Inflamm. Res. (2011) 60:163–168
DOI 10.1007/s00011-010-0250-0

Inflammation Research

ORIGINAL RESEARCH PAPER

Colocalization of substance P with tumor necrosis factor-α in the lymphocytes and mast cells in gastritis in experimental rats

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Received: 6 May 2010 / Revised: 24 August 2010 / Accepted: 2 September 2010 / Published online: 24 September 2010

Abstract
Objective Substance P (SP) elicits numerous potent neuroimmunomodulatory effects, increasing the release of tumor necrosis factor alpha (TNF-α). The study aimed to investigate immunoneural communication in experimentally-induced gastritis in rats.
Methods SP-containing nerve fibers and lymphocytes and mast cells were counted in the mucosa of the stomachs of rats using double immunohistochemical and confocal laser microscopic methods, proving colocalization of SP and TNF-α in the lymphocytes and mast cells.
Results In controls, only the nerve fibers showed SP immunoreactivity (IR). However, in gastritis the number of SP-IR fibers and TNF-α IR lymphocytes and mast cells increased significantly ($P < 0.001$); SP-IR fibers were seen in close contact with lymphocytes and mast cells. Numerous lymphocytes (13.1%) and mast cells (10.8%) showed IR for both SP and TNF-α (colocalization) within the same cells.
Conclusion SP release from nerve fibers, lymphocytes and mast cells together with TNF-α can enhance the development of gastric inflammation and participate in tissue damage in gastritis.

Keywords Substance P · Tumor necrosis factor-α · Gastritis · Lymphocytes · Mast cells · Colocalization

Responsible Editor: Makoto Katori.

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Introduction

A morphological association between lymphocytes and mast cells and neuropeptide-containing nerves has been demonstrated in many organs and tissues. Studying substance P (SP) provides important information on the bidirectional linkage between neural function and inflammatory events and, in turn, how inflammatory responses alter neural activity [1]. SP-containing nerve fibers are in intimate contact with mucosal mast cells of the rat [2, 3]. In particular, nerves associated with mast cells, lymphocytes and plasma cells contain SP, calcitonin gene-related peptide (CGRP) or both [4–9] and can activate mast cells to release histamine [10]. Release of SP can lead to vasodilation, plasma extravasation and polymorphonuclear leukocyte recruitment, thereby amplifying inflammatory responses. Noxious stimuli from viral, bacterial (*Helicobacter pylori*) infection, irritants and their consequential inflammation can stimulate retrograde events in neuropeptide-containing fibers through releasing SP from peripheral terminals by axon reflex [11]. Antagonists of pro-inflammatory peptides such as SP and corticotropin-releasing hormone (CRH) may control inflammatory diseases or processes in which these peptides have primary pathogenic roles [12]. Depletion of SP in rats by capsaicin treatment or by the administration of SP antagonist peptide reduces the number of antibody secreting cells [13]. Sio et al. [14] demonstrated that administration of specific Neurokinin-1 receptor (NK1R) antagonist (L703606) 1 h before burn injury significantly disrupted the SP-NK1R signaling pathway and reversed pulmonary inflammation.

Recently, SP has been demonstrated to induce the release of the multifunctional cytokine tumor necrosis factor alpha (TNF-α), IL-1 and IL-6 from monocytes and macrophages [15]. Stimulations of murine mast cells by SP

activates TNF-α gene expression and induces TNF-α secretion [16, 17]. In the last two decades it has been demonstrated that bidirectional communication between lymphocytes and mast cells and peripheral nerves can occur, whereby lymphocytes and mast cells transmit immunological and chemical information about the local environment to the nerves. The physiological significance of this interaction is the maintenance of homeostasis or the initiation and/or prolongation of diseases.

Mediators classically thought to be synthesized exclusively by the nervous system are now known to be produced by lymphocytes and mast cells, and vice versa. SP has been demonstrated in inflammatory cells, such as macrophages, lymphocytes and dendritic cells [18–20]. Advances in understanding the neuroimmune network and a better knowledge of the interactions between neuropeptides and cytokines could be useful in the study of mechanisms involved in inflammatory diseases. Experimental models of gastric mucosal damage have been developed that include various types of stress-induced gastritis [21], intragastric application of *Helicobacter pylori* itself [22] and iodoacetamide-induced gastritis [23]. Iodoacetamide is a sulfhydryl group blocker that depletes sulfhydryl components in the gastric mucosa, leading to gastric damage [24, 25]. In the present study we show that SP is a key mediator in the pathogenesis of gastritis via activation of TNF-α. Therefore, the aim of the present study was to extend the investigation of immunoneural communication in gastritis. Here we present evidence that SP and TNF-α can be released by the same activated immunocytes. Confocal laser microscopy was used to estimate the colocalization of SP and TNF-α in the lymphocytes and mast cells.

