertain. *In vitro* studies show an inflammation-related decrease of maximal muscle contraction on muscarinic stimulation[2,4,26]. In earlier experiments we found a marked decrease of maximum tension on CARB stimulation, which was most obvious in the transverse parts of the murine colon[21]. In the present study, DSS-induced inflammation resulted in a significant higher EC50CARB, indicating a change of muscarinic receptor binding or cellular responsiveness during inflammation. This is in contrast to studies mentioned earlier, in which no effect of inflammation on receptor susceptibility was seen[2,4,25,26]. This might in part be explained by different experimental designs, as in most cases only circular colon smooth muscles have been studied, whereas in our organ bath model longitudinal muscle changes were studied. Moreover, as we have described a marked regionality of DSS-induced smooth muscle impairment[21], results of motility studies may depend on the location of colon muscular tissue taken. SMS in saline receiving mice also showed a higher EC50CARB whereas OCT did not change this value.

However, in DSS-treated animals both SMS and OCT antagonised the DSS-induced shift-to-the-right of the dose-response curve of CARB. In guinea pig colon Corleto et al. found that SMS inhibits CARB-induced contraction in the distal colon[13]. They considered only the smooth muscle cell length on exposure to agonists or antagonists. That may not be as reproducible as alteration of EC50 values. In the same study the authors state that the contractile inhibition by SMS depends on muscle cell resting length[13]. DSS-induced diminished susceptibility of the muscarinic receptors in the transverse colon smooth muscle may reflect proximal colon stasis as is described in active human ulcerative colitis[26]. Normal human subjects show a marked decrease of colon transit time after OCT exposure[11,12]. Although co-localisation of membrane SMS and muscarinic receptors has been described[27], findings in our study do not support participation of muscarinic receptors in the OCT-derived changes of colon transit time.

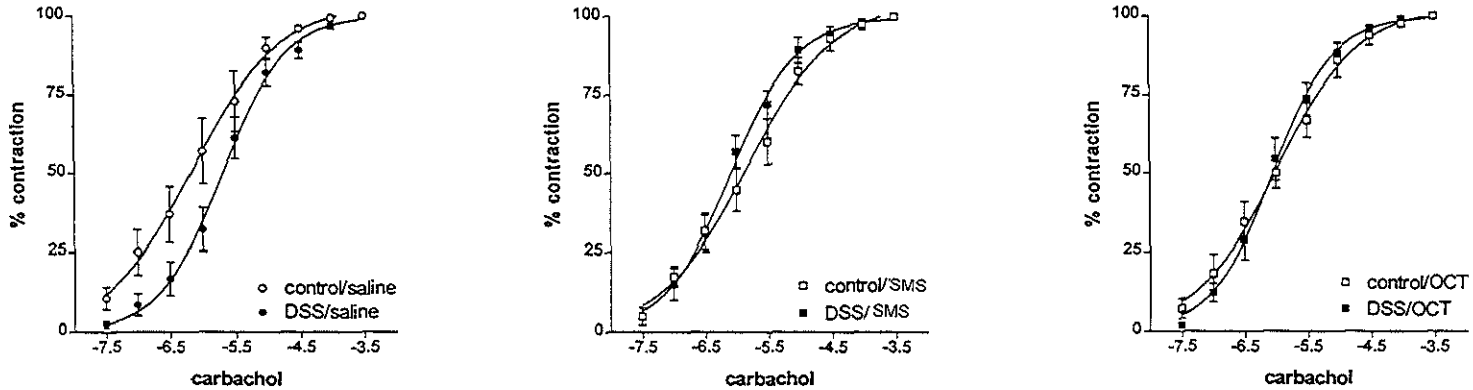

FIGURE 5.1 Dose-response curves (% of maximum contraction ± SEM) after adding increasing concentrations of carbachol (mM/L).
For abbrevations see text.

Normal mice receiving SMS, but not OCT, had a higher EC50CARB, reflecting higher thresholds for this muscle contractile agonist. SMS acts through specific membrane bound receptors and is likely not a muscarinic receptor antagonist, but interference at the level of G-proteins or second messenger systems may be anticipated[28,29].

In our model we studied isotonic contraction of the colon musculature. We found a significant higher EC50SALB on DSS exposure. This suggests a decrease of susceptibility to SALB induced by intestinal inflammation, at receptor of post-receptor level. SMS even further increased the half-maximum concentration for SALB-induced relaxation. So SMS impairs SALB induced relaxation as it does in bronchial muscle[14]. This effect is probably mediated by inhibition of the SALB-induced intracellular production of cAMP[14,30]. Relaxation curves (FIGURE 5.2) showed lower maximum relaxation after DSS exposure. As relaxation was based on maximum contraction, this difference of maximum relaxation may be due to different smooth muscle length at point of maximum contraction[21]. Otherwise, DSS-related impaired relaxation may reflect inflammation-induced diminished colon wall compliance. SMS and OCT did not improve this changed relaxation. Reduced relaxation can result in loss of colon reservoir function, thus contributing to accelerated colon transit.

In summary we have found a decreased muscarinic receptor response in DSS-induced colitis in mice transverse colon. This could be the experimental equivalent of clinically observed increased passage time of the proximal colon in IBD. The decreased response sensitivity to the muscarinic agonist CARB was normalised by treatment with SMS or its analogue OCT. Colon smooth muscle cell susceptibility to β-adrenergic stimulation by SALB was diminished in this experimental model. SMS and OCT further decreased the SALB susceptibility. Maximum relaxation length was reduced after DSS exposure, even more if DSS receiving animals were treated with SMS or OCT.

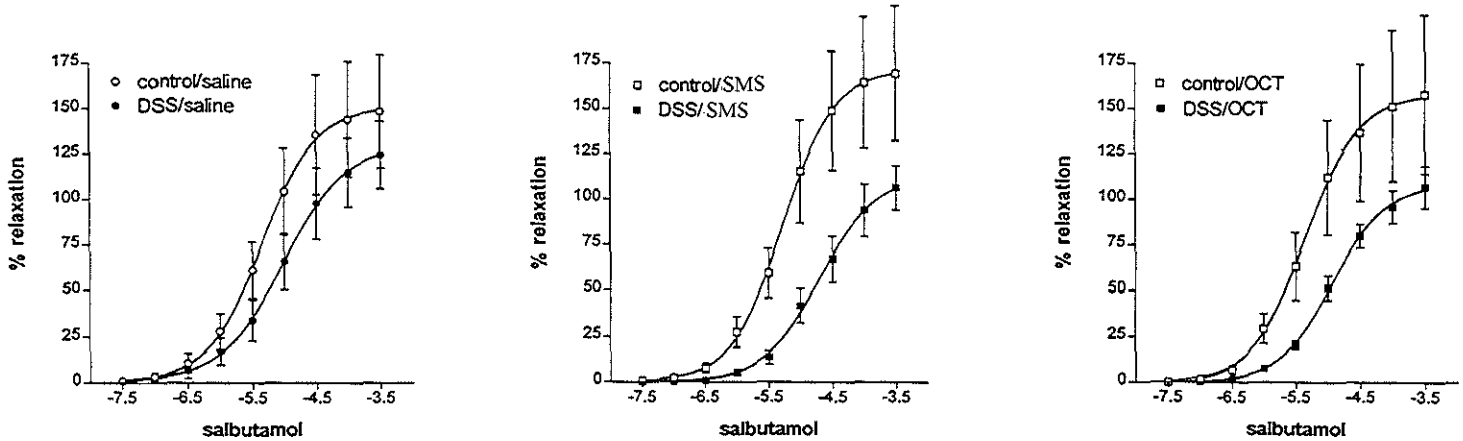

FIGURE 5.2 Dose-response curves (relaxation after maximum contraction ± SEM) after adding increasing concentrations of salbutamol (mM/L).

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CHAPTER 6

SOMATOSTATIN RECEPTORS IN INFLAMMATORY BOWEL DISEASE: *IN VITRO* DETECTION BY A POLYCLONAL ANTI-SOMATOSTATIN RECEPTOR SUBTYPE 2A ANTISERUM AND *IN VIVO* VISUALISATION WITH [^{111}IN-DTPA-D-PHE^{1}]-OCTREOTIDE SCINTIGRAPHY

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Abstract

Somatostatin (SMS) is a small neuropeptide which acts on multiple non-neural targets, including the immune system and the gastrointestinal tract. In inflammatory bowel disease (IBD) mucosal SMS is diminished and its receptor density is increased. In a combined study of endoscopy, pathology, immunohistochemistry and octreotide scintigraphy we describe the SST expression in 14 patients with clinically active IBD (11 Crohn's disease (CD), 3 ulcerative colitis (UC)), localised in colon and/or terminal ileum. Patients underwent colonoscopy and [^{111}In-DTPA-D-Phe^{1}]-octreotide scintigraphy on the same day. Abdominal images were obtained after 4 and 24 hours and were compared with 8 non-IBD control patients. Mucosal biopsy samples and in three cases also bowel resection specimens were used for immunohistochemistry, that was performed using a polyclonal rabbit anti-somatostatin receptor 2A antiserum (R2-88). Mild to moderate active of chronic inflammation was seen in colon and/or ileum specimens. Scintigraphy revealed intestinal wall [^{111}In-DTPA-D-Phe^{1}]-octreotide uptake in IBD. No correlation was found between clinical disease activity, endoscopy and histology on the one hand, and scintigraphy on the other. Immunohistochemistry showed SST2 positive staining of (sub)mucosal endothelium in both CD and UC, with a slight preference for active inflamed mucosal parts. In several CD cases superficial epithelium was positive, with a striking paranuclear staining. In UC patients SST2 positive fibroblasts and macrophages were seen.

We conclude that SST2 positive cells are found in intestinal biopsies in active IBD. These receptor expression can be visualised by immunohistochemical techniques. Although intestinal wall [^{111}In-DTPA-D-Phe^{1}]-octreotide uptake was found, specificity of these images is under discussion as is its relation with clinical and endoscopical disease activity.

Introduction

Somatostatin (somatotropin release inhibiting factor, SMS) is a small neuropeptide, which has two principal bioactive configurations consisting of 14 or 28 amino acids, SMS-14 and SMS-28. SMS acts on specific G-protein coupled transmembrane receptors in multiple targets in central nervous tissue, endocrine glands and exocrine pancreas, the immune system and the gastrointestinal tract[1]. Somatostatin receptors (SST) were demonstrated by *in vitro* radioreceptoranalysis and by *in vivo* somatostatin scintigraphy with [^{111}In-DTPA-D-Phe^{1}]-octreotide[2,3]. [^{111}In-DTPA-D-Phe^{1}]-octreotide binds with high affinity to SST2 and SST5, and has a very low biliary excretion, making abdominal imaging possible. Octreotide scintigraphy has been tested elaborately in the past years for diagnosis of haematologic malignancies, granulomatous and auto-immune diseases[4]. Receptor density correlates with disease activity and octreotide scintigraphy is proposed as a method for evaluation of these disorders[5-8].

Most *in vitro* analysis of SST was performed by autoradiographic assays. SST expression has been found in gut-associated lymphoid tissue, leucocytes, and at several intestinal mucosal and submucosal cells of the gut[9,10]. Increased density of vascular SST is observed in veins surrounding mucosal lesions in Crohn's disease (CD) and ulcerative colitis (UC), and in peri-inflammatory veins in rheumatoid arthritis, suggesting a role in regulation of inflammatory processes[11,12]. SMS has dampening effects on the immune system, which have been described in various laboratory animal experiments[10,13-15].

