Neuroimmunomodulation in the gastrointestinal tract

Ph.D. thesis

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I. INTRODUCTION

Neuroimmunomodulation is a bidirectional communication between the nervous and immune systems. It has been generally accepted that nervous system plays an important role in the pathophysiology of the peripheral inflammation and has been involved in several inflammatory diseases. The nervous system plays a role not only in the induction and the maintenance of inflammation but also in the reduction and elimination of it.

The last literature data proved the close contact between the immunoreactive nerve fibres and immune cells in some organs, that it is confirmed the bidirectional contact between the nervous and immune system. Therefore, immune and nervous systems also have a role in the development of diseases and in the modulation of the functions of immune cells. According to the literature, many immune cells (activated lymphocytes and macrophages) can produce neuropeptides and present the receptor for these neuropeptides in the different cases (such as inflammations, injuries). Thus the neuropeptides secreted by these immune cells might contribute to the effect of neuropeptides from nerve fibres increasing the inflammation, act back to the place their synthesis (axon, immune cells) and further increased the effect of neuropeptides releasing from nerve fibres. Activated immune cells are secreting many inflammatory mediators which are probably inducing the tissue damage.

Neuropeptides take place as regulators in the inflammatory events; thereby it is supposed to have a role in the development of gastritis, hepatitis and cholecystitis. Therefore, the goal of the present study was to determine the precise localisation and function of these neuropeptide-containing nerve elements and immune cells involved in the regulation of pathogenesis of these organs.

II. AIMS

Therefore in the present study neuropeptides in the sympathetic, parasympathetic and sensory nerve systems as calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), somatostatin (SOM), substance P (SP), vasoactive intestinal polypeptide (VIP) immunoreactive (IR) nerve elements and cytokines as tumor necrosis factor-alpha (TNF-α) and nuclear factor-kappa B (NF-κB) IR immune cells were examined by immunohisto- and immunocytochemical and immunofluorescence methods in the rat's and human stomach, rat, cat, guinea pig and human liver and human gall bladder.

We have tried to answer the following questions:

1. What is the distribution and localisation of neuropeptide containing nerve fibres in the control stomach, liver and gall bladder?

2. Are there similar innervation patterns of the rat's and human stomach?

3. Are there similar innervation patterns of the rat, cat, guinea pig and human liver?

4. Can ganglion cells be demonstrated in the liver?

5. Are there any alterations in the number and distribution of different neuropeptide-containing nerve fibres during inflammation (in gastritis, cholecystitis and hepatitis)?

6. Are there any alterations in the number and function of immunocompetent cells in these inflammatory conditions?

7. Can close contacts be demonstrated between the neuropeptide-containing nerve fibres and immune cells?

III. MATERIALS AND METHODS

Materials

Animal materials

Young albino Wistar male rats, young male cats and young male guinea pigs (Semmelweis University, ÁOK NET Central Animal Laboratory) were used in our experiments.

Experimental gastritis was induced in rats by adding iodoacetamide (0.1%) and sucrose (1.0%) to the drinking water for 4 and 8 days. The control group was given drinking water supplemented only with sucrose (1.0%).

All experimental procedures used conformed to the Ethical Committees on Animal Experiments, Semmelweis University, as well a specific Hungarian national law (e.g. the current version of the Hungarian Law on the Protection of Animals, No. 243/1998).

Human materials

1. Antrum biopsies were obtained from Helicobacter pylori (HP) positive patients (10 persons). Control materials were obtained from the normal histological stomach of HP negative patients having dyspepsia (10 persons).

2. Liver biopsies were obtained from patients with autoimmune hepatitis (8 persons). No person had cirrhosis or decompensated liver disease. Control samples were taken from non-tumours parenchyma adjacent to surgically removed metastasis of colorectal carcinoma (5 persons) (Transplantation and Surgical Clinics, Semmelweis University, Budapest). The sample was excised in the normal parenchyma at least 5 cm distance from metastasis.

3. Samples of gall bladder were taken from patients with chronic cholecystitis (10 persons) who underwent laparoscopic cholecystectomy because of bile stones (2nd Department of Surgery, Semmelweis University, Budapest). Normal gall bladders were obtained from healthy patients few hours after their death (3 persons), Department of Forensic and Insurance Medicine, Semmelweis University, Budapest).

