CHANGES OF THE DIFFERENT NEUROPEPTIDE CONTAINING NERVE ELEMENTS IN THE SJÖGREN’S SYNDROME AND DIABETES MELLITUS

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Introduction

It has been generally accepted that nervous system plays an important role in the pathophysiology of the peripheral inflammation and has been involved in many inflammatory diseases. The nervous system not only plays a role in the induction and maintenance of inflammation, but also in the elimination of it.

The oral health is important not only in the maintenance of the teeth and mucosa, but it is one of the determinative factors of the life quality. The number and the distribution of nerve fibres are altered in many diseases, including Sjögren’s syndrome (SS) and diabetes mellitus.

SS is a chronic autoimmune disorder of exocrine glands with unknown aetiology, affecting many organsystems, where lately the exocrine glands became infiltrated by lymphocytes (mainly by T lymphocytes), their acini are destructed and the patients have xerostomia and xerophthalmia. Two forms of SS are differentiated: primary and secondary SS, where the last one is occured with other connective tissue diseases, e.g. rheumatoid arthritis, systemic lupus erythematosus.

Diabetes mellitus is an overall name of all diseases, where the chronic hyperglycaemia as well as the disorders of glucose, lipid and protein metabolism exists. The occurrence of xerostomia is a characteristic symptom not only for SS but also for diabetes mellitus. The statistical examinations of last years determined that a variety of oral complications are frequently found to be associated with diabetes mellitus, e.g. atrophic tongue lesions, glossitis, parodontitis, sensory disorders (burning mouth syndrome, impairment of taste), leukoplakia, lichen oris planus, and tumours. According to the literature the incidents with leukoplakia occured following the second year of the manifestation of diabetes mellitus, frequently in insulin treated patients.

Peripheral neuropathy is a frequently occured disease, which could render more difficult to patients, cause the loss of achievement and come to that death. In SS peripheral neuropathy is the most common and well-known neurological complication affecting about 20-30 % of patients. According to the literature the clinical evidence of glandular involvement is often minimal or absent, when patients with SS develop peripheral neuropathy. A diffuse decrease in myelinated nerve fibre density and active axonal degeneration with marked mononuclear cell infiltration around the neurones and blood vessels was also observed. Defect in neurogenic regulation of morphologically intact gland tissue may contribute to the loss of gland function and acinar atrophy. The one of late symptoms of diabetes mellitus is a diabetic polyneuropathy, which affect the motor, sensory and autonomic nervous systems. A sensory neuropathia principally develop in paraesthetic form. In far gone cases the awry sens, the prickle, the torpidness became pronounced and pain is frequently appeared. We suppose that early events in diabetes mellitus might contribute to the development of such alterations.

On the basis that neuropeptides have a role in the inflammatory events, it is supposed that they have a role in the development of hyposalivation, xerostomia and different oral complications.
Aim

Therefore in the present study the distribution and the precise localisation of the nerve fibres containing the frequently observed neuropeptides as vasoactive intestinal polypeptide (VIP), substance P (SP), neuropeptide Y (NPY), galanin (GAL), calcitonin gene-related peptide (CGRP), somatostatin (SOM) and neural nitric oxide synthase (nNOS – enzyme, which is involved in the synthesis of NO), as well as noradrenaline (TH – tyrosine beta hydroxylase enzyme, which is involved in the synthesis of catecholamine, the marker of postganglionic sympathetic nerve fibres) were studied in the minor salivary glands of the patients with SS and in the root of the diabetic rat’s tongue.

We have tried to have an answer to the next questions:

1. What is the distribution and localisation of neuropeptide containing nerve fibres in the minor salivary glands and in the root of the tongue?

2. Are the any alterations in the number of them in the inflammatory conditions (in the SS and diabetes mellitus)?

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

4. It could be demonstrated the close contacts between the neuropeptid containing nerve fibres and immunocells?

5. Which kind of ganglion cells is found in the root of the tongue? Are the any alterations in the number of them in the inflammation?