All subtypes of SST were found in intestinal tissue[16], but in the colon mucosa and submucosa SST2 mRNA predominates[17]. We surveyed the presence of SST2 in the intestinal wall, as visualised by immuno-histochemistry with an anti-SST2A antibody and compared these findings with [^{111}In-DTPA-D-Phe^{1}]-

octreotide scintigraphy in patients with active inflammatory bowel disease (IBD). Positivity on octreotide scintigraphy correlates well with presence of the SST2[18].

Methods

Consecutive patients with clinically active inflammatory bowel disease (IBD), with ulcerative colitis (UC) or Crohn's disease (CD) of colon and/or terminal ileum, who were planned to have a colonoscopy to monitor disease activity, were enroled in the study after obtaining informed consent. Clinical disease activity was assessed using a Powell-Tuck scoring system (PTS) or a modified Crohn's Disease Activity Index (mCDAI)[19,20]. Each patient was his own control considering active inflamed and non-inflamed mucosal parts. Controls were 8 consecutive patients who underwent screening [^{111}In-DTPA-D-Phe^{1}]-octreotide scintigraphy. All scintigraphic imaging in controls were prepared through a standard laxative protocol (2 days of psyllium fibres 6 g BID, Prunacolon® 1 ml / kg on the day of radionuclide injection).

All patients were prepared for endoscopy with oral laxative solution (Klean-prep®). Colonoscopy took place 18 hrs later. The day before colonoscopy, 200 to 250 mBq of [^{111}In-DTPA-D-Phe^{1}]-octreotide (10 μg) was administered intravenously to 12 patients (2 UC)[2,21]. Planar abdominal images were obtained 4 and 24 hours after injection of the radiopharmaceutical. Single photon emission computed tomography (SPECT) was performed after 24 hrs. Scintigraphic images were reviewed in a blinded way.

Endoscopic evaluation of CD and UC was performed according to operative criteria[22-24]. A digital audio-visual endoscopy report was obtained from each colonoscopy and reviewed without knowledge of scintigraphic findings. Biopsies were taken from inflamed and non-inflamed mucosa for microscopic evaluation.

At least five biopsies were taken each from right, middle and left sided colon, rectosigmoid, and if possible from terminal ileum. After section of biopsy and staining with hematoxylin and eosin, inflammation was classified 0 (absent), 1 (mild chronic), 2 (moderate chronic and/or mild active), 3 (severe chronic, moderate active), or 4 (ulcerative).

For immunohistochemistry, biopsies were obtained from the most inflamed parts of colon or ileum of 9 patients with CD and 3 patients with UC. Moreover bowel resection specimens were obtained from 3 of these patients (2 CD, 1 UC). Immunohistochemical analysis was performed using a polyclonal rabbit anti-somatostatin receptor 2A antiserum (R2-88) (dilution 1:1000). Specificity of the antiserum was confirmed by Western Blot analysis of membrane preparations of human neuroendocrine tumors. Biopsy or bowel resection specimens were fixed in formalin 10%, dried and embedded in paraffin wax. Sections (5 µm) were mounted on uncoated glass slides. Thereafter they were rinsed in PBS and reincubated for 15 min. at room temperature with 10% normal goat serum in PBS/5% BSA. Incubation with R2-88 (dilution 1:1000) was carried out overnight at 4°C. The sections were rinsed twice in PBS and incubated for 30 min at room temperature with alkaline phosphatase-conjugated goat-anti-rabbit immunoglobulin (GaRig-AP; DAKO D0487; DAKOPATTS, Glostrup, Denmark) diluted 1:50 in PBS/5% BSA containing 2% normal human serum. After the incubation the sections were again rinsed twice in PBS. Alkaline phosphatase activity was revealed by new fuchsine as the chromogen in the presence of levamisole to block the endogenous alkaline phosphatase activity. This was followed by a haematoxylin staining. Controls for immunohistochemistry included omission of the primary antibody, and incubation with normal rabbit serum.

Results of endoscopy, microscopy and scintigraphy were descriptively analysed.

Results

Eleven patients with CD (9 female, 2 male) and 3 with UC (all male) were recruited. Patient characteristics are given in TABLES 6.1, 6.2 and 6.3. One patient had been treated with radiotherapy for prostate carcinoma (n° 9) and had developed a radiation proctitis. Six had undergone surgery for Crohn's disease, at least two years before. Six patients used 5-ASA oral monotherapy, one did not receive drug treatment at the time of this study. One patient used 5-ASA as well as corticosteroids and azathioprine, one used only corticosteroids and two were on corticosteroids and azothioprine. The patients from whom we obtained bowel resection specimens, had been treated with prednisone/SASP, and azathioprine or prednisone/cyclosporin in the month before surgery.

Results of endoscopy, histology and scintigraphy are given in TABLE 6.4. Laxation had been sufficient in 12 IBD patients. Two UC patients still had some coecal faecal stasis. Histology could be obtained from most colon segments and terminal ileum. In three patients the terminal ileum could not be reached. In one of these a small bowel barium follow through radiograph had been made, at which no abnormalities had been seen. In the two other patients ulceration of the ileo-ascendo anastomosis were observed. Histology showed only granulomata in ileum biopsies in 1 patient (n° 3). Patchy inflammation was seen in all CD patients. Inflammation was also found in biopsies of intestinal segments which had a normal appearance at endoscopy. In UC a marked chronic ulcerative inflammation was seen in the most inflamed parts.

TABLE 6.1 Clinical characteristics related to Crohn's disease (CD).

patient	age	f/m	CD history	mCDAI	therapy
1	70	f	1994 perianal disease	10	5ASA
2	48	f	1979 R.sided colitis 1985 hemicolectomy R.	12	none
3	34	f	1977 ileo-cecal resection	5	5ASA
4	46	m	1981 perianal disease 1989 sigmoidresection	10	CS azath
5	45	f	1994 ileitis / L.sided colitis	12	5ASA CS azath
6	46	f	1977 ileo-cecal resection 1994 stenosis ileo-asc.stomy 1995 resection ileo-asc.stomy	8	CS*
7	39	f	1972 ileo-cecal resection 1980 re-resection 1984 ileum stenoplasty 1986 partial ileum resection/perianal abscesses 1993 colostomy 1995 stenosis neo-terminal ileum	5	azath. CS*
8	42	f	1979 partial ileumresection 1987 re-resection	7	5ASA
9	60	m	1994 ileo-cecal resection	9	5ASA
10	43	f	1982 terminal ileitis	2	5ASA
11	35	f	1976 left-sided colitis	10	SASP

* budesonide ileal release orally

5ASA = 5-aminosalicylic acid
SASP = salicylazosulfapyridine
CS = corticosteroids
azath = azathioprine
R = right L = left
mCDAI = modified Crohn's disease activity index

TABLE 6.2 Clinical characteristics related to ulcerative colitis (UC).

patient	age	UC history	PT score	therapy
12	32	1994 left-sided colitis	11	5ASA (7 yrs) + CS (1 yr)
13	70	1967 left-sided colitis 1987 pancolitis	12	CS (10 yrs)
14	59	1991 pancolitis	9	SASP

PT score = Powell-Tuck disease activity score (maximum score: 22 points)

TABLE 6.3 Clinical characteristics of patients no. 7, 11 and 14 before surgery.

patient	diagnosis	therapy before surgery	surgical procedure
7	CD	azath	partial ileum resection, stricture plasty
11	CD	5ASA + CS (6 mo) + CsA (7 days)	subtotal colectomy
14	UC	SASP + CS (4 wks)	subtotal colectomy

Octreotide scintigraphy in controls showed positivity in the colon regions in all patients (n=8) and small bowel positivity in 4. Apart from uptake in liver, spleen and kidneys, no additional abdominal uptake was observed. In CD intestinal uptake of the radiopharmaceutical was seen at 4 hrs after injection of [^{111}In-DTPA-D-Phe^{1}]-octreotide in all patients. More than 24 hrs after injection, radioactivity was localised in the same region (FIGURE 6.1). SPECT did not give additional information. In UC one patient did not show bowel uptake. One other patient showed small as well as large bowel positivity, which had not changed after 24 hrs. In 6 patients with CD increased uptake of radioactivity was found at sites of endoscopic disease. In 5 of these patients increased uptake was also noticed at endoscopically normal sites. In 3 patients endoscopic disease was not accompanied by increased uptake. One patient showed no endoscopic

changes (patient n° 1), but scintigraphy showed uptake in the right-sided colon and small bowel. This patient had only periods of intractable diarrhoea and was known with 'silent' perianal disease. Histology and scintigraphy did not correlate well. In 4 patients, segments which were found positive at scintigraphy, biopsies showed no abnormalities (patients n° 2, 4, 7, 9). In 6 patients inflamed intestinal sections did not show up at scintigraphy (patients n° 1, 3, 4, 6, 7, 8). No correlation comes clear between either endoscopy or histology and scintigraphy.

SST2 immunohistochemistry showed posivity in all but one specimens as is listed in TABLES 6.5 and 6.6. Patient no.9 showed no SST2 positive cells, but only small biopsies had been obtained, without remarkable inflammation. SST2 positivity was specific as control staining with conjugate or anti-rabbit goat serum was negative or showed aspecific staining of non-determined cells (FIGURE 6.2). Mucosal or submucosal capillary endothelium was positive in 10 out of 11 SST2 positive patients, most pronounced in the proximity of leukocyte infiltrates. Superficial epithelium showed SST2 positivity in 6 CD patients. A remarkable paranuclear staining was seen in these cases. Crypt epithelium positivity was observed in 2 CD and all 3 UC patients. Focal positive plasma cells, ganglion cells and macrophages were seen in CD patients. UC patients showed in all cases positive macrophages.

TABLE 6.4 Results of endoscopy, histology and scintigraphy.