Light and electron microscopic examinations were done at every human sample.

All patients gave their written informed consent according to the Semmelweis University guidelines for ethics in human tissue experiments. Permission for patient studies was given by our local authorities (TUKEB No 85/2006, 36/2007) similar to the Declaration of Helsinki (Hong Kong Amendment 1989).

Methods

1. Light microscopic examination

Avidin–biotin-complex (ABC) and DAB (diaminobenzidin chromogen reaction) methods with nickel intensifications were used to show the different IR nerve elements.

2. Electron microscopic examination

To show the evidence of close contacts between immune cells and nerve fibres, to determine the type of immune cells as well as the alterations in the IR nerve fibres the sections were processed using ABC method, and were postfixed in osmium (OsO_4), dehydrated, and embedded. Ultrathin sections were examined by Jeol 100 electron microscope.

3. Confocal laser immunofluorescence microscopic examination

To show the co-localisation of neuropeptides we used double staining (SP, NPY-FITC-green and TNF-α, NF-κB–Alexa 594-red) and confirm the results obtained by the light microscopic examinations the BioRad MicroRadiance Confocal laser microscope system was used.

Control experiments

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

Quantitative analysis:

Using quantitative analysis the number of IR nerve elements and immune cells were counted in a 15-20 mm^2 tissue area and calculated for 1 mm^2. For analysis, 40 X magnifications were used with a graduated eyepiece graticule and the entire section was assessed. In every cases nerve fibres and immune cells were counted in the 15-30 numbers of sections.

Statistical analysis

Statistical analysis was performed using two sample Student t-test and ANOVA. A p value of less than 0.05 was considered to be statistically significant.

IV. RESULTS

The localisation and the distribution of different neuropeptide/transmitter containing nerve fibres in the control materials

Animal materials

Innervation of the rat gastric mucosa

The density of the VIP- and NPY-IR nerve fibres was the highest in the mucosa among the glands. The NPY-IR nerve fibres were observed mainly around the blood vessels. Only a few SP- and CGRP-IR nerve fibres were showed among the glands. The number of SOM-IR nerve terminals was only sporadical. A few SOM and CGRP positive endocrine cells were also demonstrated in the deep regions of the glands. The electron microscopic investigation showed the reaction end-products in the cytoplasm and at the membranes of these cells.

Innervation of the rat liver

Only a few NPY positive nerve fibres were found in the portal region. No IR nerve fibres were detected in the parenchyma.

Innervation of the cat liver

Some NPY-, SP- and SOM-IR nerve fibres were detected in the perivascular plexus of the portal tracts. In the intralobular areas some IR varicose fine nerve fibres were found. Some SOM- and NPY-IR nerve cell bodies were also found in the connective tissue of the porta hepatis and in the large portal tracts.

Innervation of the guinea pig liver

Dense network of NPY-IR nerve networks were observed around the blood vessels of the portal triads and in the intralobular regions. In the human liver the density of these nerve fibres was much higher than in the guinea pig, rat and cat liver. Some SP, SOM and VIP positive nerve fibres were found in both the portal and the intralobular regions. Some NPY positive nerve cell bodies were observed in the connective tissue of the porta hepatis.

Human materials

Innervation of the human gastric mucosa

The density of the VIP- and NPY-IR nerve fibres was most pronounced in the gastric mucosa. NPY-IR nerve fibres were found mainly around the vessels. CGRP, VIP and SP positive nerve fibres were detected mainly among the glands, the number of these was moderate. The electron microscopic investigations showed the reaction end-products around the membranes of vesicles and axolemma and diffused in the axoplasm.

Innervation of the human liver

The number of NPY-IR nerve fibres was most pronounced in human liver. The density of the SOM-, SP- and VIP-IR nerve fibres was moderate, but only few CGRP-IR nerve fibres were detected. The distributions of intrahepatic peptidergic innervations in human and guinea pig were similar. Some NPY-IR and a few SOM-IR nerve cell bodies were also observed around the central vein.