Materials and methods

The animal procedures included in the paper conform with the Revised Guide for the Care and Use of Laboratory Animals (ILAR 196) and with the Hungarian Law on Animal Care (1998, Hungary). The study was approved by the Ethics Committee on Animal Experiments, Semmelweis University, Budapest, Hungary. All efforts were made to minimize the number of animals used. Gastritris was induced in rats by replacing normal drinking water with distilled water containing 0.1% iodoacetamide and were allowed to drink freely for 4 (5 animals) and 8 days (5 animals). Control rats (5 animals) received normal drinking water.

Immunohistochemical analysis

Adult male Wistar rats were transcardially perfused under deep ketamine/xylazine (75 mg/7.5 mg/kg, i.m.) anaesthesia with Zamboni's fixative consisting of 4% paraformaldehyde, 0.1% glutaraldehyde in 250 ml 0.1 M phosphate buffer, 150 ml saturated picric acid (pH 7.3) for 6 h. The stomachs were dissected out, cut along the greater curvature and some pieces of the corpus mucosa were cut and placed overnight in glutaraldehyde-free fixative containing 20% sucrose at 4°C. Sections (40 μm thick) were treated for 1 h with 1% Triton X-100 to increase membrane permeability and for 15 min with 3% hydrogen peroxide in order to remove endogenous peroxidase activity. The incubation with primary antisera was performed for 48 h at 4°C. Avidin–biotin immunoperoxidase technique was employed using a commercially available kit (Vectastain Elite ABC, Vector Laboratories, Peterborough, UK) for immunostaining. All steps were performed at room temperature. The immunoreactivity (IR) was visualized with diamino-benzidine (DAB) chromogen reaction (Dako, Milan, Italy) (0.025% 3,3-diamino-benzidine, 0.0015% H_2O_2 in 0.05 M Tris–HCl buffer, pH 7.5) for 8 to 10 min, at room temperature. For light microscopic examination the sections were mounted on gelatinized slides, air-dried, cleared and covered with Depex.

Antibodies

Polyclonal antibody to SP was developed in rabbit (Peninsula Lab. Inc., CA, USA T-4107.0050 Antigen sequence: H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), dilution: 1:10,000. TNF-α monoclonal antibody was developed in rabbit (Sigma–Aldrich), dilution: 1:8,000.

For double staining, the sections were also examined by confocal laser microscopy (Nikon Eclipse 800 microscope, Japan; Radiance 2,100, Bio-Rad, LaserSharp2000 Software, Bio-Rad House, Hertfordshire, UK). Frozen sections were washed in phosphate-buffer saline (PBS) at room temperature and permeabilized for 20 min in PBS (2× NaCl) containing 0.3% Triton X-100 and 2% normal serum; the same solution was used to dilute the antibodies. Afterwards, they were sequentially incubated with anti-SP antiserum at dilution 1:5,000 overnight. Slides were washed in PBS and then incubated for 3 h at room temperature with a secondary fluorescein (FITC, 1:100) conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA). The sections were washed with buffer and incubated with anti-TNF-α antiserum (1:5,000) for 24 h, followed by secondary antiserum raised in donkey (fluorescein-labeled anti-rabbit IgG, Alexa 594, diluted in 1:500, Molecular Probes, Eugene, OR, USA) for 3 h, mounted in anti-fade medium (Vectashield, Vector Laboratories, Peterborough, UK) and stored at −20°C. The cell bodies and the nerve fibers that could be identified were then scanned with a confocal laser microscope equipped with a krypton–argon laser.

Fluorescent signals from FITC (green) and Alexa 594 (red) were sequentially detected on a Bio-Rad Micro Radiance confocal laser system (Bio-Rad MRC1024) and scanned through a 4× lens on a Nicon Eclipse E 800 microscope attached to a Bio-Rad Radiance 2100 Rainbow confocal laser scanning system.

