Correlation and Immunolocalization of Substance P Nerve Fibers and Activated Immune Cells in Human Chronic Gastritis

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ABSTRACT

Neuropeptides are able to modulate cytokine production by macrophages in response to various stimulators and have a major role in inflammation of different organs. Mammalian poly (ADP-ribose) polymerase (PARP) and nuclear factor kappa B (NF-κB) both have been suggested to play a crucial role in inflammatory disorders. Unregulated increase of tumor necrosis factor-α (TNF-α) may also be pathogenic in inflammatory diseases. The aim of this study was to investigate the correlation between the number of Substance P (SP) containing nerve fibers and activated immune cells using immunohisto-, immunocytochemical (EM) and confocal laser microscopic methods. To investigate expression and activation of immune cells gastric biopsy samples from patients with chronic gastritis were used. The number of SP containing nerve fibers and activated immune cells increased significantly in gastritis. Using monoclonal p65 antibody, activated NF-κB was found in inflamed mucosa but was absent in uninflamed mucosa. Immunobinding for the activated form of p65 of NF-κB was found in 22% of macrophages and 45% of lymphocytes. The number of immune cells showing IR for NF-κB, PARP and TNF-α correlated with the increasing number of SP containing fibres. Confocal laser microscopy was used to confirm the colocalization of SP in TNF-α and NFκB positive lymphocytes and mast cells in inflamed mucosa. Immunoelectronmicroscopic investigation confirmed that these cells belong to lymphocytes, mast cells and macrophages. Conclusions: The increase of SP in nerve fibers and in activated immune cells further activate the production of other proinflammatory mediators (e.g. TNF-α) and therefore generate the chronic inflammation. Anat Rec, 291:1140–1148, 2008. © 2008 Wiley-Liss, Inc.

Key words: substance P; gastritis; neuroimmunomodulation; TNF-alfa; NFkappaB

Neuropeptides are able to modulate cytokine production by macrophages in response to various stimulators. Substance P (SP) and calcitonin gene-related peptide (CGRP) are important mediators of neuroimmunomodulatory activity (Veronesi et al., 1999; Azzolina et al., 2003; Yarace et al., 2003). It was also demonstrated that SP has a major role in inflammation of the stomach (Mózsik et al., 2001). Previously we have shown that the

number of the SP-immunoreactive (IR) nerve fibers was increased significantly and that they were morphologically close to the immune cells during chronic gastritis (Sipos et al., 2006). The increased number of SP IR nerve fibers suggests that SP specifically stimulates the chemotaxis of lymphocytes, monocytes, neutrophils, and fibroblasts (Kahler et al., 1993; Schratzberger et al., 1997; O'Connor et al., 2004). Lymphocyte extravasation is facilitated by neurogenic inflammation and plasma extravasation that are dependent on the release of SP from capsaicin sensitive primary sensory nerve endings by means of an axon reflex. SP selectively activates tumor necrosis factor-alpha (TNF-α) gene expression in different mast cells (Ansel et al., 1993; Cocchiara et al., 1997). Unstimulated peritoneal mast cells spontaneously released a small quantity of TNF-α, whereas after SP stimulation the amount of released TNF-α was approximately four times higher and this effect was inhibited only by pretreatment with SP antagonist (SPA, P101, CP-96345; Cocchiara et al., 1999). It is well known that TNF-α plays a key role in the immunopathogenesis of inflammatory bowel diseases, a fact which is overtly confirmed by clinical effects observed in refractory Crohn's disease patients treated with a chimeric monoclonal antibody against TNF (infliximab; Blam et al., 2001).

The binding of SP to tachykinin-1 receptor can result in the up-regulation of proinflammatory cytokines, such as TNF-α and interleukin-8 (IL-8), whose expression is controlled by the transcription factor NF-κB (Lieb et al., 1997; Marriott et al., 2000). Activation of the NF-κB/Rel transcription family, by nuclear translocation of cytoplasmic complexes, plays a central role in inflammation through its ability to induce transcription of proinflammatory genes (Baldwin, 1996). NF-κB exists in the cytoplasm in an inactive form associated with regulatory proteins called inhibitors of κB (IκB). Phosphorylation of IκB an important step in NF-κB activation, is mediated by IκB kinase. The dimer, typically composed of a p50 and p65 subunit, is translocated to the nucleus after degradation of the inhibitory I-κB in response to a wide variety of stimuli (Ghosh et al., 1998). Varro et al. (2004) also demonstrated that in unstimulated cells, p65-dsRed was located in the cytosol, and stimulation by H. pylori caused translocation to the nucleus. The activation of transcription factor nuclear factor-κB (NF-κB) regulates various genes involved in the proliferation, invasion, angiogenesis, and metastasis of cancer cells. Substantial in vitro data suggest that activation of NF-κB is a critical initial step in the inflammatory response. Several studies demonstrated a link between in vivo NF-κB activation accompanied by cytokine production and the generation of inflammation in animal models of inflammatory diseases (Sakurai et al., 1996; Blackwell et al., 1997; Ellis et al., 1998). The identification of NF-κB as a key player in the pathogenesis of inflammation suggests that NF-κB targeted therapeutics might be effective in diseases like gastritis. Inflammation in any part of the gastrointestinal tract can profoundly influence the function of the mucosal layer that lies closest to the luminal contents. The inflammatory response is coordinated to a large extent by an array of chemical mediators that are released from nerves, from the immune cells and epithelium.