Innervation of the human gall bladder

The VIP-, NPY-, SP- and CGRP-IR nerve fibres were observed in all layers of the gall bladder. Sometimes few IR nerve cells were showed in the myenteric plexus.

Changes in the number of neuropeptide/transmitter containing nerve fibres in pathological conditions

Animal materials

Iodoacetamide-induced gastritis in rat

In gastritis the numbers of SP, NPY and VIP positive nerve fibres were increased significantly ($p<0.05$), while the number of CGRP-IR nerve fibres was decreased slightly, compared to the control. The numbers of SOM and CGRP positive cells were increased significantly in gastritis ($p<0.001$).

Human materials

Human gastritis

In gastritis the numbers of SP-, NPY- and VIP-IR nerve fibres were increased significantly ($p<0.05$), while the number of CGRP positive nerve fibres was decreased slightly, compared to the control. The electron microscopic analysis showed that IR nerve fibres were wrapped in Schwann cell processes, whereas in other cases, the terminals had completely lost their association with Schwann cells and were located in a very close association with the immune cells. The varicosity of SP IR nerve terminals showed a large number of large granulated and small clear synaptic vesicles.

Human autoimmune hepatitis

In the inflamed liver the number of NPY-IR nerve fibres was increased significantly ($p<0.05$), compared to the control. The numbers of SOM-, SP- and VIP-IR nerve fibres were also increased, but the difference was not significant. Only very few CGRP-IR nerve fibres were detected. The numbers of SOM and NPY positive nerve cell bodies were also increased in hepatitis.

H*uman cholecystitis*

The number of VIP-IR nerve fibres was significantly increased in cholecystitis ($p<0.05$). A large number of nerve fibres were observed in the mucosa right under the epithel cells. The number of NPY positive nerve fibres was increased slightly, but the difference was not significant. However, the number of SP-IR nerve fibres was decreased significantly in chronic cholecystitis ($p<0.05$). In the myenteric plexus the number of VIP positive nerve cell bodies was also increased.

Changes in the number of immunocompetent cells and their contact with the immunoreactive nerve fibres in pathological conditions

Animal materials

Iodoacetamide-induced gastritis in rat

In the experimental gastritis the numbers of SP- and TNF-α-IR immune cells were significantly increased ($p<0.001$). Fluorescent double-labelled immune-staining showed that the part of SP positive immune cells also showed immunostaining for TNF-α. Immunofluorescence double staining also revealed numerous immune cells close to the SP-IR nerve fibres.

Human materials

Human gastritis

A large number of NPY- and SP-IR immune cells was observed in gastritis. The electron microscopic investigation showed that these cells belong to lymphocytes. The distance between the IR nerve fibres and immune cells was about 1μm or very often less than 20 nm. In gastritis the number of TNF-α positive immune cells (lymphocytes, macrophages) was increased significantly ($p<0.001$). In gastritis the NF-κB immunoreactivity was observed in the nucleus of the cells. Confocal laser microscopic investigations revealed that some of the SP positive immune cells were both immunoreactive for TNF-α and NF-κB. The electron microscopic examinations showed that the reaction end-products were distributed in the cytoplasm of the TNF-α-IR cells, while in the NF-κB-IR cells the reaction end-products were located in the nucleus of the cells.

Human autoimmune hepatitis

Many lymphocytes, mast cells and some Kupffer cells showed IR for NPY and SP. The NPY positive lymphocytes and mast cells were in close contact with the NPY-IR nerve fibres. In hepatitis the number of TNF-α– and NF-κB-IR immune cells was significantly increased ($p<0.001$). The electron microscopic examinations proved that these cells belong to lymphocytes, plasma cells and a few Kupffer cells were also observed. Fluorescent double-labelled immunostaining showed that these NPY-IR immune cells had no colocalization with TNF-α and NF-κB. However, some of the SP positive immune cells exhibited inmunostaining for NF-κB.

Human cholecystitis

In cholecystitis a large number of immunocompetent cells showed IR for SR, CGRP and VIP next to IR nerve fibres. The electron microscopic investigations proved that these cells belong to different immunocompetent cells (lymphocytes, plasma cells and mast cells). The distance between IR nerve fibres and immune cells was about 1 μm or even less.