Electron microscopy

Small pieces of the same biopsy materials were placed overnight in glutaraldehyde-free fixative containing 10% sucrose and 4°C, then 30 μm sections were cut by Vibroslices and plunged rapidly into liquid nitrogen, thawed in 0.1 M PBS. From this point on, the immunocytochemistry was performed as described above with the exceptions that Triton X-100 was omitted from all solutions, and the sections were incubated for 48 h at 4°C in the antibody. Furthermore, the sections were osmicated in 0.5% OsO_4 containing PBS solution for 1 h and dehydrated by ascending alcohols and propylene oxide. The sections were then flat embedded in Epon. The selected sections were reembedded, and ultrathin sections were obtained with an ultramicrotome using diamond knife. The sections were counterstained using uranyl acetate and lead citrate. The materials were observed using Jeol 100 electron microscope (all morphological examinations were performed by the same investigator, E.F.).

Control experiments

To verify the specificity of the immunohistochemical detections, non-immune serum or primary antibody solution with specific antigens added in microgram quantities (preabsorption), were applied on serial sections where no immunostaining appeared.

For each staining procedure we used positive controls (i.e., colonic mucosa), while the negative control staining was conducted without primary antibody or with preabsorbed antibody, i.e., with diluted antibody that had been mixed with the antigen peptide (1 μg/mL) and incubated at room temperature. No IR was observed in the negative control sections.

Activation score

To assess the activity of SP and TNF-α in situ in the rat stomach, specimens from all rats were stained. All sections visualized the entire axis from the superficial epithelium to the muscularis mucosae. In order to quantify the frequency of colocalization of SP with TNF-α, we classified SP-IR cells from five sections from each animal ($n = 5$) as either labeled or double-labeled cells. For quantitative analysis, the number of IR nerve fibres and the lymphocytes and mast cells counted in a 15–20 mm^2 tissue area and calculated for 1 mm^2 tissue area were taken as the average. For analysis, 40× magnification was used with a graduated eyepiece graticule and the entire section was assessed. Microphotographs were also taken ($n = 15$–25 per rat), digitalized and then analysed using the PC-based image analysing software IMAN (beta) 2.0 (MFA, Budapest, Hungary). The studies were carried out by two investigators as a double blind trial.

Statistical analysis

Analysis of variance (ANOVA) was employed to determine overall differences in the quantity of IR nerve terminals and in the immunocytes. In order to evaluate the statistical significance of differences between materials from healthy controls and gastritis, the data were also analysed with Student's two-sample *t*-test. A value of $P < 0.05$ was considered as statistically significant.

Results

In the control mucosa of the stomach, only the nerve fibers showed IR for SP; the lymphocytes and mast cells were not stained, however they were positive for TNF-α. Iodoacetamide intake in drinking water resulted in marked leukocyte and mast cell infiltration in the mucosa (moderate gastritis). The injury was more pronounced after 8 days, where the gastric gland cells appeared pale, edematous with congestive blood vessels in the mucosa (severe gastritis). In the experimental gastritis group the lamina propria was infiltrated by leukocytes (mainly lymphocytes), plasma cells, mast cells, and a moderate number of neutrophil granulocytes. There was no difference between the number of IR elements in the moderate and the severe gastritis samples. In several cases these IR cells were in close contact with the SP-containing nerve fibers. Experimental gastritis caused a large increase in the number TNF-α IR lymphocytes. This increase was associated with a concomitant increase in the number of SP-IR nerve fibres. A large number of lymphocytes and mast cells showed IR both for SP and TNF-α (Fig. 1c, e). Table 1 shows the percentage distribution of either SP−, TNF-α or both SP+, TNF-α positive lymphocytes and mast cells in the mucosa of normal rats and of those suffering from gastritis. Confocal laser microscope investigations revealed that the dense green reaction end product indicating SP-IR was distributed throughout the cytoplasm of lymphocytes and mast cells. Nerve fibres also contain SP-IR. In addition, TNF-α IR (a red end product) was observed in the cytoplasm of lymphocytes and mast cells (Fig. 1a, b, d, e). Figure 1c and f are merged images. Colocalization of SP