Besides of NF-κB the mammalian poly (ADP-ribose) polymerase (PARP) has been also suggested to play a crucial role in inflammatory disorders (Hassa and Hot-

tinger, 2002). The activation of PARP is now considered as a final common effector in various types of tissue injury including systemic inflammation, circulatory shock and ischemia/reperfusion (Virág and Szabó, 2002). The obligatory triggers of PARP activation are DNA single string breaks, which can be induced by a variety of environmental stimuli, most notably hydroxyl radical and peroxyntirite. Direct and indirect experimental evidence demonstrated that activation of PARP importantly contributes to the pathophysiology of various forms of inflammation (Szabó, 1998). PARP inhibition is a successful therapeutic concept in the treatment of a wide variety of inflammatory diseases (Szabó et al., 2004).

It is now clear that *Helicobacter pylori* (HP) activates the transcription factor NF-κB and this event plays a central role in the induction of the inflammatory reaction often associated with colonization with this bacterium. The increase of SP-IR in nerve fibers and immune cells that parallel with the grade of inflammation in the stomach has never been described previously. Although, in recently similar changes in other inflamed organs has been published, the correlated increase in the substances described was not observed. Therefore, we have examined in this study the expression and activation of NF-κB, TNF-α, PARP and the correlation of SP immunoreactive nerve fibers and immune cells in the control stomach and HP-associated gastritis using light and electron microscopy as well as confocal laser microscopy.

MATERIALS AND METHODS

Endoscopic biopsies of gastric antrum were obtained from 10 HP-positive patients (4 male; 6 female, ageing from 30 to 67 years old) who underwent esophagogastroduodenoscopy for dyspeptic symptoms over a period of 1 year. None of the patients had neoplasmic disease or peptic ulcer. Antral samples of 5 dyspeptics (2 male; 3 female, ranging from 27 to 58 years) obtained with endoscopy and histologically normal HP negative stomach samples were used as controls. HP positivity, assessed by rapid urease test, had been confirmed by histology and serology in all patients. Biopsies from HP positive patients showed histologically chronic gastritis with moderate or severe activity according to the Sydney's system (Price, 1991). All patients and healthy volunteers gave their informed consent according to Semmelweis University guidelines for ethics in human tissue experiments (No: TUKEB 85/2006).

Immunohistochemical Analysis

Biopsy materials were fixed in Zamboni's fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde in 250 mL of 0.4 M phosphate-buffer, 150 mL picric acid (pH 7.3) for 6 hr and placed overnight in glutaraldehyde-free fixative containing 20% sucrose at 4°C. Sections (40 μm thick) were treated for 1 hr with 1% TRITON-X 100 to increase membrane permeability and for 15 min with 3% hydrogen peroxide to remove endogenous peroxidase activity. Incubation with primary antisera was performed for 48 hr at 4°C. The Avidin-Biotin technique was employed using a commercially available kit (Vectastain Elite ABC, Vector Laboratories, Peterborough, UK) for the immunostaining. All manipulations were performed at room temperature. Immunoreactivity was visualized with diamino-benzidine (DAB) chromogen reaction (Dako, Milan, Italy) (0.025% 3, 3-diamino-benzi-
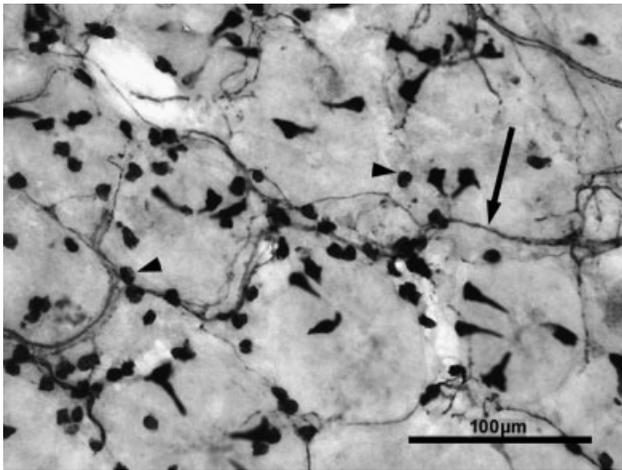
Fig. 1. Cross-section of the stomach glands from gastritis. Numerous immunoreactive SP nerve fibers (arrow) and SP-positive immunocytes (arrowheads) are located among the glands. Scale bar = 100 μm.
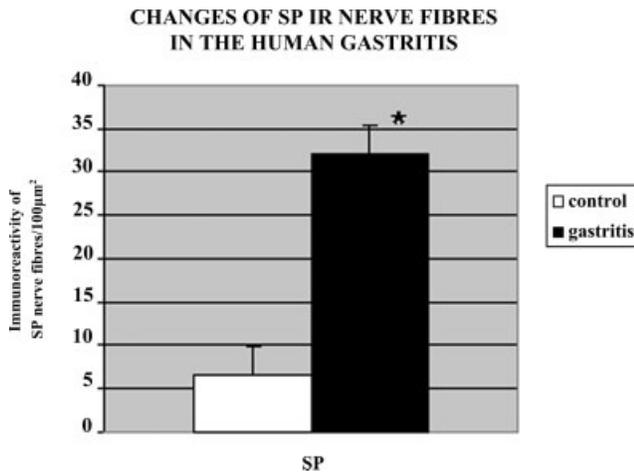

Fig. 2. Changes of the SP-IR nerve fibers in the human gastritis.