Patient		Endoscopy	Histology*	Scintigraphy	
1	ileum	NI	1	pos	
	R. colon	N	1	pos	
	M. colon	N	1	neg	
	L. colon	N	1	neg	
	rectosigmoid	N	1	neg	
2	ileum	N	0	neg	
	ileoascendostomy	ulcers	2	pos	
	R. colon	N	0	pos	
	M. colon	N	0	neg	
	L. colon	N	0	pos	
	rectosigmoid	N	0	pos	
3	ileum	N	2	neg	
	ileoascendostomy	ulcers	2	pos	
	R. colon	N	2	pos	
	M. colon	N	2	neg	
	L. colon	N	2	pos	
	rectosigmoid	N	2	neg	
4	ileum	N	0	pos	
	R. colon	N	1	neg	
	M. colon	N	1	neg	
	L. colon	erythema	1	neg	
	rectosigmoid	ulcerative stenosis	2	neg	
5	ileum	aphthae	0	neg	
	R. colon	N	1	pos	
	M. colon	N	1	pos	
	L. colon	aphthae	1	pos	
	rectosigmoid	N	1	pos	
6	ileum	NI	1	neg	
	ileoascendostomy	ulcerative stenosis	1	pos	
	R. colon	N	1	pos	
	M. colon	N	1	pos	
	L. colon	aphthae	1	pos	
	rectosigmoid	aphthae	1	pos	
7	ileum	ulcerative stenosis	1	neg	
	R. colon	N	0	pos	
	M. colon	N	0	pos	
	L. colon	N	1	pos	
	rectosigmoid	N	1		
8	ileum	NI		neg	
	ileoascendostomy	ulcers	2	pos	
	R. colon	N	0	pos	
	M. colon	N	0	neg	
	L. colon	N	0	neg	
	rectosigmoid	N	0	neg	
9	ileum	N	0	pos	
	ileoascendostomy	aphthae	0	pos	
	R. colon	N	0	pos	
	M. colon	N	0	neg	
	L. colon	N	0	neg	
	rectosigmoid	radiation proctitis	0	neg	
10	ileum	NI		neg	
	R. colon	pseudopolyps	1	pos	
	M. colon	N	0	neg	
	L. colon	N	0	neg	
	rectosigmoid	N	0	neg	
13	ileum	N	1	neg	
	R. colon	erythema	2	neg	* see Methods
	M. colon	erythema	2	neg	
	L. colon	atrophy	2	neg	NI = not inspected
	rectosigmoid	sporadic ulcers	2	neg	N = normal
14	ileum	NI		pos	
	R. colon	superficial ulcers	1	pos	pos = positive
	M. colon	ulcers	2	pos	neg = negative
	L. colon	ulcers	2	neg	
	rectosigmoid	ulcers	2	neg	

TABLE 6.5 Results of SST2 immunohistochemistry 1 (biopsy and bowel resection specimens).

Patient	colon/intestines	stain	positive	negative
7	anastomosis ileo-asc.	SST2	superficial epithelium (paranuclear), esp. near infiltrate (endothelium not available)	
		conj.	none	
7	ileum resection	SST2	superficial epithelium (paranuclear) endothelium (near infiltrate or ulcers)	submucosal endothelium leukocyte-infiltrate
		rabbit	endothelium (focal) (neg. were SST2 is pos.)	epithelium
		conj.	none	
11	colon biopsy	SST2	crypt epithelium (cell membrane) macrophages (focal) capillary endothelium (++)	
		rabbit	aspec.	
		conj.	non-determined cells (paranuclear)	
11	colon resection	SST2	crypt epithelium endothelium (mucosa) endothelium (musc. ext.) and surrounding infiltrate	macrophages
		conj.	none	
14	colon biopsy	SST2	endothelium	
		conj.	none	
14	colon resection	SST2	endothelium (++) (submucosal: focal) fibroblasts / macrophages (near musc. mucosae) crypt epithelium (focal)	
		conj.	none	

Discussion

Somatostatin is a small neuropeptide, which acts in various tissues and has mainly inhibitory effects[1]. Its specific receptors are found in the the central nervous system, the immune system and the digestive tract. Five distinct SST have been identified (SST1-5), each with different binding characteristics for somatostatin and different SMS analogues including octreotide[25,26]. *In vivo* SST expression can be revealed by scintigraphy with indium-labelled octreotide. This

[^{111}In-DTPA-D-Phe^{1}]-octreotide (pentreotide) is mainly bound to subtype 2 and 5[18]. The application of [^{111}In-DTPA-D-Phe^{1}]-octreotide scintigraphy has been well established in the diagnosis and staging of neuroendocrine tumours[27]. Receptor positivity is also found in breast cancer, lung tumours, malignant lymphoma and granulomatous disease[27,28]. Normally, uptake of [^{111}In-DTPA-D-Phe^{1}]-octreotide is seen in the pituitary, thyroid, liver, spleen and both kidneys[28]. It has a largely renal clearance and only small amounts show up in the faeces[21]. [^{111}In-DTPA-D-Phe^{1}]-octreotide scintigraphy is used for staging and monitoring abdominal lesions, like gastrinomas and other neuroendocrine tumours, because these diseases show high receptor density[29]. However, a small amount of intestinal background acitivity due to faecal elimination of [^{111}In-DTPA-D-Phe^{1}]-octreotide is seen in most cases[18,30-33].

We are not aware of other studies concerning intestinal SST scintigraphy *in vivo* in IBD. In controls mainly large bowel activity was seen. This could be due to ineffective bowel cleaning, bowel imaging being related to faecal radioactivity content. In all CD patients and in one UC patient intestinal somatostatin scintigraphy with [^{111}In-DTPA-D-Phe^{1}]-octreotide was found positive. As scintigraphy and colonoscopy were performed on the same day, the effectiveness of the laxative protocol could be visually checked. In our study laxation had been sufficient in all but two patients who underwent colonoscopy. No unequivocal relation of receptor imaging and either clinical or endoscopic disease activity was observed. Normal endoscopic findings often went together with increased uptake of radioactivity. In patients with clinical IBD symptoms scintigraphy was found abnormal. Intestinal [^{111}In-DTPA-D-Phe^{1}]-octreotide uptake did not show an unequivocal relation with inflammatory changes at histologic evaluation of mucosal biopsies. Octreotide scintigraphy therefore is unsuitable for monitoring the localisation of involved intestinal segments in IBD as well as disease activity. The specificity of increased intestinal uptake of [^{111}In-DTPA-D-Phe^{1}]-octreotide in active IBD is disputable. Granulomatous

diseases as well as diseases with activated mononuclear leukocytes show SST expression. Receptor density is correlated with disease activity[5,6].

A new polyclonal anti-somatostatin receptor antibody was used in our study. This antibody is specifically bound to the SST type 2A. We were able to show that this method can be applied to formalin fixated and paraffin waxed tissue sections. In the biopsy specimens the mucosal and often also the submucosal endothelium was SST2 positive. Most IBD specimens also showed SST2 positivity at superficial epithelium and crypt epithelium. At many spots this receptor positivity was seen near leukocyte infiltrates. SST2 could not be reveiled at leukocytes, despite reported positivity *in vitro*, most likely concerning a different SST[34]. In patients consuming corticosteroids SST2 was seen in qualitively comparable amounts as in those on 5ASA monotherapy. This indicates that corticosteroids do not per se downregulate SST expression. Epithelium positivity was not only seen at the cell membranes, but also paranuclear. This could be a non-specific finding, as some of the anti-rabbit goat antibodies also showed paranuclear staining in non-determined cells. However, earlier studies also describe an increased density of SST in intestinal epithelium at autoradiography[35,36]. The effects of SMS on intestinal epithelium is associated with inhibition of regeneration, but might also have an effect on mucosal permeability or secretion[37,38].

SST are also located in the intestinal lymphoid tissue, like palatine tonsils, germinal centres of intestinal lymph nodes and Peyer's patches[39]. This receptor expression is seen in normal intestinal tissue, without inflammatory changes, and concerns most likely SST type 1 receptors[36].

TABLE 6.6 Results of SST2 immunohistochemistry 2 (biopsy specimens).

patient	biopsy	stain	positive	negative
1	colon	SST2	endothelium (++) endothelium submucosa plasma cells (paranucl.) (focal) superficial epithelium (paranucl.) crypt epithelium (focal) submucosal lymphe vessel	
		conj.	none	
2	ileo-asc. anast.	SST2	superficial epithelium (paranucl.)	
		conj.	none	
3	colon	SST2	endothelium plasma cells (paranucl.) (focal) superficial epithelium (paranucl.)	infiltrate
		conj.	none	
4	colon	SST2	endothelium	epithelium
		rabbit	non-determined cells (paranucl.)	
		conj.	none	
8	ileo-asc. anast.	SST2	endothelium superficial epithelium (paranucl.) ganglion cells (focal) fibroblast-like cells (focal)	
		conj.	none	
9	ileo-asc. anast.	SST2	none	
		conj.	none	
10	colon	SST2	endothelium superficial epithelium (paranucl.)	
		conj.	none	
12	colon	SST2	endothelium submucosal endothelium crypt epithelium fibroblasts / macrophages	
		conj.	none	
13	colon	SST2	endothelium (focal) crypt epithelium (focal) fibroblast-like cells (focal)	
		conj.	none	

In IBD, increased expression of these receptors is obvious in walls of intramucosal veins, but not in arterial walls[12]. The role of this increased receptor content still has to be disclosed. Speculations on interference with or counterregulation of pro-inflammatory, vasodilatative effects of other neuropeptides (vasoactive intestinal peptide, substance P) have been made[9]. Regulation of local blood flow in intestinal inflammation is anticipated. In our biopsy series endothelial SST positivity could be a comparable phenomenon. Although selection bias might play a role, the SST positive endothelium we saw in the bowel resection specimens was particularly located near active inflamed sites. A role in local inflammatory control is suggested, as described in intestinal granulomata in *Schistosoma mansoni* induced inflammation[40]. Inhibition of endothelial permeability by octreotide has been described[41]. Increased SST density could also result from a negative feedback effect from the diminished mucosal SMS content[42-44]. However, the same phenomenon is observed in studies of colon carcinoma. A marked density of SST has been described in surrounding vessels of human colon adenocarcinoma, without signs of inflammation[45]. In colon adenocarcinoma this increased receptor density can not be visualised by *in vivo* scintigraphy, because of interference with radioactivity in intestinal faecal content.

An interesting observation is the receptor positive fibroblast cells in UC bowel wall. SST2 have been described in lung and intestinal fibroblasts[46]. This might reflect the inhibitory function of effect of SMS on fibroblasts and histiocytes[47], but in other studies such an effect is not observed[48]. This variation of observations could be a result of the presence of different SST subtypes.

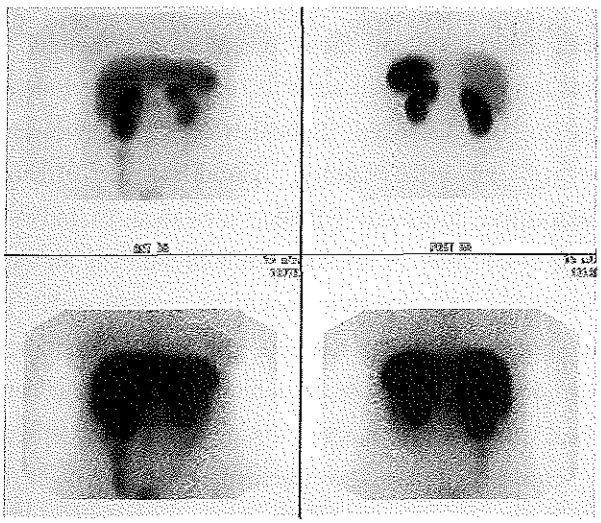

FIGURE 6.1 Octreotide scintigraphy 24 hours after injection in patient with Crohn's disease. Left panel: anterior view. Right panel: posterior view.