6. Does the insulin treatment prevent the alterations in the number of neuropeptide containing nerve fibres?
Materials and Methods

Materials:

1. **Human biopsies:**
   Labial salivary glands were obtained from 5 patients with primary SS and 8 SS patients with sensory neuropathy as well as from 12 healthy controls. Primary SS was diagnosed if any four of the six classification criteria have been fulfilled. All the patients and the healthy volunteers gave their informed consent according to the guidelines for ethics in human tissue experiments.

2. **Animal materials:**
   Albino Wistar male rats (weight 120-450 g) were used in our experiments. Diabetes mellitus was induced by a single dose of streptozotocin (65mg/kg) injected into the tail vein. Blood was withdrawn from the tail vein and glucose was measured by the glucose oxidase method. Rats displaying glucose levels > 16 mmol/l was considered diabetic. The diabetic animals of the insulin replacement study received injection of Ultralente insulin 2 times daily. One group of insulin treated animals received the treatment immediately on the morning that hyperglycaemia was first identified; the others were treated only 1 week after, where the diabetes was manifested. Insulin was given intramuscularly in an individual dose to keep blood glucose level between 4.5-12.5 mmol/l. All groups were maintained under identical conditions. After one, two and four weeks duration of diabetes mellitus the animals were terminally anaesthetised and perfused by Zamboni, later the pieces of the tongue root of the rats were obtained.

   All experimental procedures used conformed to the “Principles of laboratory animal care” (NIH publication No. 86-23, revised 1985), as well a specific Hungarian national law (e.g. the current version of the Hungarian Law on the Protection of Animals, No. 243/1998).

Methods:

1. **Light microscopic examination**
   Avidin – biotin - complex (ABC) and DAB (diaminobenzidin reaction) method was used to show the different IR nerve elements.

2. **Electronmicroscopic examination**
   To show the evidence of close contacts between immunocells and nerve fibres, to determine the type of immunocells as well as the alterations in the IR nerve fibres the sections were processed using ABC method, and were postfixed in OsO₄, dehydrated, and embedded. Ultrathin sections were examined by Jeol 100 electronmicroscope.

3. **Confocal microscopic examination**
   To show the co-localisation of neuropeptides (SP-FITC and VIP – Alexa 594) in the intralingual ganglion cells and confirm the results obtained in the light microscopic examinations the Bio Rad 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 was appeared.
Quantitative analysis:

Using quantitative analysis the number of IR nerve elements, immunocells and contacts between IR nerve fiber and mast cells were counted in a 15-20 mm² tissue area and calculated for 1 mm². For analysis, 40 X magnifications were used with a graduated eyepiece graticule and the entire section was assessed. Microphotographs were also taken, digitalized and then analysed using a PC-based image analysing software.

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.
Results

1. The localisation and the distribution of different neuropeptide containing nerve fibres in the control materials:

In the labial salivary glands of control volunteers:

All neuropeptides investigated were present in the glands with different density and distribution. The numbers of VIP, NPY, TH and NOS IR nerve fibres were more pronounced. Large number of VIP and NOS IR nerve fibres was seen around the acini. The number of NPY and TH positive nerve fibres was less and mainly found around the blood vessels. The number of SP IR nerve fibres was moderate and SOM and CGRP IR nerve fibres were found in the lowest number. Under the electronmicroscopic examination the IR nerve fibres had many small clear synaptic vesicles and large granulated ones. No immunocompetent cells were IR.

In the root of the control rat’s tongue:

In the root of the rat’s tongue IR nerve fibres were found with different variety in all layers. The density of SP IR nerve fibres was higher in the mucosa beneath the epithelium. NPY and TH IR nerve terminals were mainly observed around the blood vessels. A large number of VIP IR nerve fibres and some SP, NPY IR nerve fibres were distributed around the serous and mucous acini of the glands. The density of TH, SOM and GAL IR nerve fibres was the lowest.