Fig. 3. Changes of the activated immune cells in the human gastritis.

Fig. 4. A part of the tunica mucosa from gastritis. Arrows point TNF-α–IR immunocytes beneath the glands. Gl, glands; MM, lamina muscularis mucosae. Scale bar = 100 μm.

dine, 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. To block nonspecific binding of antibody, sections were preincubated with 1:10 diluted normal goat serum.

Antibodies

Antibody to Substance P (SP) was developed in rabbit (Peninsula Lab. Inc., San Carlos, CA), dilution: 1:10,000). Antisera to NF-κB subunits, anti-p65 mouse monoclonal IgG (Chemicon, International Inc., Temecula, CA), dilution: 1:1,500; which recognizes an epitope overlapping the nuclear location signal of the p65 subunit of NF-κB heterodimer. The anti-human TNF-α polyclonal antibody developed in rabbit (Sigma-Aldrich), dilution: 1:8,000, anti-PARP mouse monoclonal antibody (Biomol Int. LP, Plymouth Meeting, PA), dilution:1:500.

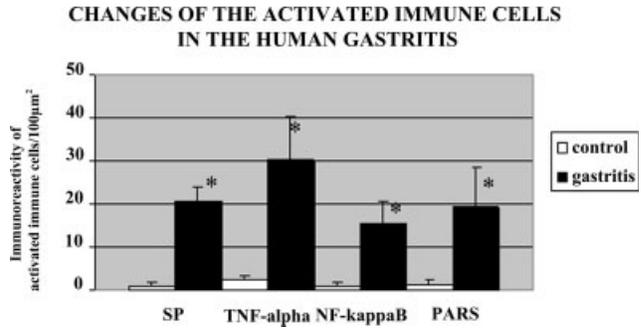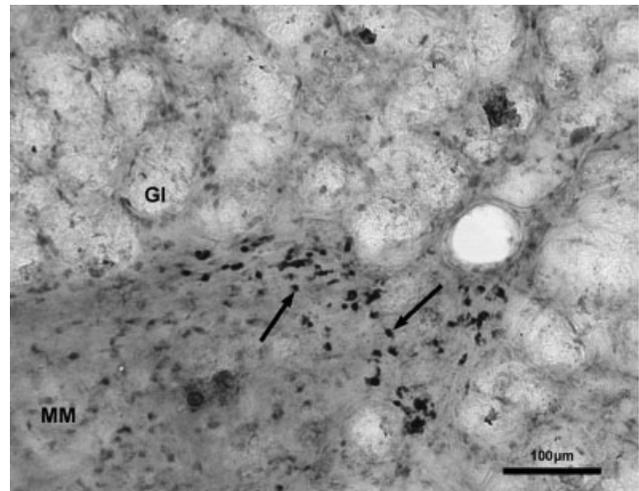For double staining the sections were also examined by confocal laser microscopy (Nikon Eclipse 800 microscope, Japan, Radiance 2100, Bio-Rad, LaserSharp2000 Software, Bio-Rad House, Hertfordshire, UK). Frozen sections were washed in phosphate buffered saline (PBS) at room temperature and permeabilized 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. Afterward, they were sequentially incubated with anti-SP antiserum at dilution 1:5,000 overnight. Slides were washed in PBS and then incubated for 3 hr at room temperature with a secondary fluorescein (FITC, 1:100) conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA). The sections were washed with buffer and incubated with anti–TNF-α (1:8,000) for 24 hr, followed by secondary antiserum raised in donkey fluorescein-labeled anti-rabbit IgG, Alexa 594, diluted in 1:500, Molecular Probes, Eugene, OR) for 3 hr, mounted in anti-fade medium (Vectashield, Vector Laboratories, Peterborough, UK) and stored at −20°C until needed. The cell bodies and the nerve fibers that could be followed 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 MicroRadiance confocal laser system (Bio-Rad MRC1024).