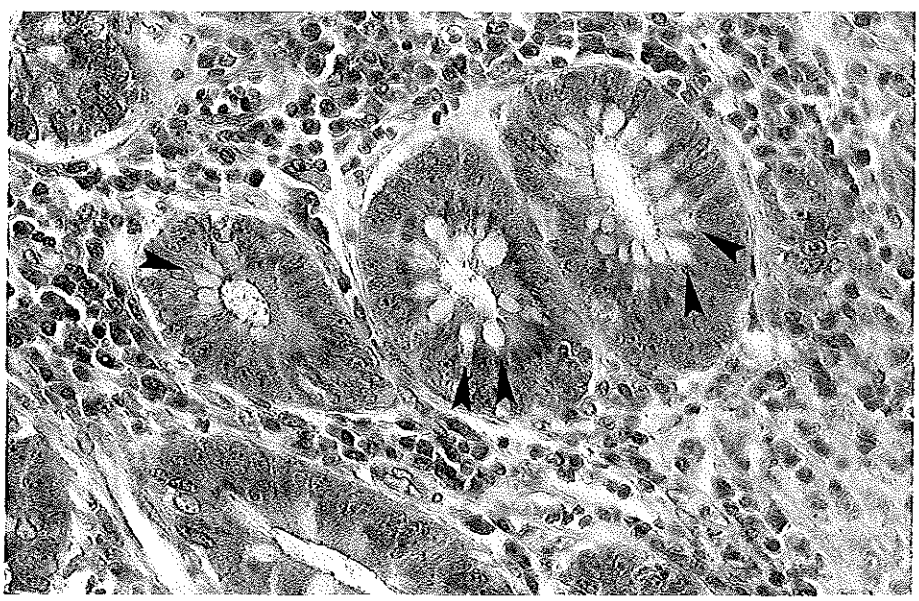

FIGURE 6.2 Colon biopsy specimen of Chrohn's disease patient with example of positive SST2A staining in crypt epithelium

In conclusion, we have demonstrated that a polyclonal anti-SST antibody can be used to demonstrate the SST type 2A positive cells, i.e. mucosal and submucosal endothelium at inflamed bowel wall biopsies, as well at superfical and crypt epithelium in active IBD. In UC a striking receptor positivity is seen at mucosal fibroblasts and macrophages. The presence of SST at these cells probably opens up new perspectives for IBD therapy with SMS or SMS analogues, which could have inhibitory effects on target cells. Intestinal uptake of [^{111}In-DTPA-D-Phe^{1}]-octreotide in IBD was observed, particularly in CD, possibly indicating increased SST type 2 density. As no relation with clinical, endoscopical or histological disease was observed, octreotide scintigraphy is not suitable for monitoring disease activity or localisation.

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CHAPTER 7

OCTREOTIDE IN PATIENTS WITH ACTIVE ULCERATIVE COLITIS TREATED WITH HIGH-DOSE CORTICOSTEROIDS (OPUS 1)

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Abstract

In ulcerative colitis the intestinal somatostatin (SOM) content is reduced. The neuropeptide SOM has several immune-inhibitory effects. In vitro SOM diminishes activity of intestinal lymphocytes and peripheral blood monocytes. Its long-acting analogue octreotide has beneficial effects on mucosal damage in acute experimental acetic acid colitis in rats.

The aim of the study was to determine the potential benefits of octreotide as treatment of patients with severe ulcerative colitis treated with high-dose corticosteroids.

We studied 42 patients with severe ulcerative colitis (more than 10 points on Powell-Tuck scoring system and mucosal disease Heatly grade III or IV). In a multi-center, double blind, placebo-controlled trial all patients were treated with oral 5-ASA (1.6-2.4 g daily) and high-dose cortico-steroids (tapering off from 60-80 mg daily). They were randomly assigned to receive subcutaneous placebo (n=22) or octreotide 500 μg (n=20) thrice daily during 21 days. Clinical and endoscopic disease activity, histology and laboratory parameters were obtained during the study period.

Clinical disease activity for both octreotide and placebo were not significantly different at baseline and after 21 days of treatment. Endoscopic disease activities (mean ± SD) changed both signficantly from 12.5 ± 4.7 to 7.2 ± 5.3 for octreotide and from 11.5 ± 5.0 to 5.0 ± 3.4 for placebo (octreotide vs placebo: NS). Seven patients from both groups received additional treatment (colectomy (n=6), cyclosporin (n=1)). Adverse events occurred equally in both groups.

Subcutaneous administration of octreotide 500 μg thrice daily is not of additional benefit as adjuvant therapy to high-dose corticosteroids in severe ulcerative colitis.

Introduction

Standard medical treatment of ulcerative colitis (UC) is based on 5-aminosalicylates (5-ASA) and corticosteroids. Many patients however fail to respond adequately to standard therapy and this has stimulated the search for other strategies[1]. Most new approaches are still experimental, but immunosuppressive drugs as cyclosporin and azathioprine are increasingly being used in clinical practice for patients who do not improve on standard therapy. Although immunosuppressives are effective in colitis, up to 35% of patients with severe UC will require a colectomy within one year[2]. Most new drugs for inflammatory bowel disease (IBD) are aimed at suppressing cytokine or eicosanoid production or effects, or at scavenging free radicals.

Recently, attention has been drawn to the possible role of neuropeptides such as substance P, vasoactive intestinal peptide, calcitonin gene related peptide and somatostatin in inflammation of the gut. Somatostatin (SMS) is particularly interesting, as it has several inhibitory effects of the immune system[3]. SMS levels in mucosa and submucosa of IBD patients were reported to be reduced[4,5]. The long acting SMS analogue octreotide attenuated colonic mucosal damage in an experimental animal model[6]. These experiments support the possible protective role of SMS or octreotide in mucosal inflammation in IBD.

Octreotide, in combination with standard therapy, should influence a broader range of inflammatory mediators and hence could be beneficial in patients with IBD. We therefore performed a multicentre, double-blind, randomised, placebo-controlled trial of octreotide added to standard treatment regimens of 5-ASA and corticosteroids in patients with severe UC, to determine whether octreotide could attenuate symptoms and avert colectomy or cyclosporine.

Patients and methods

Patient selection

Patients with UC fulfilling the in- and exclusion criteria were recruited for participation from in- and outpatient populations of participating hospitals. Hospitals qualified if they had at least 8 patients with severe UC per year, the possibility of videotaped colonoscopy and proven experience with GCP clinical studies. All hospital Ethical Committees approved the protocol. Patients were selected for study if they were aged 18 to 65 years, suffering from clinical active UC with $\geq$ 10 points on Powell-Tuck scoring system[7] and mucosal disease activity grade III or IV (Heatly) at baseline[8]. Not more than 2 days elapsed between endoscopy at baseline and the start of therapy. Patients with other causes of colitis, especially proven Crohn's disease (biopsy diagnostic for Crohn's disease, and/or small bowel involvement, and/or intestinal fistulae, and/or skip lesions on colonoscopy), and patients with a history of alcohol or drug abuse, or with concomitant microbial infection (Salmonella, Shigella, Yersinia, Campylobacter jejuni, presence of parasite eggs or cysts), heart, lung, kidney or liver disease were excluded from the study. Patients who used other medication for treatment of IBD were excluded, except for 5-ASA and corticosteroids, or at least 3 months of constant dose of azathioprine. Pregnancy was excluded and patients were encouraged to take contraceptive measures.

Treatment protocol

After obtaining informed consent patients were randomly assigned to one of two treatment groups. In a double blind fashion either octreotide (500 μg) or placebo was administered subcutaneously three times daily. After baseline assessments the trial drugs were given during a maximum period of 21 days. Concomitant treatment consisted of an oral daily dose of 1.6 to 2.4 g 5-ASA and prednisone or prednisolone once daily intravenously or orally, starting with 60 - 80 mg in the first week of treatment, tapering off by 10 to 20 mg per week. If needed, at any

point surgery would be performed or therapy with immunosuppressive drugs (e.g. cyclosporin) would be added. Patients were allowed to take all medication outside the centre, provided that they were able to self-administer the subcutaneous injections and that sufficient medical control on this procedure could be warranted.

Clinical disease activity

Clinical disease activity was evaluated at baseline and during therapy (study day 1 to 10 and 21). Grading was done according to the Powell-Tuck scoring system, including the rating off well being, abdominal pain, bowel frequency, stool consistency, bleeding, anorexia, nausea or vomiting, abdominal tenderness, eye, mouth, joints or skin complications, body temperature and biochemical parameters[7]. Safety assessments included recording of all adverse events, clinical laboratory tests and vital signs.

Endoscopy

Disease activity was assessed at baseline and during therapy (study day 10 and 21) by video or photo-recorded colonoscopy or sigmoidoscopy. In a standardised procedure the endoscope was introduced as far as the level of transition between normal and abnormal mucosa. Endoscopic disease activity was analysed by a blinded end-point committee, comprised of three experienced endoscopists, that reviewed all videotapes. Endoscopic grading of the most inflamed part involved erythema, vascular pattern, friability, granularity, spontaneous bleeding, occurrence and severity of ulcers, extent of ulcerated surface and presence of mucopurulent exudate. All parameters were scored from 0 to 2 points. Four grades of activity were recognised according to the sum of all parameters: inactive disease (0-3), mild disease (4-7), moderate disease (8-12) and severe disease (13-18).

Laboratory

Histologic disease activity by biopsy for confirmation of UC was assessed at baseline and during therapy (study day 21). Biochemical and haematological parameters indicating disease activity were measured at baseline and during therapy (study day 1, 3, 7, 10 and 21). These parameters consisted of haemoglobin, leukocyte count (including differential counts), orosomucoid or C-reactive protein, and albumin.

Final analysis

All clinical evaluable patients were included in the standard efficacy analysis. Primary parameters of efficacy were time to reduction (more than 3 points for at least 3 consecutive treatment days) in clinical disease activity during the first 10 days of therapy, and endoscopic and clinical disease activity as assessed on day 10 and 21. Secondary parameters of efficacy were time to surgery decision within the study period (op to day 21) and time to addition of therapy with immunosuppressives. Efficacy was also evaluated in an additional intention-to-treat analysis, including all patients in whom the study was initiated. Data were descriptively analysed. ANOVA or Kruskal-Wallis analysis of variance were used to test for overall differences between the groups. Further testing was done by t-test or Mann-Whitney (U) test / robust rank-order test.

TABLE 7.1 Patients characteristics.

	octreotide	placebo
N	20	22
M / F	13 / 7	13 / 9
mean age ± SD (yr)	35 ± 10	42 ± 12
therapy at study start		
5-ASA / SASP	20	22
topical corticosteroids	1	1
oral corticosteroids	4	9
azathioprine	1	1

Results

Patients characteristics

Inclusion criteria were met in 42 patients. Twenty patients were randomised to octreotide treatment, 22 received placebo (TABLE 7.1). All patients had been treated with oral 5-ASA, 15 also had been taken corticosteroids and 2 had been on azathioprine. Three patients from the placebo group were withdrawn from study: 1 patient on her own request due to unbearable disease activity, 1 for Campylobacter enteritis, 1 for oliguria. Adverse events were reported in 12 and 9 patients from octreotide and placebo treated groups respectively (TABLE 7.2). Two severe adverse events (disease exacerbation and non-toxic colon dilatation) were seen.