V. DISCUSSION

The process and the therapy of the diseases may be influenced by not only the immune system but also the nervous systems. The most frequent neuropeptides involved in the neuroimmunomodulation are the next: NPY, SP, CGRP, VIP and SOM, and they influence in the function and inflammation of different organs.

The function of the stomach is regulated by vegetative and sensory nerve fibres together. In human being the neuropeptides released from both the extrinsic and intrinsic nerve fibres take place in the regulation of the gastric acid secretion and the integrity of the mucouse membrane. In the inflammation the number of the different neuropeptide-containing nerve fibres changed differently. The number of SP, NPY and VIP IR nerve fibres increased significantly, while the number of CGRP IR nerve fibres decreased in the inflamed stomach compared to the control. SP and CGRP are synthesized in the sensory nerve elements, making axon reflex and located around the small vessels, participate in development of inflammation and pain as neurogenic mediators. In the chronic gastritis the IR nerve fibres were located in a very close situation to the immune cells, suggesting that these neuropeptide-containing nerve fibres took place in the immunomodulation. In the inflammation lymphocytes, plasma cells, mast cells and eosinophil granulocytes were accumulated in the inflamed area. These immune cells also express different neurotransmitter receptors. **Our results proved that some of the lymphocytes were IR for NPY and SP in the inflamed stomach suggesting that these immune cells expressed these peptides and participate in the local immune reactions of the stomach.** It is widely accepted that bidirectional interactions exist between the nervous and immune systems in the gastrointestinal tract. Changes in immune function might influence the distribution of nerves and expression of neural transmitter receptors in immune cells.
Acute and chronic psychological stress might also cause inflammatory reactions in the stomach. These inflammatory processes may occur by release of neuropeptides (especially SP) from lymphocytes and primary capsaicin sensitive sensory nerves and amplifying the inflammatory response via an axon reflex. Therefore, it can be concluded, that better treatment/control of disease activity and pain can be achieved by blocking the cascade leading to amplification of inflammatory process. We therefore propose that neuropeptides in gastritis act as endogenous factors that regulate immune homeostasis.

In our experiments we compared the innervation pattern of liver from human being, guinea pig, cat and rat. The density and distribution of NPY IR nerve fibres were similarly high in the guinea pig, cat as in the human liver, however, in the rat liver only few IR nerve fibres were found mainly around the portal vessels. The similarity of the peptidergic innervation from guinea pig and human being was

documented by other authors. Our light- and immunocytochemical investigations proved that the innervation of the human liver is the most similar to the guinea pig ones. The density and distribution of the neuropeptide-containing nerve fibres were similar in both samples. The most numerous NPY-IR nerve fibres were found in the human liver and they were located not only around the portal triads but also between the hepatic cells and sinusoids, like the aminergic nerves. These fibres play a very important role in the blood supply, bile secretion, in the metabolism of the glycogen and lipids. Transection of the extrinsic nerves resulted in a marked decrease in NPY IR nerve fibres; however, a significant number of them remained intact. These findings suggest that hepatic NPY nerve fibres arise from the coeliac ganglion, as well as from other sources. Some NPY-IR nerve cell bodies were noted in the connective tissue of the port hepatis, indicating that they may originate from the intrinsic ganglia located around the great vessels. In contrast to extensive previous immunohistochemical studies using serial sections, **intraparenchymal nerve cell bodies in the human, feline and guinea pig liver we shown only in our studies.** These intrinsic nerve elements might be very important for the functions of the transplanted liver.

The role of the different neuropeptides was investigated in autoimmune diseases; however, the changes of the different neuropeptide-containing nerve fibres in the autoimmune hepatitis were publicated by our laboratory. The number of the NPY IR nerve fibres was increased significantly in hepatitis. NPY might take a very important role in the blood supply of the liver, making very dense perivascular plexus around the portal vessels and central vein. A large number of SP IR nerve fibres were observed around the vessels and in the Disse space, their number also increased in autoimmune hepatitis. The number of the NPY IR nerve fibres was increased significantly in the inflammation, and they were located in a very close situation to the lymphocytes and macrophages which were also immunoreactive for NPY. This morphological close relationship suggests the direct interaction between the nerve fibres and immune cells in human hepatitis. The NPY might modulate the functions of the immune cells. It is proved that NPY inhibits the neurogenic inflammation presynaptically. So NPY might be a new possibility in the therapy of the citokin-mediated autoimmune and inflammatory diseases, for example in autoimmune hepatitis.