For electron microscopic investigations the sections were post-fixed in osmium and embedded in Epon.
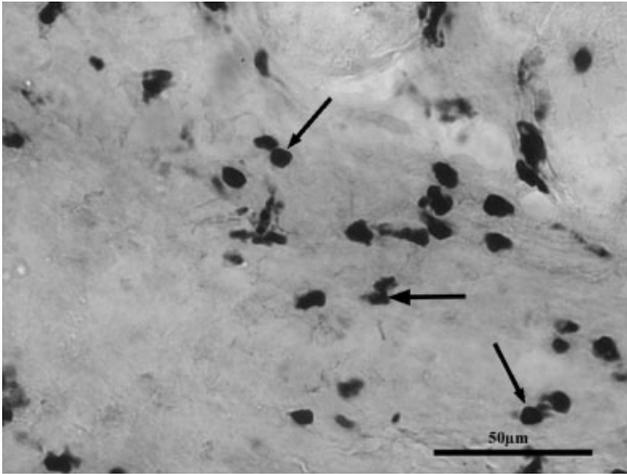
Fig. 5. Tunica mucosa from human gastritis. Arrows show the strong positive poly(ADP-ribose) staining in the cell nuclei of immune cells. Scale bar = 100 μm.

Ultrathin sections were cut and stained with uranyl acetate and lead citrate and photographed in a Jeol 1OO electron microscope.

Activation Score

To assess the activity of NF-κB, PARP and TNF-α in situ in the human stomach, specimens from all patients were stained for the previous antibodies. Three view fields of each section were investigated at ×200 magnification (0.0325 mm^2/viewfield), and positive cells were counted, blind to the clinical diagnosis. The average of the three fields was taken as the activation score of the specimen. To be able to compare the results between patients, it was ensured that all sections visualized the entire axis from the superficial epithelium to the muscularis mucosae.

Control Experiments

Specificity of the immunoreactivity was controlled by omission of the primary antiserum or, when the sections were incubated in antisera, preabsorbed with excess antigen, where no immunostaining appeared.
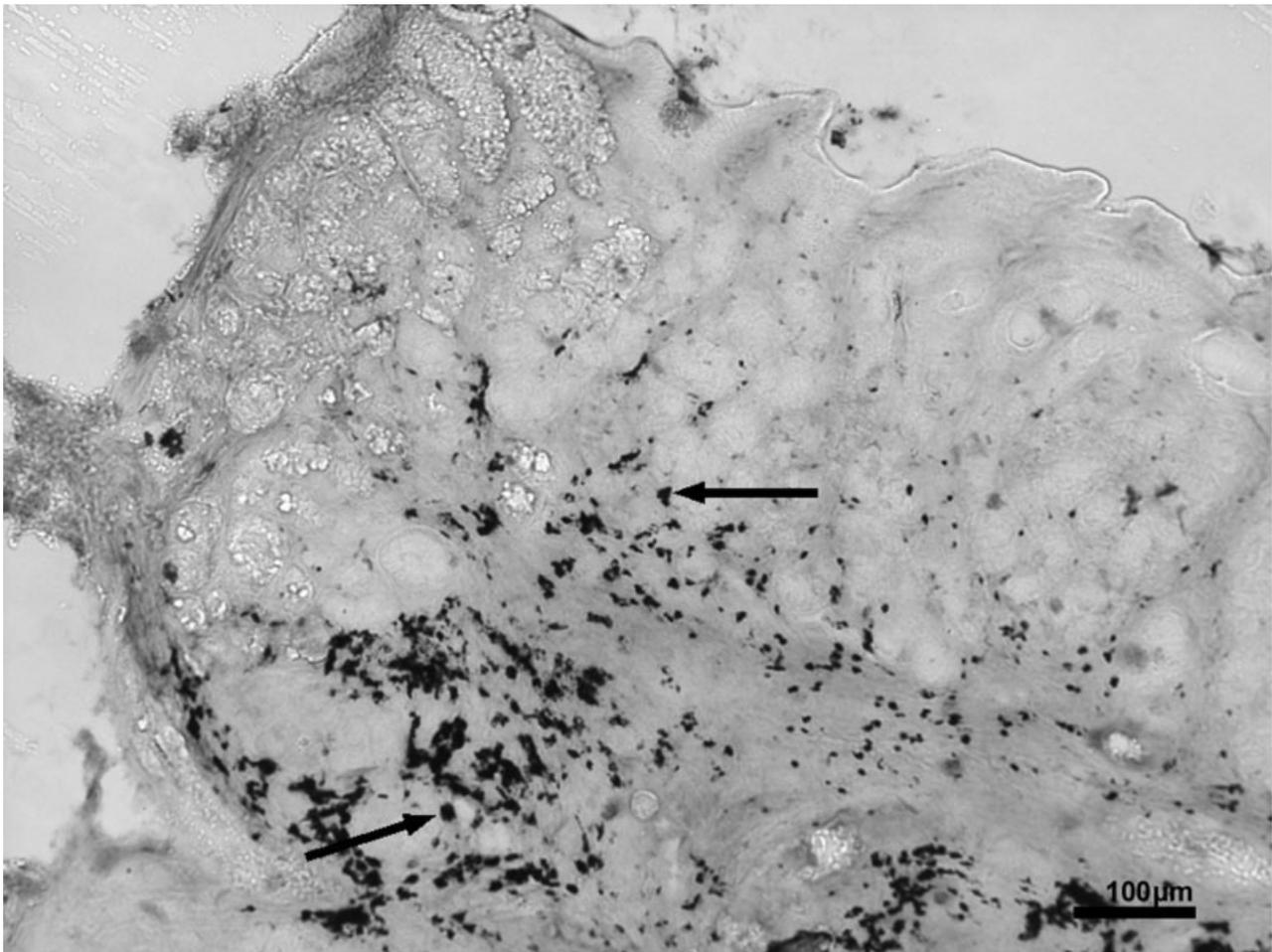