TABLE 7.2 Adverse events.

octreotide (n=12)	placebo (n=9)
hyperglycaemia	urticaria
pain, redness and swelling around injection site	progressive diarrhoea
tonsillitis	ancle oedema
abdominal bloating	blurred vision
non-toxic dilatation transverse colon	nausea
paresthesia in the tongue	headache
swelling of cervical lymph nodes	oliguria
flushing	Campylobacter enteritis
erythema nodosum	progressive abdominal cramps and diarrhoea
pyoderma gangrenosum	

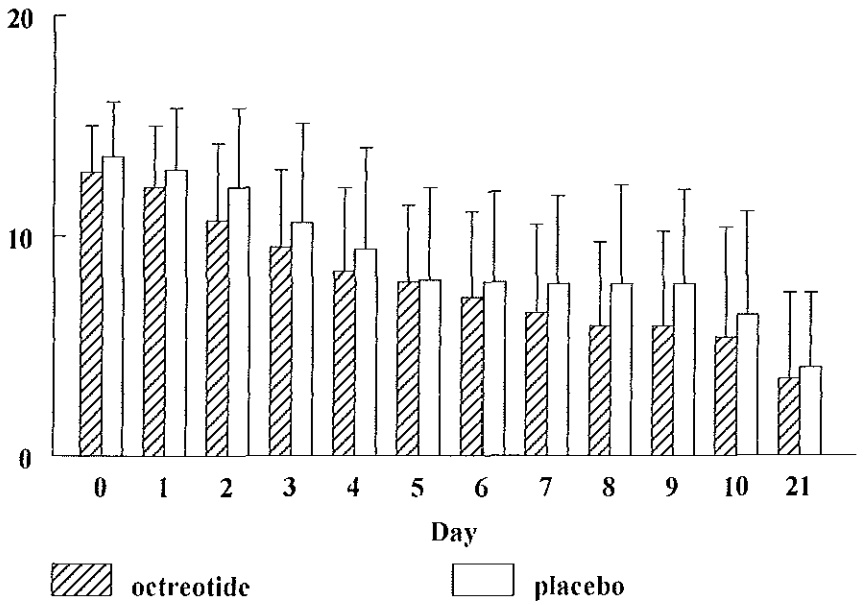

FIGURE 7.1 Clinical disease activity, Powell-Tuck Score, means and standard deviation per assessment day for total scores.

Clinical disease activity

Mean clinical disease activity (95 % confidence interval) at first assessment was 12.9 (12.0-13.8) for octreotide and 13.6 (12.6-14.6) for placebo treated patients (NS). During the treatment period 7 patients received additional treatment for unresponsive disease to conservative management (octreotide n=3, placebo n=4). No differences in time to additional treatment were seen between the 2 groups. One of these patients received cyclosporin after 2 weeks (octreotide group), all others underwent surgery after 2 to 3 weeks. A similar decrease of clinical disease activity was seen in both groups (FIGURE 7.1). At day 10 mean clinical disease activity (95 % confidence interval) was 5.4 (3.1-7.7) for octreotide and 6.4 (4.1-8.7) for placebo, at day 21 these scores were 3.5 (2.1-4.9) and 4.0 (2.3-5.7) respectively (NS). No significant differences of individual scoring parameters were seen, even not for bowel frequency (FIGURE 7.2).

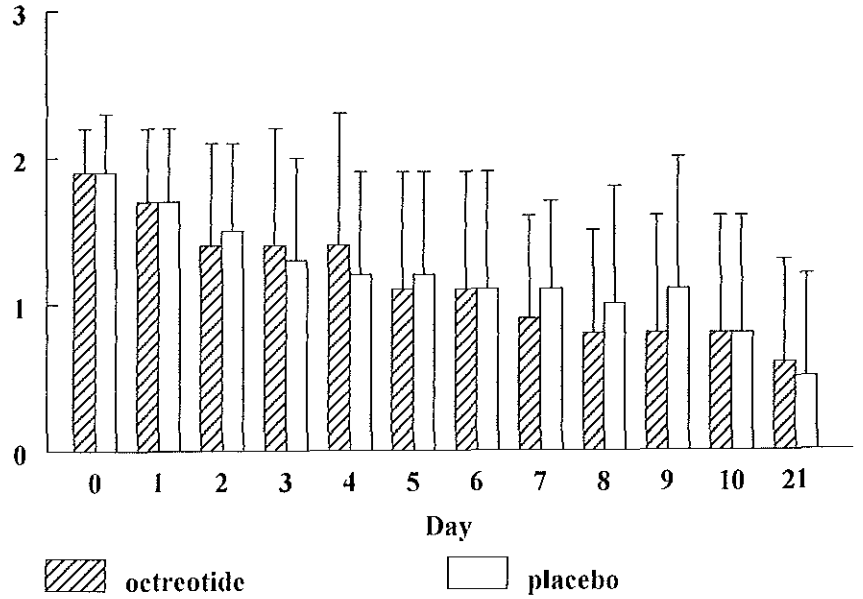

FIGURE 7.2 Powell-Tuck Scoring system, means and standard deviation for bowel frequency.

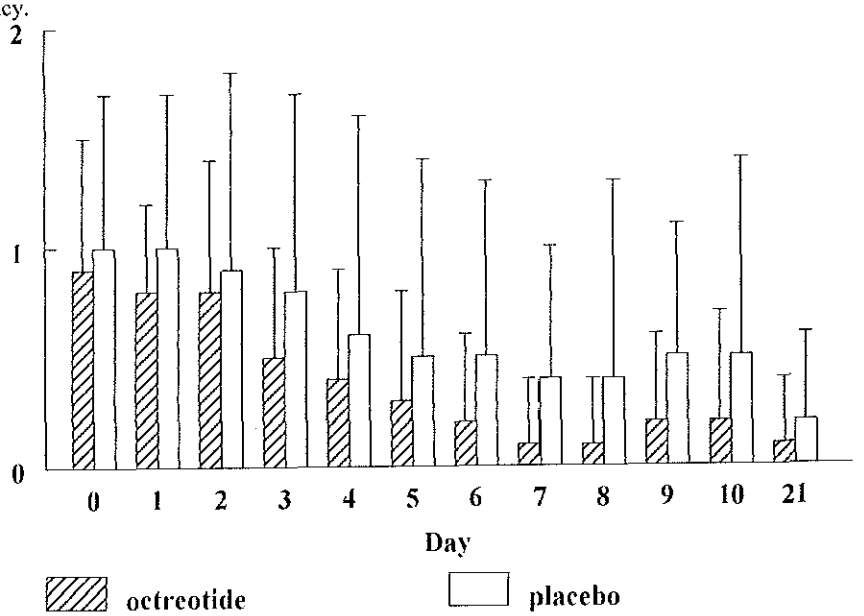

FIGURE 7.3 Powell-Tuck Scoring system, means and standard deviation for abdominal tenderness.

Abdominal tenderness tended to decline more rapidly in the octreotide group (FIGURE 7.3, NS). Significant reduction of clinical disease activity (more than 3 points for at least 3 consecutive treatment days) was seen in 17 octreotide and 15 placebo treated patients (NS). Time to reduction in clinical disease activity during the first 10 days of therapy was 5.6 (4.5-6.7) and 5.7 (4.9-6.5) days respectively (NS).

Endoscopic disease activity

From 24 patients (11 octreotide) endoscopy videos were obtained at baseline, at day 10 and 21. Mean endoscopic scores decreased during treatment. No significant differences between the 2 treatment groups were seen on either point of evaluation (TABLE 7.3).

TABLE 7.3 Endoscopic disease activity score mean scores and 95 % confidence intervals.

	octreotide	placebo
baseline	12.5 (10.4-14.6)	11.5 (9.4-13.6)
day 10	9.2 (6.6-9.6)	7.8 (6.0-9.6)
day 21	7.2 (4.9-9.5)	5.0 (3.6-6.4)

Laboratory assessments

Increase of fasting glucose levels was seen in 1 octreotide and 1 placebo patient, values respectively 8.9 and 11.6 mmol/L. Two patients in the octreotide group showed slight increase of ALAT/ASAT and gamma GT (> 2x upper limit of normal). Mean C-reactive protein decreased during treatment. No significant differences between the two groups were seen concerning haemoglobin, leukocytes, albumin, C-reactive protein or orosomucoid.

Discussion

The treatment of severe UC is a challenge for clinicians. High-dose corticosteroids, administration of other immunosuppressive drugs, like cyclosporin, are often inevitable. Despite intensive immunosuppressive therapy up to 30% of these patients are non-responders or poor-responders and need colectomy, immediately or after several months[2,9,10]. Therefore, developing new therapeutic strategies for acute severe UC is warranted.

Pro- as well as anti-inflammatory actions of neuropeptides have been described. SMS is a key immunoinhibitory neuropeptide and its clinical anti-inflammatory properties have been demonstrated[3,11]. Proliferation and cytokine release of intestinal lymphocytes is inhibited by SMS and SMS analogues[12-15]. An increase of SMS receptors has been observed in germinal centres of the gut associated lymphoid tissue[16] and there is close contact of receptor bearing cells with developing immunocytes[17]. A decrease of mucosal SMS concentration in active IBD has been observed[4,18,19]. This only holds for the colon in UC or Crohn's colitis, as no differences of SMS content was seen in normal and diseased terminal ileum in Crohn's disease[4]. Disease activity and SMS content are inversely related[4,5]. Octreotide was found to be effective in inhibiting the intestinal inflammation in acetic acid-induced colitis in rats giving a significant decrease of primary as well as secondary inflammatory mediators[6]. Other clinical and experimental studies provide evidence for an anti-inflammatory effect of SMS analogues, which may potentiate the effects of corticosteroids[11,20,21].
Case reports on successful treatment of intestinal inflammatory disorders have been published[22,23]. However, as far as we know, controlled clinical trials of octreotide in the treatment of IBD have not been performed. In our trial we administered octreotide as an adjuvant therapy to high dose corticosteroids in severe UC. Effects of adjuvant octreotide did not differ from placebo, as measured by a decrease of clinical disease activity, laboratory parameters and

endoscopic disease. Octreotide did not affect leukocyte count. Hyperglycaemia was only observed in 1 patient, who was previously known to have a corticosteroid-induced diabetes. When comparing all individual parameters in the clinical disease activity score (Powell-Tuck) a trend towards a more rapid decline of abdominal pain and tenderness was observed. However, statistical significance was not reached. As octreotide changes visceral pain perception and reduces inflammatory hyperalgesia, this could be an explanation for this phenomenon[24,25]. An interesting finding is that octreotide did not affect bowel frequency and stool consistency, suggesting a lack of interaction with colon resorptive capacity and muscular activity.

In conclusion, we found no additional benefit of octreotide administration in patients with severe UC, who are treated with high-dose corticosteroids. Octreotide did not improve response to corticosteroid therapy. The already low incidence of surgical procedures and administration of cyclosporin did not decrease.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION AND CONCLUSIONS

Somatostatin (somatotropin release inhibiting factor, SMS) has a large number of biological effects, acting mainly as an inhibitory neuropeptide. SMS is present in the central and peripheral nervous system, the digestive tract and the immune system. Several *in vitro* and *in vivo* studies have shown that SMS is involved in inflammation. The studies described in this thesis were aimed at determining whether SMS plays a role in the modulation of intestinal inflammation and whether SMS analogues might be of benefit in the treatment of human idiopathic inflammatory bowel disease. As reviewed in chapter 2, there is good evidence that SMS inhibits cytokine release by several types of immunocytes, including the intestinal epithelial lymphocytes. SMS or SMS analogues may modulate intestinal inflammation, at least in animal models[1-3]. In human inflammatory bowel diseases (IBD), the mucosal SMS content is decreased, and this could possibly contribute to ongoing inflammation. Human white blood cells, including intestinal lymphocytes, have SMS receptors, and it is likely that suppressive effetcs of SMS on leukocyte function are at least in part specific receptor-mediated effects. Intestinal leukocyte proliferation and gammaglobulin production are inhibited by SMS. As most studies concern test tube conditions, the overall effects of SMS in intestinal inflammation when administered to human subjects is not well known. In an animal model, the SMS analogue octreotide attenuates acetic acid-induced colon inflammation[3]. Although in that study the colonic level of secondary immunologic mediators (platelet activating factor, leukotriene B_4) was decreased, no evidence is given of direct interaction between SMS or octreotide and immunocytes. It is possible that a non-immunological mechanism might be involved in the beneficial effect of octreotide in acute acetic acid-induced colitis. Pancreas proteolytic enzyme level is increased in faeces of patients with active colitis[4]. Octreotide suppresses pancreas secretion[5], resulting in lower intraintestinal levels of proteolytic enzymes. This could make the colon mucosa less susceptible to acetic acid.