The release of the bile is influenced by different factors. The neuropeptide-containing nerve fibres act on the synthesis of the bile in the liver, influence the contraction of the gall bladder, as well as the m. sphincter Oddi. The absorption and secretion in the gall bladder is influenced by the nerve fibres being parasympathetic and sympathetic. In the inflamed gall bladder the number of the VIP IR nerve fibres increased significantly. These fibres were mainly located in the inner circular layer of this organ causing the relaxation, stagnation of the bile, might play a role in the bile stone development. In cholecystitis the number of the

VIP IR also increased significantly. The electron microscopic investigations showed that these VIP IR nerve fibres were in a very close situation to the membrane of smooth muscle cells, suggesting the direct effect on it. Increased number of VIP IR nerve fibres after irritation of the mucouse membrane of the gall bladder might play a very important role in the function of the epithelial lining, might influence the blood supply, increasing the permeability of the capillaries. NPY IR nerve fibres are located in the perivascular plexus of the arteries and veins and play a very important role in the regulation of the blood supply. In the inflamed gall bladder the number of the CGRP and SP IR nerve fibres was significantly decreased which might also influence the bile stasis. In the cholecystitis the immune cells were also immunoreactive for SP, CGRP and VIP suggesting that these IR immune cells also involve in the inflammation, because the SP and CGRP increase the proinflammatory cytokine synthesis. The effect of them might be also opposite, while they inhibit the production of the free radicals and influence the inflammatory cytokine productions. Naturally, their effects depend on the balance of pro- and anti-inflammatory factors released from immune cells.

Our results supported that neuropeptides play a very important role in the physiological and pathological functions of the stomach, liver and gall bladder. During inflammation changes of the IR nerve fibres in these organs (regeneration and degenerations parallel) might act in the function of the organ and of the immune system.

The neuropeptide-containing nerve fibres were observed in a very close situation to the immune cells (20 nm-1 µm) suggesting the direct effect between the nervous and immune systems in the inflamed stomach, liver and gall bladder of the investigated animals and human being. Some of the immune cells were also immunoreactive for SP, NPY and VIP, suggesting that these neuropeptides might play a very important role in the development and maintenance of the pathological processes of these organs.

VI. CONCLUSIONS

The nerve system plays a crucial role in pathophysiology of the peripheral inflammation and has been taken place in many inflammation diseases. Neuropeptides play a very important role in these cases. The adequate volume and quality of gastric juice and bile secretion is important in protection of mucosa and digestion. The changes in the neuropeptide-containing nerve elements play a role in the pathogenesis of gastritis, hepatitis and cholecystitis and take place in the development and maintenance of inflammatory processes. Immunocompetent cells may also produce neuropeptides, thereby enhance the inflammation, and have back effect on nerve elements. Locally released bioactive mediators from immune cells act directly on surrounding tissue cells, may cause apoptosis, necrosis, nerve regeneration and proliferation. All these factors have a role in pathological alterations (metaplasia, tumours) of the stomach, liver and gall bladder.

Balance between the pro- and anti-inflammatory factors may also be important in the control of inflammation. According to the last data neuropeptide agonists and antagonists could be useful therapeutic agents for treatment of some inflammatory and autoimmune diseases.

VII. PUBLICATIONS

The theses is based on the following publications

1. **Pongor É,** Fehér E, Lászik A, Sipos P. A különböző neuropeptid tartalmú idegelemek számának változása humán gyulladt epehólyagban. Orvosi Hetilap 2006; 32: 1513-1518.
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2. **Pongor É,** Baán Sz. A neuropeptid tartalmú idegelemek mennyiségének változása humán epehólyagban gyulladás hatására. TDK konferencia, Budapest, 2005.
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