Fig. 6. A part of the mucosa of the inflamed human stomach. Arrows show the active NF-κB immunoreactivity in the nucleus of the immune cells. Scale bar = 100 μm.
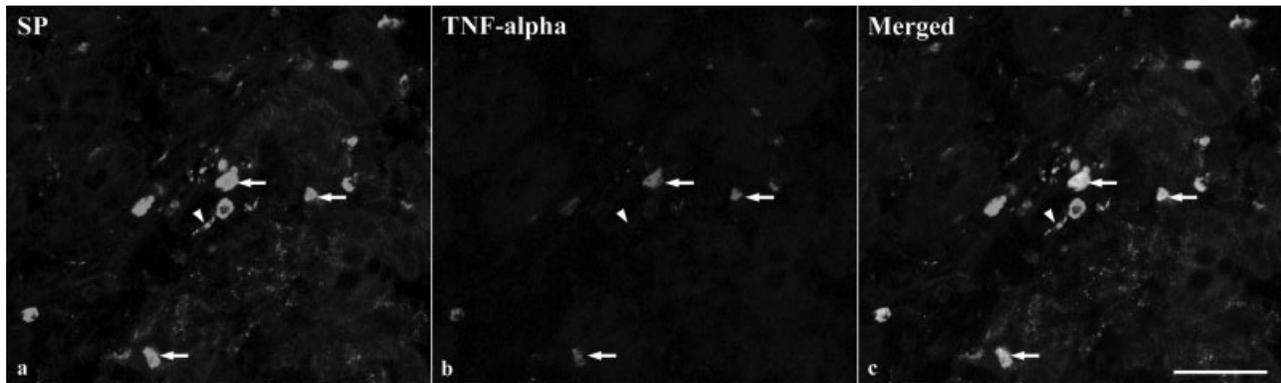
Fig. 7. Confocal images illustrating of SP-IR nerve cells and activated immune cells in the human gastritis. **a:** SP-IR immune cells (arrows) in the lamina propria among the glands. Arrowhead shows the SP-IR nerve fibers close to the activated mast cells. **b:** The arrows show the same mast cells showing immunoreactivity for TNF-α. **c:** Arrows indicate double-labeling immune cells for SP and TNF-α. Scale bar = 50 μm.
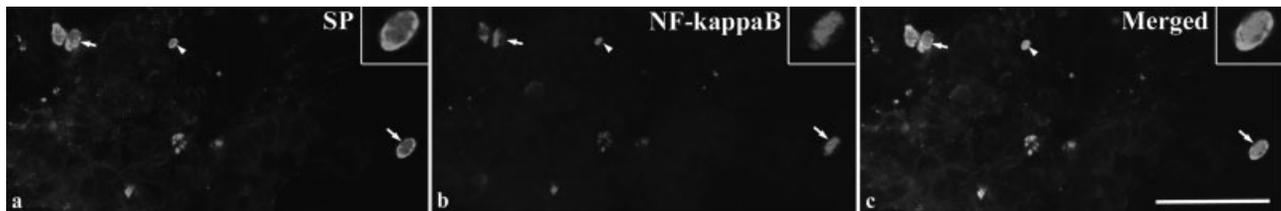

Fig. 8. Confocal images illustrating of SP and NF-κB immune cells in the human gastritis. Fluorescent double-labeled immunostaining showed that SP (FITC) fluorescence-positive immune cells were also labeled for NF-κB, where the immunostaining (Alexa 594, red) was located in the nucleus of these cells. In the insert, one of the double-labeled mast cell. **a:** Arrows point the SP mast cells and arrowhead show a SP lymphocyte. **b:** The arrows and arrowhead show the same cells showing immunoreactivity for NF-κB. **c:** Arrows indicate the double-labeling cells for SP and NF-κB. Scale bar = 50 μm.

Statistical Analysis

Data are presented as mean ± scanning electron microscopy. Comparisons between groups of data were made using a one-way analysis of variance followed by a Tukey post hoc test. P values < 0.05 were considered statistically significant.

RESULTS

All subjects were of adult age and no predominance of male or female sex was present. At endoscopy gastric hyperemia was the main feature of HP-related chronic gastritis.

The number of SP IR nerve fibers was increased significantly, also some immune cells (lymphocytes, plasma cells, macrophages, and mast cells) showed immunoreactivity for SP (Fig. 1). The gap between the nerve terminals and immune cells was approximately 1 μm or even less. The change of the number of SP-IR nerve fibers is shown on Figure 2.