Cytoprotective effects of SMS, possibly related to diminished pancreas secretion, have been reported in experimental pancreatitis[6]. Other protective effects may result from increased blood perfusion, as has been described in several experimental pancreatitis, liver ischemic models and experimental intestinal ischemia studies[7-9].

Table 8.1 Dextran sulphate sodium-induced colitis: relevance of animal model compared to human inflammatory bowel disease.

	DSS-colitis	ulcerative colitis	Crohn's disease
bacterial involvement	+	+	+?
genetic influence	+	+	+
primary macrophage dysfunction	+	?	?
TH lymphocyte pattern in subacute phase	TH_2	TH_2	TH_1
patchy inflammation of colon	+	-	+
small bowel lesions	-	-	+
malignant degeneration in chronic phase	+	+	+

Dextran sulphate sodium (DSS)-induced colitis in rodents is a very reproducible model of experimental colitis. Colitis is induced by dissolving DSS 10 % (weight/volume) in the drinking water of laboratory animals (BALB/c mice). The model has several similarities with ulcerative colitis (TABLE 8.1). DSS-induced colitis is characterised by neutrophil infiltration, loss of crypt architecture and drop-out of mucosal goblet-cells. Granulomatous inflammation is not seen and inflammatory changes are mild and superfical. The entire colon is inflamed, but inflammatory changes can be patchy, in contrast to ulcerative colitis. As in human ulcerative colitis, DSS colitis is associated with low colon levels of interleukin-10, probably reflecting TH_2-lymphocyte dysfunction. In a recent study administration of IL-10 was found to reduce inflammation in DSS-induced colitis, supporting a TH_2-lymphocyte dysfunction[10]. Others report an increase of predominantly TH_1 activity in chronic DSS colitis[11]. The exact

mechanism of DSS-induced colon damage is still not entirely understood. It has been suggested that DSS interferes with colon macrophage function, which is impaired by DSS (FIGURE 8.1)[12,13].

In chapter 3 and 4 we found supportive evidence for this, reflected by low colon IL-6 levels. This is in contrast with the elevated intestinal mucosal and intestinal fluid IL-6 levels reported in human ulcerative colitis[14,15]. Macrophage dysfunction results in an inadequate response to bacterial invasion and subsequent inflammation of the colon mucosa[16,17]. Other mechanisms may be involved. DSS might change mucosal epithelial integrity and permeability, but this is rather speculative[18]. Activation of non-intestinal parts of the immune system could be involved, inducing intestinal inflammation from peripheral blood. Our results do not support commitment of peripheral leukocytes as cytokine content of thymus and spleen did not change in DSS treated mice.

Neuropeptides may be important in development of DSS-induced colitis. Nerve damage by capsaicin intensifies colonic inflammation by DSS[19]. It is not known whether colon SMS content is affected in DSS-induced colitis. In a different experimental colitis model (trinitrobenzene sulphonic acid) reflecting acute inflammation and severe mucosal damage, colonic wall SMS immunoreactivity was only slightly affected[20]. Other neuropeptides (substance P, vasoactive intestinal polypeptide, calcitonin gene related peptide) promote inflammation in different experimental inflammatory models and are involved also in IBD[21-26]. SMS could possibly oppose the pro-inflammatory activity of other neuropeptides like substance P[27].

In acute DSS-induced colitis the inflammation score, which is largely determined by mucosal neutrophil infiltration, was diminished by SMS if administered before DSS exposure, as described in Chapter 3. The mechanism of this protective effect is not known. Leukocyte migration may hampered by SMS[28,29], although conflicting data exist[30,31].

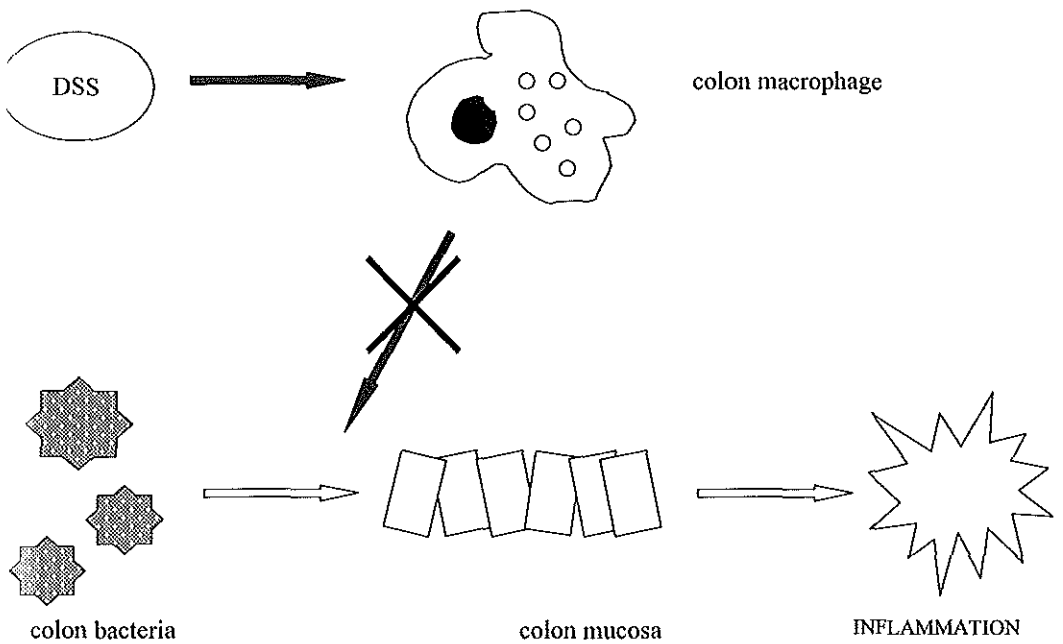

FIGURE 8.1 Proposed pathophysiological mechanism of dextran sulphate sodium-induced colitis: DSS hampers colon mucosal macrophage opsonisation, so that luminal bacteria are able to enter colon mucosa and induce mucosal inflammation (shaded arrows: inhibition).

Interference of SMS with leukocyte adhesion may be involved[32-34], probably by decrease of adhesion molecules[35,36]. Direct inhibition of intestinal lymphocytes is not likely as T-cell derived interleukin levels are not affected by SMS. We found that SMS further reduced already low colon IL-6 concentrations in DSS-induced colitis, probably reflecting macrophage inhibition added to the effect of DSS. Inhibition of macrophage activity by SMS has been reported before[37,38].

In chapter 4, neither SMS nor its long-acting analogue octreotide were found to attenuate intestinal neutrophil infiltration in sub-acute DSS-induced colitis, when administered simultaneously with DSS during seven days. Animals receiving SMS or octreotide without DSS even showed enhanced mucosal and submucosal neutrophil content. Although SMS under certain circumstances promotes *in vitro* leukocyte migration, the mechanism of this phenomenon is not well understood. Colon wall interferon (IFN)-γ was not detectable, probably not playing an important role at the studied stage of DSS-induced colitis. In the subacute DSS model, the already low IL-1β, IL-6 and IL-10 levels were further reduced by SMS and octreotide. That may imply that prolonged SMS administration impairs TH_2-lymphocyte function. IL-10 is a key inhibitory cytokine in intestinal inflammation[39] and severe colitis develops in IL-10 knockout mice[40]. IL-10 treatment may be of benefit as well in DSS-induced colitis as in human IBD[10,41,42]. Low SMS-induced colon tissue IL-10 levels could possibly aggravate DSS-induced inflammation in the post-acute phase. Future research could focus on the influence of SMS or SMS analogues on the release of secondary mediators like prostaglandins, leukotrienes and nitric oxide in the chronic stage of DSS-induced colitis, and the evolution of chronic DSS colitis, which develops after repeated exposure to DSS alternating with normal drinking water. In that scope colon carcinoma development after several months of DSS-exposure is interesting[43,44], as inhibition of human colon carcinoma growth by SMS analogues has been reported[45,46].

Frequent bowel movements are common in active IBD, and are often difficult to manage. Several clinical studies have shown a beneficial effect of octreotide on intractable diarrhoea of various causes[47]. SMS may modulate bowel secretion and activity of enteric nerves, smooth muscle cells and immune cells. Most research in this field concern SMS affecting small bowel secretion and motility. In Crohn's disease of the small bowel SMS certainly can be of value as antidiarrhoeal agent[48]. Its role in ulcerative colitis still has to be clarified. Human

studies showed increase of transit time in the normal colon during administration of SMS or octreotide. As far as we know, no studies of SMS treatment in colitis have been published before. The DSS model is suitable for studying inflammation-induced muscle dysfunction, without (visible) muscular damage. In earlier studies in DSS-induced colitis we found a marked decrease of contraction by carbachol (CARB) and salbutamol (SALB)-derived relaxation in longitudinal colon segments[49].

In chapter 5, in an organ bath model with longitudinally mounted transverse colon segments from BALB/c mice with superficial, sub-acute DSS-induced colitis, a diminished susceptibility for CARB and SALB was found, as reflected by an increase of the dose inducing half-maximum contraction or relaxation ($EC50_{CARB}$ and $EC50_{SALB}$). This may contribute to proximal colon stasis, as is observed in human ulcerative colitis. The DSS-induced decrease of contractibility may reduce colon reservoir function, which largely depends on longitudinal muscle length (cylinder volume = $\pi r^2 \cdot 0.5h$). SMS in the normal mice diminished $EC50_{CARB}$ values. In the inflamed colon SMS or octreotide returned $EC50_{CARB}$ values to normal, thus preserving smooth muscle contraction susceptibility. $EC50_{SALB}$ increased by one week of DSS exposure. Octreotide did not influence this value. SMS enhanced $EC50_{SALB}$ both in control and inflamed mice, reducing β-adrenergic receptor sensitivity. SMS-induced increase of $EC50_{SALB}$ value might promote smooth muscle contractile activity in DSS-induced colitis as well. As the muscular layer in DSS-induced colitis is intact, this modulation of smooth muscle contraction agonist and antagonist susceptibility should concern the muscarinic and β-adrenergic receptor or post-receptor events. SMS has its own receptors (SSTR type 1 to 5) and interference with muscarinic or adrenergic receptors is most likely at the post-receptor level, like competition for same cell membrane bound G-proteins or intracellular cyclic AMP or cyclic GMP[49-51].