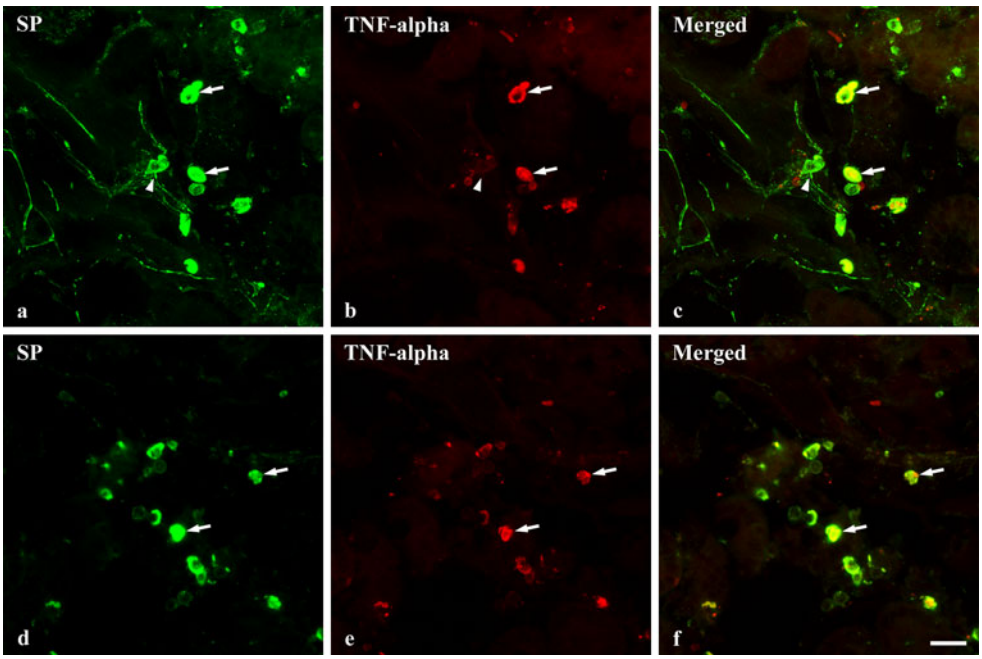

Fig. 1 Confocal laser micrograph of the inflamed mucous membrane of the rat's stomach. **a** Confocal images illustrating of SP-IR nerve fibers (*arrowhead*) close to an activated lymphocyte and activated mast cells (*arrows*) in experimental gastritis. **b** The *arrowhead* shows the TNF-α negative nerve fibers; *arrows* point the lymphocytes and mast cells showing immunoreactivity for TNF-α. **c** *Arrows* indicate the double-labeled immunocells for both SP and TNF-α; *arrowhead* shows the SP-IR nerve fibers close to a mast cell having SP and TNF-α in colocalization. **d** Fluorescent double-labeled immunostaining shows the SP fluorescence-positive lymphocytes (*arrows*) in the inflamed gastric mucosa. **e** Some of the lymphocytes were also labeled for TNF-α (*arrows*), where the immunostaining was located in the cytoplasm of these cells. **f** *Arrows* point to the lymphocytes showing immunoreactivity for both SP and TNF-α. *Bar scale* 20 μm

Table 1 Percentage distribution of SP-, TNF-α or both in the mucosa of normal rats of those suffering from gastritis

	Lymphocytes		Mast cells	
	Control	Gastritis	Control	Gastritis
Only SP-stained	0	29.5 ± 1.30	0	23.4 ± 1.68
Only TNF-α-stained	0.53 ± 0.15	15.1 ± 0.85	0	12.6 ± 0.92
Both SP + TNF-α-stained	0	13.1 ± 1.35	0	10.8 ± 1.25

and TNF-α was found in 23.9 ± 1.30% of lymphocytes and mast cells. Immunofluorescence double staining also revealed numerous lymphocytes and mast cells close to the SP-IR nerve fibers in the gastritis.

The electron microscopic investigation proved that some of the nerve fibres and the lymphocytes and mast cells showed IR for SP. The reaction end-products were distributed in the cytoplasm and at the membranes of these cells (Fig. 2). The varicosity of SP-IR nerve terminals possessed a large number of granulated and small clear synaptic vesicles and was located in a very close association to a mast cell (Fig. 3). The distance between IR nerve fibres and lymphocytes and mast cells was about 1 μm and very often less than 200 nm. In some instances, degranulated mast cells were in the vicinity of IR nerve fibres.