In the control materials the number of PARP- and TNF-α–IR cells was very low, in some sections totally missing. However, in sections from chronic gastritis their number was significantly increased ($P < 0.001$) (Fig. 3). PARP and TNF-α staining in gastritis were localized primarily to the mononuclear cells and fibroblasts in the lamina propria, with no detectable staining of the epithelium (Figs. 4, 5). These IR immune cells were in close contact with epithelial cells. The reaction end-product of PARP was seen in the nuclei of the cells, while TNF-α immunoreactivity was observed in the cytoplasm of the positive cells. According to the size and form of these IR cells they belong to the lymphocytes, macrophages and polymorphonuclear cells (PMN).

In the normal stomach, low and moderate immunoreactivity of NF-κB (p65 subunit) was found in the cytoplasm of gastric glandular epithelial cells located in the deep region of the glands. In gastritis, the active NF-κB was observed in clusters of inflammatory cells, where NF-κB was detected not only in large amounts in the cytoplasm but also in the nucleus, suggesting activation of NF-κB in these cells (Fig. 6). The number of active NF-κB IR cells was markedly increased, as compared with uninflamed tissue, and they were mainly detected among the glands. 22% of mast cells and 45% of lymphocytes were immunoreactive in gastritis.

Confocal laser microscope investigations showed that dense green reaction end products (FITC) were distributed in the SP containing nerve fibers and throughout the cytoplasm of immune cells (SP); some of these showed a red reaction (Alexa 594) for TNF-α (Fig. 7). Fluorescent double-labeled immunostaining showed that SP (FITC, green) fluorescence positive immune cells also had immunostaining for p65 of NF-κB (Alexa 594, red), where their labeling was located in the nucleus of these cells (Fig. 8).

The electron-microscopic investigation proved that these cells belong to different immunocompetent cells (lymphocytes, macrophages and mast cells). The reaction
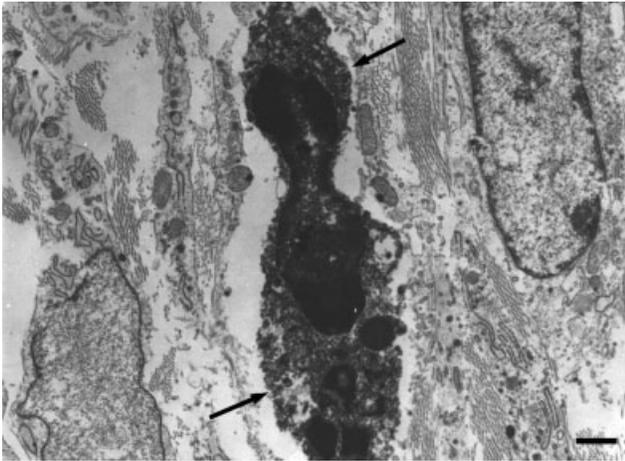
Fig. 9. Electron microscopic photograph of a TNF-α–IR granulocyte from the gastritis. Note that the reaction end-products are visible in the cytoplasm of the cell (arrows). Scale bar = 1 μm.
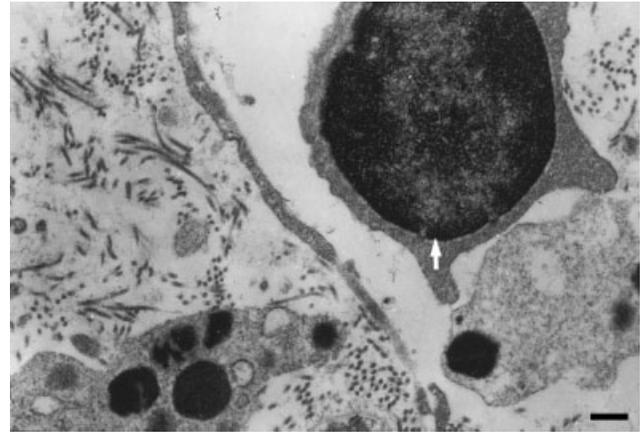

Fig. 11. Electron microscopic photograph of a NF-κB immunoreactive lymphocyte from the inflamed stomach. The reaction end-products are located in the nucleus (arrow) showing the activation of this cell. Scale bar = 1 μm.
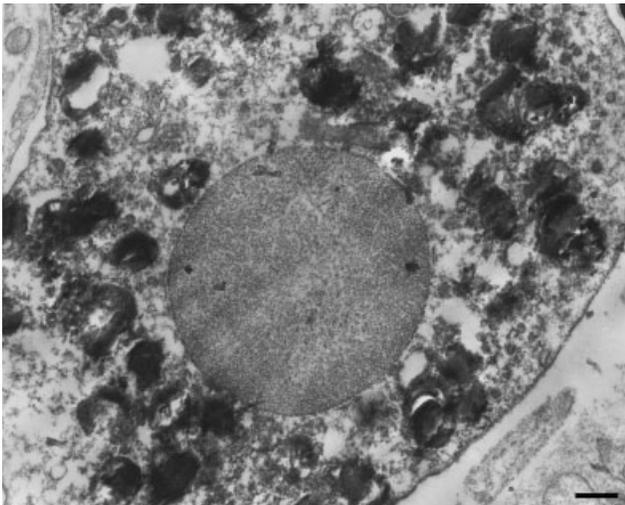

Fig. 10. Electron microscopic photograph of a TNF-α–IR mast cell from the gastritis. Scale bar = 1 μm.

end products were distributed in the cytoplasm and at the membranes of the TNF-α–IR cells (Figs. 9, 10), while in NF-κB–IR cells, the reaction end products were located in the nucleus of the cells (Fig. 11) reflecting activation of the NF-κB heterodimer.