Parallel to a decreased concentration of SMS an upregulation of SMS receptor (somatostatin receptor, SSTR) expression has been found at specific sites in the inflamed intestinal wall. SSTR are cell membrane bound. In autoradiographic studies these SSTR are found at vascular structures near sites of inflammation in the IBD gut wall[52,53], not specifying receptor subtypes. Although it is not fully understood yet, the type 2 and 3 receptor might be the most important SSTR in gastrointestinal tissue, and octreotide has high affinity for SSTR2[54]. SSTR type 2 can be visualised by octreotide scintigraphy, which is by now a valuable method of *in vivo* SSTR detection in neuroendocrine tumors and several granulomatous diseases. Results of receptor studies are described in chapter 6. Scintigraphy was performed with [^{111}In-DTPA-D-Phe^1]-octreotide, that normally does not show intestinal wall uptake. Enhanced bowel radioactivity was observed in patients with active IBD. No relation was found between clinical, endoscopic or histologic disease activity. *In vitro* immunohistochemistry using a polyclonal rabbit anti-somatostatin receptor 2A antiserum reveiled SSTR at the intestinal superficial and crypt epithelium cells and endothelial cells of active inflamed intestinal sites. SSTR were also found at fibroblast-like cells, especially in ulcerative colitis. Upregulation of SSTR can be a result of feedback from the decreased intestinal SMS concentration in inflammation. As these studies were purely descriptive, there only can be speculation on the function of this SSTR expression. SMS could impair endothelial proliferation[55] or endothelial leukocyte adherence in IBD[56]. Vascular SSTR may play a role in SMS-derived prevention of intestinal ischemia[7], which could benefit intestinal regeneration.

In chapter 7, in a multi-centre trial the effect of octreotide in severe human ulcerative colitis, treated with high-dose corticosteroids, was studied in 42 patients. Subcutaneous administration of 500 μg octreotide thrice daily during the first three weeks of therapy did not show differences from placebo. No differences were seen between octreotide and placebo treated subjects in clinical or endoscopic response. A tendency towards quicker resolving of abdominal

tenderness was seen in the octreotide group. Based on these results, at this point octreotide can not be recommended as adjuvant to corticosteroids in severe ulcerative colitis, although it may possibly be helpfull in reducing abdominal pain in these patients[57-59]. The benefit of SMS analogues on prevention of IBD exacerbations, fistula and fibrosis development and diarrhoea in IBD could possibly be evaluated in future trials. The recent introduction of the microencapsulated octreotide acetate, which provides long-term octreotide release from a single intramuscular injection once in a month, is an attractive alternative to the present formulation and could make future studies more easy to perform.

A weave of immune processes, including a very large number of cytokines, eicosanoids, neuropeptides and other soluble messengers, mediates intestinal inflammation. SMS is a single component of these combined, intricate processes. In active IBD, SMS receptor expression seems enhanced, as shown either by immunohistochemistry or octreotide scintigraphy. Octreotide scintigraphy does not appear to be suitable for monitoring disease acitivity, as enhanced uptake does not seem related with endoscopic or histologic grading of inflammation. SMS may prevent acute intestinal inflammation. However, both in animal and in human colitis, treatment with SMS or the long acting SMS analogue octreotide did not attenuate already active inflammation. SMS therefore cannot at present be recommended for the treatment of ulcerative colitis. Its potential in modulation of disturbed motility and the application in patients with intestinal fistulae deserve further study, as does the more speculative possible indication as preventing rather than treating relapses and influencing malignant degeneration in chronic colitis.

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CHAPTER 9

SAMENVATTING EN DISCUSSIE

Samenvatting en discussie *

Het neuropeptide somatostatine (SMS) heeft een voornamelijk remmend effect op tal van biologische processen. Het komt vooral voor in het centrale en perifere zenuwstelsel, het immuunsysteem en het spijsverteringsstelsel. Uit verscheidene studies, zowel *in vitro* als *in vivo*, blijkt dat SMS betrokken is bij diverse ontstekingsprocessen. In dit proefschrift wordt onderzocht of SMS een rol speelt bij ontstekingsprocessen van het darmstelsel, meer in het bijzonder van het colon. Daarbij is uitgegaan van de gedachte dat SMS of analoge preparaten wellicht van belang kunnen zijn bij behandeling van inflammatoire darmziekten (inflammatory bowel disease, IBD), zoals colitis ulcerosa en de ziekte van Crohn. Uit de literatuurstudie in hoofdstuk 2 blijkt dat SMS de vrijmaking van cytokines uit diverse immunocyten, waaronder intestinale epitheliale lymfocyten, vermindert. SMS en SMS-analoga remmen ontstekingsprocessen in diverse diermodellen[1-3]. De hoeveelheid SMS in het slijmvlies van de darm is verlaagd bij humane IBD, wat mogelijk bijdraagt aan voortgaande slijmvliesontsteking. Receptoren voor SMS worden gevonden op de celmembranen van humane leukocyten, zowel in het perifere bloed als in het darmslijmvlies. De proliferatie en de gammaglobulineproductie van intestinale leukocyten wordt door SMS geremd, zoals blijkt uit verscheidene *in vitro* experimenten. In een diermodel waarin colitis wordt veroorzaakt door azijnzuur, brengt het lang-werkend SMS-analogon octreotide de intensiteit van darmontsteking terug[3]. Daarbij daalt de concentratie van secundaire ontstekingsmediatoren (platelet activating factor, leukotriene B_4) in het colon. Dit kan het gevolg zijn van directe interactie van octreotide met immunocyten, maar ook door invloed op niet-immunologische processen. Octreotide onderdrukt namelijk ook de secretie van proteolytische pancreasenzymen, waardoor de gevoeligheid van colonslijmvlies voor azijnzuur wellicht afneemt[4,5]. Daarnaast zijn uit onderzoek bij experimentele pancreatitis cytoprotectieve effecten van SMS bekend[6]. Ook de invloed op doorbloeding kan

* voor referenties en figuren: zie Chapter 8

een beschermende rol spelen, zoals bij experimentele pancreatitis, in modellen van leverischemie en bij experimentele darmischemie[7-9].

Wanneer knaagdieren via hun drinkwater worden blootgesteld aan dextraan-natriumsulfaat (dextran sulphate sodium, DSS) in bepaalde concentraties, ontstaat een vrij milde colitis. Dit colitismodel is goed reproduceerbaar en heeft kenmerken van colitis ulcerosa (TABEL 8.1). De studies uit dit proefschrift betreffen een muismodel (BALB/c muis), waarin colitis wordt veroorzaakt door DSS 10 % (gewicht/volume) in drinkwater. DSS-colitis wordt gekenmerkt door toegenomen mucosale influx van neutrofiele granulocyten, verstoorde cryptarchitectuur en verlies van slijmbekercellen. De ontsteking is doorgaans mild en betreft de oppervlakkige slijmvlieslagen. Granulomen worden niet aangetroffen. Meestal is het gehele colon ontstoken, maar in tegenstelling tot colitis ulcerosa wordt aangedaan met normaal slijmvlies frequent afgewisseld. Net als bij humane colitis ulcerosa is bij acute DSS-colitis de concentratie van interleukine-10 (IL-10) colonslijmvlies verlaagd. Dit ontstaat mogelijk door verminderde activiteit van T-helper$_2$ (T_{H_2})-lymfocyten[10]. Bij chronische DSS-colitis blijkt een verhoogde T_{H_1}-functie[11]. Nog niet alles is bekend van de pathogenetische mechanismen van DSS-colitis. Aangenomen wordt dat DSS de functie van colonmacrofagen beperkt (FIGUUR 8.1)[12,13]. In hoofdstuk 3 en 4 wordt steun gevonden voor deze hypothese. Er treedt een verlaging op van het voornamelijk door macrofagen geproduceerde IL-6 in het colonslijmvlies bij DSS-colitis. Dit staat in contrast met verhoogde IL-6 concentraties in colonmucosa en darminhoud bij colitis ulcerosa[14,15]. Verminderde activiteit van macrofagen geeft darmbacteriën kans de colonmucosa binnen ter dringen, wat leidt tot slijmvliesontsteking[16,17]. Daarnaast bestaat de mogelijkheid dat DSS de permeabiliteit van het colonslijmvlies vergroot[18]. Ook zouden extra-intestinale delen van het immuunsysteem bij het ontstaan van DSS-colitis betrokken kunnen zijn, waarbij darmontsteking door invloeden vanuit het perifere bloed ontstaat.

Betrokkenheid van organen als thymus en milt komt echter niet naar voren uit onze studies.

Er zijn aanwijzingen voor betrokkenheid van het enterale zenuwstelsel c.q. enterale neuropeptiden bij DSS-colitis. De ernst van deze colitis neemt toe wanneer capsaïcine de uiteinden van enterale zenuwcellen beschadigt[19]. Het is evenwel niet bekend of SMS betrokken is in DSS-colitis. In een ander ontstekingsmodel, met acute, hevige colitis door trinitrobenzeen sulfonzuur, is het SMS-gehalte van het colon slechts licht verlaagd[20]. Andere neuropeptiden (substance P, vasoactive intestinal peptide, calcitonin gene related peptide) hebben een ontstekingsbevorderend effect in verscheidene ontstekingsmodellen en mogelijk ook bij IBD[21-26]. SMS antagoneert in sommige opzichten het pro-inflammatoire effect van substance P[27].

Zoals in hoofdstuk 3 wordt beschreven, heeft SMS een preventief effect op de influx van neutrofiele granulocyten in het colonslijmvlies. Hierdoor daalt de histologische ontstekingsscore van DSS-colitis. Het onderliggende mechanisme is nog onbekend. Uit de literatuur komen tegengestelde berichten over de invloed van SMS op migratie van leukocyten naar voren. Zowel remmende als stimulerende effecten worden beschreven[28-31]. SMS kan mogelijk interfereren met leukocytenadhesie[32-34], zoals door verlaging van adhesiemoleculen[35,36]. Uit onze studies blijkt geen direct remmend effect van SMS op intestinale lymfocyten, omdat T-lymfocyt-afhankelijke cytokineproductie niet wordt beïnvloed. Reeds door DSS verlaagde IL-6 waarden in het colonslijmvlies worden nog lager na toedienen van SMS. Dat kan samenhangen met een remmend effect op macrofagenactiviteit, zoals eerder door anderen gerapporteerd[37,38].

SMS noch octreotide zijn van invloed op ontstekingsactiviteit in sub-acute DSS-colitis, die ontstaan is na 7 dagen DSS toediening (hoofdstuk 4). Er is zelfs enige

toename van neutrofiele granulocyten in het colonslijmvlies van muizen die SMS of octreotide kregen zonder blootstelling aan DSS. Zoals hierboven reeds vermeld, is dit een merkwaardige tegenstelling tot eerdere bevindingen bij de acute DSS-colitis. In dit stadium van DSS-geïnduceerde colitis lijkt interferon (IFN)-γ geen belangrijke rol te spelen, getuige de niet te detecteren waarden in het colonslijmvlies. SMS en octreotide brengen de reeds verlaagde spiegels van IL-1β, IL-6 en IL-10 bij sub-acute DSS-colitis verder terug. Dit kan wijzen op TH_2-dysfunctie door toediening van SMS. IL-10 speelt een rol bij darmontstekingen[39,40] en heeft mogelijk een heilzame werking bij DSS-colitis en humane IBD[10,41,42]. Een verlaagde IL-10 concentratie zou mogelijk in de post-acute fase van DSS-colitis een ontsteking in de hand kunnen werken. Toekomstig onderzoek zou de invloed van SMS op secundaire ontstekingsmediatoren (prostaglandines, leukotriënen, stikstofoxide) bij DSS-colitis kunnen belichten, evenals de invloed op ontstekingsintensiteit bij chronische DSS-colitis, die ontstaat door herhaalde blootstelling aan DDS alternerend met normaal drinkwater. In dit verband is ook de ontwikkeling van coloncarcinomen tijdens langer bestaande DSS-colitis interessant[43,44]. SMS-analoga remmen groei van adenocarcinoma[45,46].