Some ganglia were observed in the vicinity of vessels, in the connective tissue of the gland, in the striated muscle and beneath the epithelium. These cells were IR for NPY, VIP, SOM and SP having a large (30-50 \(\mu\)m in diameter) and medium (20-30 \(\mu\)m in diameter) size with round and ovoid shape, they had large nuclei and long neuronal processes. The VIP and NPY IR ganglion cells were found mainly in the vicinity of muscle and in the connective tissue of the glands, they often had numerous neuronal processes. The SP IR neurons were placed beneath the epithelium and in the muscle layer. The confocal examination showed that these SP IR cells didn’t show the colocalisation with VIP and had mainly one neuronal process. The SP IR neurons were placed beneath the epithelium and in the muscle layer. Under the electronmicroscopic examination showed that SP IR nerve fibres are found in close contacts with both IR and immunonegative neurons of the ganglion.

2. Changes in the number of IR nerve fibres in pathological conditions:

In the labial salivary glands of the patients with SS:

In the area of lymphoepithelial lesion the acini were destroyed and the number of nerve fibres was significantly reduced comparing to the control. In the inflammation area some degenerated nerve fibres were found having dark degenerated mitochondria.

The morphological structure of labial salivary glands from SS patients with sensory neuropathy was similar to those in the control (neither apoptosis nor fibrosis were seen). The number of the SP, NPY IR nerve fibres was decreased, however the number of GAL, VIP and TH IR nerve terminals was increased compared to the control. The SP, VIP, TH and NPY IR nerve fibres become thicker in SS. Under the electronmicroscopic examination some nerve terminals showed signs of degeneration.

In the diabetic rat’s tongue:

After one week of the streptozotocin treatment the total number of IR nerve fibres was decreased. However, after four weeks duration of diabetes the number of IR nerve fibres increased significantly compared to the control. The increased number of SP IR nerve processes was located mainly in and
below the epithelium. The diameter of SP, NPY, TH IR nerve fibres has been also markedly increased. In the electronmicroscopic examination some of the nerve fibres were found in degeneration in the early stage of diabetes mellitus.

**In the insulin treated diabetic animals:**

After immediate insulin treatment the decrease of the IR nerve fibres has prevented. However the late administration of it the number of IR nerve terminals was further enhanced significantly compared to the untreated diabetic data.

3-4. **The immunocompetent cells and their contacts with IR nerve fibres:**

**In the labial salivary glands of the SS patients:**

Lymphocytic infiltration was found in the labial salivary glands of the patients with primary SS. In the SS patients with sensory neuropathy no real lymphocytic infiltrates were found, however some areas were infiltrated by different immunocompetent cells. Counting of all immunocompetent cells in whole sections showed that 46.2% of them were IR for SP and 34.4% was IR for NPY. The electronmicroscopic investigation proved that these cells, e.g. lymphocytes, plasma cells and mast cells had IR for these peptides. Sometimes the nerve fibres had a close contact with lymphocytes and plasma cells. The synaptic gaps between immunocells and nerve fibres were 40-200 nm.

**In the tongue of the diabetic rats:**

After the streptozotocin treatment the oral mucosa seemed normal, however the light- and electronmicroscopic examination revealed that the lamina propria was infiltrated diffusely by inflammatory cells. Immunohistochemical examination showed that mast cells were found in all areas of the tongue. In the control materials no immunocells were found having IR for any antisera. However in the diabetes mellitus many of them became IR for SP and NPY. These cells were lymphocytes, plasma cells and mast cells. After 4 weeks of the treatment significantly (p < 0.05) increased number of mast cells were observed in all layers of the tongue, especially around the blood vessels and in the lamina propria below the epithelial lining. The number of contacts between SP IR nerve fibres and mast cells was increased even more significantly (p < 0.01) in diabetes in all layers of the tongue. Counting of all immunocompetent cells in whole sections showed that 12.3% of them were IR for SP and 25.4% was IR for NPY. The electronmicroscopic investigation showed that SP IR nerve fibres were in close situation (20-200 nm) to the membrane of the mast cells and proved that these immunocompetent cells are lymphocytes, plasma cells and mast cells.