DISCUSSION

SP is involved in the biological activities of the immune system, including the induction of cytokines in immune cells (Lee et al., 1994; Ho et al., 1996; Cocchiara et al., 1997; Maggi, 1997). It was demonstrated that the number of SP-IR nerve fibers and SP IR immune cells increased significantly during inflammation in the tongue and in the stomach (Batbayer et al., 2004; Sipos et al., 2006). SP degranulates mast cells and, therefore, further amplifies the inflammatory reactions. Capsaicin

pretreatment (which decreases or eliminates the functional contribution of C-fiber nociceptors) decreases phagocyte migration into inflammation sites and prevents degranulation of mast cells (Perretti et al., 1993). Antagonists of pro-inflammatory peptides such as SP may control inflammatory diseases or processes in which these peptides have a primary pathogenic role (Frieri, 2003).

SP-stimulation of murine mast cells activates TNF-α gene expression and induces TNF-α secretion (Ansel et al., 1993). It was also demonstrated by Azzolina et al. (2003) that the NFκB pathway is involved in the transcriptional regulation of the TNF-α and IL-6 over expression in SP-stimulated mast cells. SP also activates the transcription factor NFκB, a threefold increased nuclear translocation being observed in alveolar macrophages from healthy smokers (Bardelli et al., 2005). Our previous data and this study showed that Helicobacter pylori caused continuous infiltration of inflammatory cells and significant increase of SP IR nerve terminals as well as the SP immunoreactive immune cells (Sipos et al., 2006). Therefore, we have postulated that during the early events of an inflammatory process induced by neuropeptide SP could be the cause of the increase in TNF-α in the inflamed organs. The increased production of TNF-α might activate the NF-κB in the cells of the mucosa as well as it further activates the PARP which causes injury to the mucosal lining (Fig. 12).

PARP activation was identified as a key pathway in different pathophysiological conditions and disease states. Recent reports have shown that PARP can act as a coactivator of NFκB (Hassa and Hottinger, 2002). The cleaved form of PARP is catalytically inactive. Depending on the severity of DNA damage, genotoxic stimuli can trigger three different pathways. In the case of mild DNA damage, PARP facilitates DNA repair and thus survival. More severe DNA damage induces enzymes of the apoptotic cell death during which caspases, the main executor enzymes of apoptotic process, inactivate PARP cleaving into two fragments (p89 and p24). The most severe DNA damage may cause excessive PARP activa-
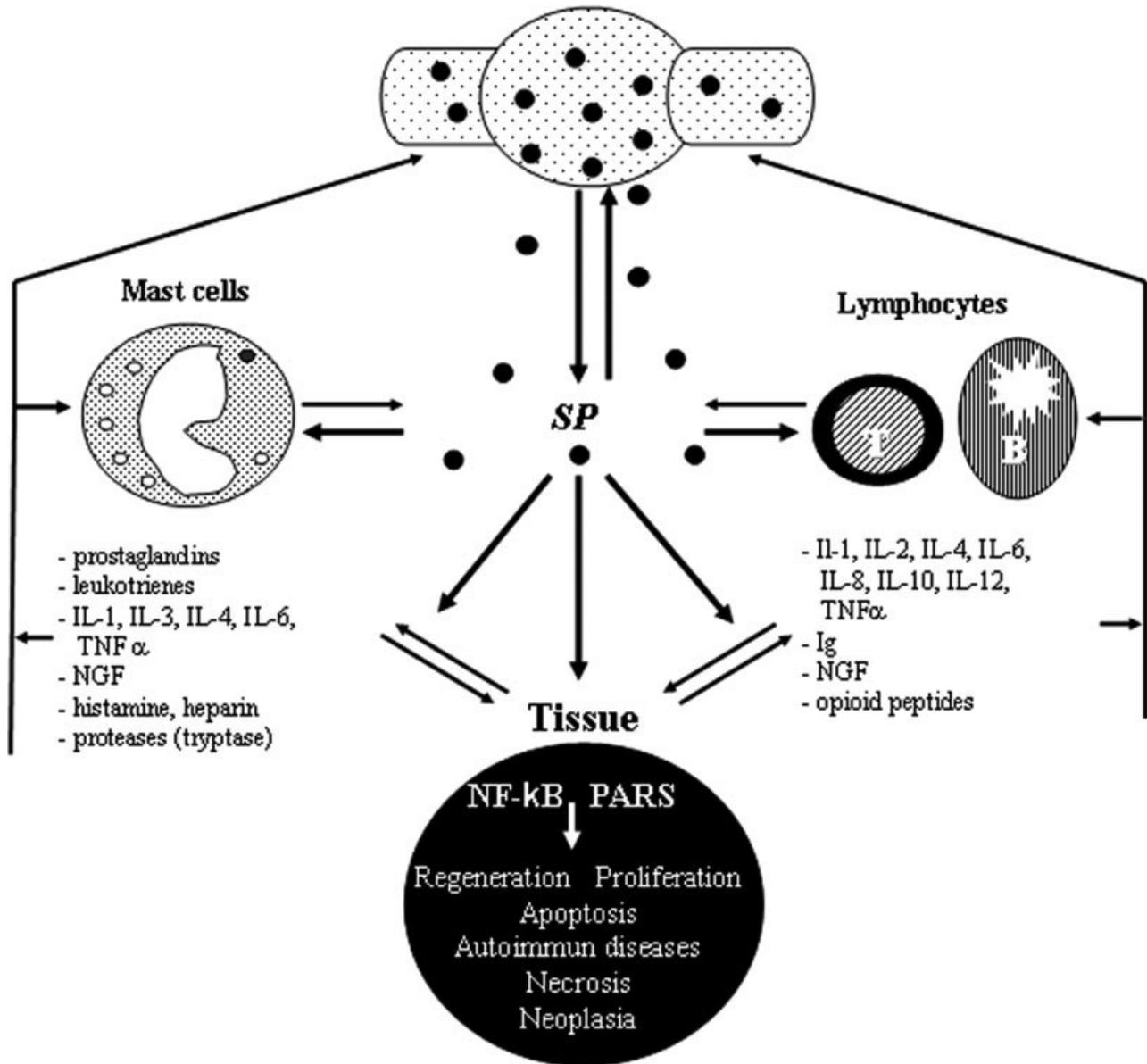
Fig. 12.   Postulated mechanisms of gastric mucosal injury.