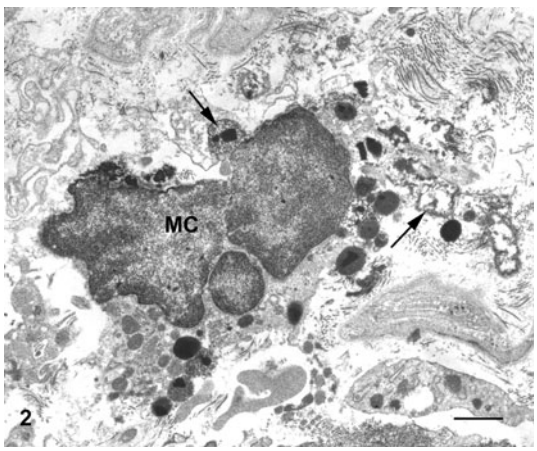

Fig. 2 Electron micrograph of a mast cell (MC) showing immunoreactivity for SP. *Arrows* show the reaction end-products locating in the cytoplasm and cell membrane. *Bar scale* 1 μm

Discussion

In this study we confirmed previous observations showing that SP modulates gastric inflammatory processes in rats and in human beings via inducing neurogenic inflammation [20, 26]. Double immunostaining for SP and TNF-α revealed that considerable evidence exists for a consistent anatomical association between lymphocytes and mast

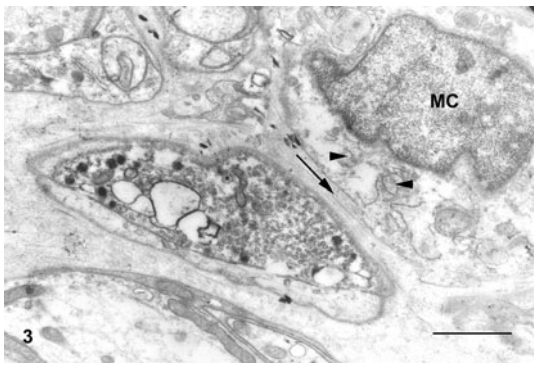

Fig. 3 Electron micrograph of a SP-IR nerve fibres containing a large number of synaptic vesicles close to a degranulated immunonegative mast cell (MC). *Arrow* points the synaptic gap, *arrowheads* show the degranulated vesicles in the mast cell. *Bar scale* 1 μm

cells and nerves in tissues throughout the body [27]. The immune cells (neutrophils, eosinophils, lymphocytes, monocytes, macrophages, mast cells) express membrane-bound NK1R [8, 9]. Therefore, upon activation of SP-NK1R complex, numerous immunocytes are rapidly triggered to release a cascade of proinflammatory mediators, which progress to cause exacerbated gastritis. Moreover, co-release of SP together with TNF-α from lymphocytes and mast cells into the surrounding tissue may also enhance the development of gastric inflammation.

In vitro studies have demonstrated that mast cells produce increasing amount of TNF-α mRNA after SP activation [28], and this expression is down-regulated by SP antagonist P101 [29]. Castagliuolo et al. [30] demonstrated that pretreatment of rats with the SP antagonist CP-96, 345 inhibits toxin A-mediated TNF-α release from isolated intestinal macrophages in the mucosa.

Increased SP release leads to many of the features typical of gastric inflammation including lymphocyte and mast cell activation and degradation, hypersecretion of the glands and smooth muscle contraction. SP induces proinflammatory cytokine secretion from inflammatory cells, which amplifies local inflammation [31, 32]. SP produced by the nervous system can influence the function of the stomach, and the behavior of the immune cells and the materials (proinflammatory cytokines, chemokines, SP, hormones) produced by lymphocytes and mast cells themselves can each influence the production of the other materials [33, 34]. Elevated SP level is postulated to be a significant pathophysiological factor in the development of gastritis. Studies with neurokinin receptor antagonists suggest that blocking the binding of SP to the NK1R interrupts the inflammatory cascade that triggers and maintains intestinal lesions of inflamed bowel diseases [35]. The strong inhibition of TNF-α production with SP antagonists could also be important in future therapy of inflammatory diseases, since these antagonists might interrupt the axon reflex-mediated neurogenic inflammation and decrease SP and TNF-α production in the lymphocytes and mast cells. Capsaicin-induced depletion of nociceptive afferent nerves completely inhibited the development of colitis [36].

In conclusion, this study adds further data to the existing evidence for direct communication between the mucosal immune/inflammatory system and the nerve fibers in experimental gastritis. Bidirectional neuroimmunomodulation of the lymphocytes and mast cells and SP nerve fibers have an important effect on the development of iodoacetamide-induced gastritis. Our new findings demonstrated the colocalization of SP and TNF-α in activated immunocytes lymphocytes and mast cells during exposure to inflammation.

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