Bij IBD patiënten is frequente defaecatie vaak lastig te behandelen. Uit diverse klinische studies blijkt een gunstig effect van octreotidetoediening op moeilijk te behandelen diarree[47]. Daarbij wordt mogelijk de secretie van darmsappen, de activiteit van enterale zenuwcellen of intestinale gladde spiercellen beïnvloed. Het meeste onderzoek heeft zich geconcentreerd op dunne darmsecretie en –motiliteit. SMS wordt toegepast bij behandeling van diarree bij de ziekte van Crohn van de dunne darm[48]. Een eventuele toepassing bij colitis ulcerosa is nog onbekend. Het DSS-colitismodel is geschikt voor onderzoek naar functie van gladde spieren van het colon door ontsteking, vanwege de oppervlakkige weefselschade, waarbij de colonmusculatuur geheel intact blijft. Uit eerder onderzoek bij DSS-colitis blijkt een duidelijke afname van contractie door

carbachol (CARB) en relaxatie door salbutamol (SALB) van longitudinale colonsegmenten[49].

In een orgaanbadopstelling worden longitudinale colonsegmenten uit een muis met sub-acute DSS-colitis onderzocht, zoals beschreven in hoofdstuk 5. Er is een verminderde gevoeligheid voor CARB en SALB, zoals blijkt uit een toename van de dosis die 50% van de maximale contractie respectievelijk relaxatie geeft ($EC50_{CARB}$ en $EC50_{SALB}$). Een relatieve ongevoeligheid voor contractie-agonisten geeft stase van de coloninhoud, wellicht equivalent aan de toename van proximale colon transit tijd bij humane colitis ulcerosa. Daarnaast leidt vermindering van contractiliteit van het colon transversum tot verminderde colonreservoirfunctie, die onder meer afhankelijk is van longitudinale spierlengte (cilinder volume = $\pi r^2 \cdot 0{,}5h$). SMS verlaagt de $EC50_{CARB}$ bij controle muizen zonder DSS-colitis. De bij DSS-colitis verlaagde waarde van deze parameter normaliseert door SMS en octreotide, zodat de gevoeligheid voor contractie bewaard blijft. $EC50_{SALB}$ neemt toe na een week DSS toediening. Octreotide is hierop niet van invloed. SMS verhoogt de $EC50_{SALB}$ in normale muizen en muizen met DSS-colitis, daarmee de gevoeligheid voor β-adrenerge middelen verlagend. Deze door SMS verlaagde gevoeligheid voor relaxerende invloed kan ten goede komen aan de coloncontractiliteit bij colitis. Omdat de spierlaag van het colon bij DSS-colitis optisch intact is, zijn de waargenomen veranderingen niet veroorzaakt door beschadigde spiercellen, maar lijken ze samen te hangen met receptordisfunctie of met post-receptoreffecten. SMS wordt gebonden aan eigen, specifieke receptoren (SST type 1 tot 5). Interferentie met muscarine- of adrenerge receptoren geschiedt waarschijnlijk op post-receptorniveau, door bijvoorbeeld competitie voor dezelfde celmembraangebonden G-proteïnen of het intracellulaire cyclisch AMP of GMP[49-51].

In ontstoken darmweefsel wordt naast een verlaagde SMS-concentratie een verhoogde hoeveelheid SMS-receptoren (SST) gevonden op de membranen van

diverse celtypes. SST worden met autoradiografie, waarbij receptorsubtypering meestal niet mogelijk is, gevonden op vaten rond ontstekingshaarden in de darmwand bij IBD[52,53]. Alhoewel nog niet alles daarover duidelijk is, lijken de SST typen 2 en 3 (SST2 en SST3) het meest voor te komen in gastro-intestinaal weefsel. Octreotide heeft grote affiniteit voor SST2[54]. SST2 kan gevisualiseerd worden door middel van octreotidescintigrafie. Dit onderzoek is een gevalideerde methode voor *in vivo* detectie van SST-positieve haarden bij neuro-endocriene tumoren en diverse granulomateuze ziekten. Resultaten van receptorstudies worden beschreven in hoofdstuk 6. Scintigrafie werd uitgevoerd met [^{111}In-DTPA-D-Phe^{1}]-octreotide, dat normaliter niet in de darmwand wordt opgenomen. Toegenomen activiteit van [^{111}In-DTPA-D-Phe^{1}]-octreotide is zichtbaar in het darmstelsel van patiënten met actieve IBD. Er is geen relatie gezien tussen de klinische, endoscopische of histologische activiteit enerzijds en de scintigrafische activiteit anderzijds. Immunohistochemie *in vitro*, met behulp van een polyklonaal konijnen anti-somatostatine receptor 2A antiserum, liet aanwezigheid van SST zien op het intestinale oppervlakkig- en cryptepitheel en endotheel nabij ontstekingshaarden. SST worden ook gezien op fibroblast-achtige cellen, in het bijzonder bij colitis ulcerosa. Toename van SST zou een gevolg kunnen zijn van een positieve feedback door dalende SMS concentratie bij ontsteking. Vanwege de descriptieve aard van de studie kan slechts gespeculeerd worden over de functie van deze SST expressie. Van SMS wordt inhibitie van endotheliale proliferatie[55] en –leukocytenadherentie[56] bij IBD beschreven. Wellicht spelen de vasculaire SST een rol bij de aan SMS toegeschreven preventie van ischemie[7], daarmee intestinale regeneratie bevorderend.

In hoofdstuk 7 wordt een multi-centre studie beschreven waarin octreotide werd toegediend bij 42 patiënten met ernstige colitis ulcerosa, tevens behandeld met hoge doses corticosteroïden. Driemaal daagse, subcutane toediening van 500 µg octreotide gedurende de eerste drie weken van behandeling toont geen

verschillen met placebo, zowel in klinisch, als in biochemisch of endoscopisch opzicht. Wel bestaat er bij de met octreotide behandelde groep een trend tot snellere vermindering van buikpijn. Omdat de studiegroep relatief klein is, kan op grond van de resultaten van deze studie het gebruik van octreotide als aanvulling bij de behandeling van ernstige colitis ulcerosa met corticosteroïden vooralsnog niet worden aanbevolen. Interessant is wel een mogelijk gunstig effect op de buikpijn bij IBD, dat misschien perspectieven biedt voor verder onderzoek[57-59]. In toekomstig onderzoek zou ook het potentiële effect van SMS-analoga bij preventie van IBD-exacerbaties, preventie van fistelvorming en ontwikkeling van fibrose en diarree bij IBD onderzocht kunnen worden. De recente introductie van het ultralang-werkende octreotide-acetaat, dat eenmaal per maand intramusculair kan worden toegediend, kan daarbij belangwekkende ontwikkeling zijn.

Intestinale ontsteking wordt onderhouden door een ingewikkeld en bijzonder uitgebalanceerd netwerk van immuunprocessen, waarin naast cellulaire onderdelen, diverse cytokines, eicosanoïden, neuropeptiden en andere moleculaire mediatoren een rol spelen. SMS neemt maar een kleine plaats in in dit geheel. Bij actieve IBD lijkt er een verhoogde SST2 expressie te bestaan. Octreotidescintigrafie is niet geschikt om bij IBD de ziektelokalisatie of –activiteit te duiden, wegens ontbreken van duidelijke relaties met histologische of endoscopische bevindingen. Acute ontsteking kan met SMS wellicht voorkomen worden. Daarentegen heeft het SMS-analogon octreotide geen bewezen gunstig effect bij behandeling van reeds aan de gang zijnde colitis, zowel in het DSS-muismodel als in de mens. Daarom kan SMS niet worden aanbevolen als ontstekingsremmende therapie bij IBD. Potentiële beïnvloeding van verstoorde darmmotoriek en bijvoorbeeld fistelvorming verdient nader onderzoek, evenals preventieve effecten bij exacerbaties van IBD en maligne degeneratie van colonslijmvlies bij chronische colitis.

Nawoord

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Curriculum vitae

De auteur van dit proefschrift werd geboren op 1 april 1962 te Barendrecht. Na het examen VWO-B aan het Christelijk Atheneum te Arnhem in 1980 volgde de studie Geneeskunde aan de Katholieke Universiteit van Nijmegen (doctoraal examen: maart 1985, artsexamen: februari 1988). In 1987 werd het co-assistentschap Sociale Geneeskunde vervuld in Lesotho, Afrika. Na het artsexamen was de auteur werkzaam als AGNIO bij het Universitair Longcentrum Nijmegen (prof.dr.C.L.A.van Herwaarden) van maart 1988 tot juni 1989. In die periode was hij tevens tijdelijk werkzaam als wetenschappelijk docent bij de Vakgroep Fysiologie, sectie Inspanningsfysiologie van de Medische Faculteit, Katholieke Universiteit te Nijmegen (prof.dr.R.A.Binkhorst). Daarna volgde de opleiding tot internist in ziekenhuis Rijnstate te Arnhem van juni 1989 tot mei 1993 (dr.C.van Gastel, dr.J.M.Werre). Deze opleiding werd voortgezet in de Kliniek voor Inwendige Ziekten van het Academisch Ziekenhuis te Nijmegen (prof.dr.J.W.M.van der Meer) tot mei 1994, met een stage gastro-enterologie in Rijnstate te Arnhem (dr.C.J.J.Mulder), en bij de Afdeling Interne Geneeskunde II van het Academische Ziekenhuis Rotterdam (prof.J.H.P.Wilson). Inschrijving als internist in het specialisten register vond plaats op 12 juni 1995. In het Academisch Ziekenhuis Rotterdam werd ook de opleiding tot maag-, darm- en leverarts gevolgd bij de gastro-enterologen M.van Blankenstein en prof.dr.S.W.Schalm (registratie gastro-enterologie op 1 september 1997). In deze periode nam hij als internist deel aan het levertransplantatieteam van het Academisch Ziekenhuis Rotterdam. Tijdens de opleiding gastro-enterologie werd een aanvang gemaakt met het onderhavige promotieonderzoek (prof.J.H.P.Wilson), dat deels onder leiding van dr.F.J.Zijlstra werd uitgevoerd op de Vakgroep Farmacologie van de Faculteit Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit te Rotterdam. Betrokkene was daarna werkzaam als tijdelijk staflid op de Afdeling Interne II, Sectie Gastro-enterologie en waarnemend gastro-enteroloog op de Afdeling Interne Oncologie, locatie DDH, van het Academisch Ziekenhuis Rotterdam. Vanaf 1 februari 1998 maakt hij als gastro-enteroloog deel uit van de Maatschap Interne Geneeskunde en Gastro-enterologie in het ziekenhuis Gelderse Vallei, locatie Wageningen/Bennekom. Hij is getrouwd met Jacqueline Heitink. Zij hebben 3 zonen: Thomas, Jorne en Stijn.