tion inhibiting glycolysis and mitochondrial respiration and depleting NAD+ and ATP stores (Szabó et al., 1997, 2002). Our histological examination clearly demonstrated that significant PARP activation occurs in the mucosa of the stomach during chronic inflammation. Inhibition of PARP reduces the infiltration of neutrophils, decreases inflammatory infiltrate, which would be associated with a reduction in both oxygen and nitrogen centered free radical production (Szabó et al., 1997).

Our data indicate that immune cells (mast cells, lymphocytes, macrophages) are the most prominent cell types exhibiting activated NF-κB in the inflamed mucosa, where their number was increased in chronic gastritis. This is in agreement with the findings of Rogler et al. (1998) in patients with inflammatory bowel dis-

eases, who showed that NF-κB was mainly activated in macrophages and epithelial cells. There is evidence of NF-κB activation in inflammatory bowel disease, where the lamina propria macrophages display activated p50, c-Rel, and p65 (Neurath et al., 1996). Reed et al. (2005) demonstrated that NF-κB activation increased significantly biphasically (temporal activation) in experimental colitis. *Helicobacter pylori*-associated gastritis in previous studies exhibited increased NF-κB activity in gastric epithelial cells, where the number of NF-κB–positive cells correlates with the degree of gastritis (Van Den Brink et al., 2000). It is now recognized that activation of NF-κB induces many different genes, some of which are associated with inhibition of apoptosis and others with innate immunity and inflammatory

responses (Hassa and Hottinger, 2002). It was also demonstrated that PARP-1 can act as a coactivator of NF-κB. Secretion of pro-inflammatory cytokines by recruited inflammatory cells leads to continued stimulation of epithelial and endothelial cells that in turn might activate the NF-κB/PARP-1 complexes. Thus, a positive autoregulatory loop might be established that can amplify the inflammatory response and increase the duration of chronic inflammation resulting in a persistent activation of NF-κB/PARP-1 complexes characterized by overexpression of pro-inflammatory mediators. It was proposed that disrupting the physical interaction of PARP-1 and NF-κB might inhibit the abnormal transcriptional activity of NF-κB, thereby reducing the inflammatory response at the level of transcription in instances where this process becomes chronic or dysregulated (Hassa and Hottinger, 2002). NF-κB directed therapy was demonstrated to be effective in a model of inflammatory bowel disease induced by 2,4,6-trinitrobenzene sulfonic acid. It was also shown that selective TNF receptor inhibition may be advantageous with anti-TNF treatments in combating chronic inflammatory disease (Kollias, 2005). The treatment of patients with rheumatoid arthritis with antibodies against TNF-α can control refractory disease (Elliott et al., 1994). Single intraperitoneal injection of anti–TNF-α monoclonal antibody treatment significantly reduced serum/tissue TNF-α and improved indomethacin-induced enteropathy in rats by modulating iNOS expression (Saud et al., 2005). Our data indicate and suggest that TNF signaling system mediates mucosal damage by the enhancement of NF-κB activity parallel with increased number of SP nerve fibers and immune cells and that they may be the key pathogenetic factor of gastritis. TNF-α may influence the severity of disease, possibly by the persistent activation of NF-κB.

ACKNOWLEDGMENT

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