**In the insulin treatment** the number of IR immunocells decreased.
Discussion

The morphology and histology of minor salivary glands of rats are similar to the human minor salivary glands. Therefore, rat’s salivary glands are widely used experimental model. Our light and electronmicroscopical examinations also demonstrate that no alterations are found in the innervation of human and rat’s salivary glands. The number and density of neuropeptide containing nerve fibres were similar in both of tissue.

From the localisation and the density data of VIP IR nerve fibres we suggest that these fibres have a major role in the regulation of minor salivary gland secretion and blood flow. The minor salivary glands have NPY and SP IR nerve fibres around the acini and blood vessels, suggesting that these nerve fibres are mainly involved not only in the regulation of the blood flow, but also in the modulation of salivary secretion.

The SP IR nerve fibres are mainly found in and under the epithelium as well as around the blood vessels of tunica propria. For the first time we have presented the distribution of IR nerve fibres in the different areas of the root of the rat’s tongue. The intralingual ganglion cells IR for SP, VIP, GAL, and NADPH were previously demonstrated by others. The electronmicroscopic examinations demonstrated that in the local ganglion the SP IR nerve fibres are found in a very close contact and sometimes in the synapse. The confocal microscopic double staining examination showed that SP IR perycaryon doesn’t show immunoreactivity for VIP. On the basis of these results we suggest that these SP IR neurons and nerve fibres belong to the afferens branch of the intralingual reflex. On the other hand, the VIP, NPY IR nerve elements, which are affect the salivary secretion and bloodflow, might have a function in the efferens branch of this reflex.

It has been suggested by many literatures that neuropeptides have an important role in the formation of autoimmune and inflammatoric events. According to the latest data of literature focal adenitis may cause the degeneration of local nerve fibres as well as the atrophy of gland cells, which in turn might contribute to the decrease of salivary secretion. In our experiments we could not found IR nerve fibres in the inflamed areas, only degenerated nerve fibres have been detected by electronmicroscopic examination. In our experimental materials from Sjögren syndrome (SS) patients with sensory neuropathy, nor focal lymphocitic infiltration neither fibrosis could be detected, where different type of immunocells have been observed and the number of neuropeptide containing nerve fibres showed alterations. Some of immunocells showed IR for SP and NPY.

According to the literature and our results we suggest that imbalance in the neuropeptide level might play a role in the pathogenesis of SS. Neuropeptides as local neurogenic components of inflammation have effect on the functions and the activation of other immunocompetent elements. Therefore, they could be involved in the formation of lymphocitic infiltration. The neuropeptides released from nerve terminals and immunocells might have a direct effect on acini, blood vessels and other immunocells. Inflammatory factors released from activated immunocells might exacerbate the inflammation, might cause atrophy, apoptosis and necrosis of the acini.

Diabetes mellitus induced alterations in the number and localisation of neuropeptide containing nerve fibres has been reported. In our experiments the number of different IR nerve fibres decreased in one week duration of diabetes and significantly increased after 4 weeks duration of it. The increasing in the number of IR nerve fibres and the enhancing in the diameter of them suggest that these neuropeptides are involved in the early events of the diabetic inflammation and it has happened because the synthesis of neuropeptides has been increased and/or possibly the proliferation of nerve fibres occured.

In our experimental diabetes the number of different neuropeptide containing nerve fibres decreased, which have been followed by significant increase of them. One of complications of diabetes mellitus is neuropathy, which is involved all kind of nerve fibres including the motor, sensory and autonomic nervous system. Nerve regeneration coexists with degeneration and contributes to nerve function as well as nerve pathology in diabetes mellitus. The decreased nerve regenerative capacity in diabetes has been associated with impaired neurotrophic tone. In diabetic patients and experimental models, peripheral tissue NGF synthesis as well as retrograde axonal transport of it has been reported to be decreased. In the present study significantly increased number of neuropeptide containing nerve fibres was observed after 4 weeks duration of
diabetes, suggesting regeneration, sprouting, proliferation of nerve fibres, as well as increased synthesis of neuropeptides (mRNA upregulation).

There are conflicting literature data about the effect of insulin on the nerve elements and their content. Authors have been observed that oral diseases are frequently found among the insulin treated patients. According to the literature data insulin treatment has different effect on the quantification of neurotransmitters: stimulates the synthesis of them in different regions of central nervous system, or downregulates the production of them. In our experimental models after immediate insulin treatment the changes of the IR nerve fibres has prevented. However, after the late administration of insulin the number of IR nerve terminals was further enhanced significantly compared to the untreated diabetic data. It has been described that insulin has neurotrophic effect. In the diabetic inflammation, where the effect of free radicals, changes in the NGF level, the infiltration of immunocells occured the late insulin treatment perhaps doesn’t have preventive effect. The insulin treatment perhaps moderates the inflammatory events (by decrease of IR immunocells). We suggest that insulin administration enhances the synthesis of neuropeptides and the proliferation of nerve elements.

It has been known for many years that psychic conditions and the nervous system affect the function of immunosystem. It has been suggested that mast cells, as well as SP may be involved in the axonreflex responsible for the flare reaction (vasodilatation, neurogenic inflammation) to noxious stimuli. Increased number of mast cells and mast cell-nerve fiber contacts were found in the different pathological conditions, including rheumatoid arthritis, peripheral neuropathy, gastrointestinal diseases and infections. Neurogenic inflammation results the degranulation of mast cells, and many vasoactive proinflammatory and nociceptive mediators as histamine, cytokines, proteolytic enzymes may cause the tissue inflammation. It has been also described that other immunocells have a response for the different pathological changes. Mast cells might have an inductor role in the inflammatory events, activate other immunocells, and cause aggregation of them. In both materials from SS patients and diabetic animals some of immunocells were IR for SP and NPY, suggesting that these cells might be activated. According to the literature, many immunocells (activated immunocells, macrophages) can produce neuropeptides and present the receptor for these neuropeptides. Thus the neuropeptides secreted by these immunocells might contribute to the effect of neuropeptides from nerve fibres. Activated immunocells are secreting many inflammatory mediators which are probably inducing the tissue damage.

The neurotransmitters and neuropeptides are stored in secretory vesicles and after the activation they are released and reach all the effector cells. On the basis of physiological and morphological examinations the pre- and post-synaptic elements of neuroefferct junctions may be separated by distance of 200-300 nm, or up to 2 \( \mu \)m in some instances. IN our examinations, neuropeptide containing nerve fibres and immunocells are found in a very close contacts (20nm-1\( \mu \)m); suggest that direct association occured between the neuro- and immunosystems. As an effect of inflammation some of immunocells were IR (for SP and NPY), suggesting that these neuropeptides are might enhance the pathological events, and change in the nerve elements (degeneration, regeneration, proliferation).
Conclusion

The adequate volume and quality of salivary secretion is important in the maintenance of oral homeostasis; hyposalivation lead to the difficulties in the formation of mould and swallow, disturb the speech, caries, gingivitis, periodontitis, candidiasis are frequently develop in the patients. Changes in the neuropeptide containing nerve elements have a role in the formation and maintenance of inflammatory events, might implicated in the pathogenesis of Sjögren syndrome and diabetes mellitus. Immunocompetent cells may also synthesise neuropeptides, hereby enhance the inflammation, and have back effect on nerve elements. Locally released bioactive mediators from immunocytes act directly on surrounding tissue cells, may cause apoptosis, necrosis, nerve regeneration and proliferation. All these factors have a role in the oral pathological alterations (lichen oris planus, leukoplakia, tumours). Many other factors as environment, nicotine, alcohol and other agents may also contribute to the diseases.
The theses is based on the following publications

**Articles:**

   **IF= 0.309**
   **IF= 0.309**
   **IF=1.098**
   **IF=2.633**

**Abstracts:**

   **IF=0.837**
   **IF=1.076**
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