CORRELATIONS BETWEEN THE MICROSCOPIC AND MACROSCOPIC ASPECTS OF THE ORAL MUCOSA AND THE MICROBIAL FLORA IN PATIENTS WITH PERIODONTOPATHIES AND DIABETES MELLITUS

- ABSTRACT -

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CRAIOVA
2012
INTRODUCTION

GENERAL CONSIDERATIONS

Chapter I – Histology of oral cavity.

Chapter I – Microbiologic aspects regarding the biocenosis of the oral cavity.

Chapter III – Diabetes mellitus. Disease complications.

PERSONAL CONTRIBUTIONS

Chapter IV – The microbiologic research of the microbial flora in patients with periodontopathies and diabetes mellitus

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   II. Material and method
   III. Results of microbiologic research
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Chapter V – The histologic research of periodontopathies in patients with diabetes mellitus
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INTRODUCTION

Recent breakthroughs revealed the incontestable relation between diabetes mellitus and the periodontal disease. It is well known that the periodontal disease is responsible for the destruction of connective tissues that support the tooth into the alveolus and the diabetes is in fact the inadequate use of sugars (determining the decrease of the immune response to infection, the increase of the blood viscosity, tissue hypoxia, etc.). Although the periodontal disease and the diabetes involve different determinism, they aggravate mutually through immune-biochemical mechanisms.

Taking into consideration the interrelation between diabetes - oral health - periodontal diseases, the thesis concentrates its attention towards the prevention of these diseases and complications through the performance of a screening that is easy to achieve even in the dentist’s practice.

As the periodontal disease represents a disorder having impact on population (especially on adults and seniors) it is responsible for pain, dento-maxillary imbalance and in the end leads to the loss of teeth.

The most important chronic complications caused by the metabolic imbalance of the diabetic disease are the result of the microangiopathic disorders (retinal, renal, neurologic), the typical injury being the thickening of the basal membrane of the vascular epithelium.

Glycoproteins, the basic constituents of the basement membranes are responsible, next to the high level of fibrinogen, for the growth of the blood viscosity (an important element in the pathogenesis of the retinopathy and diabetic glomerulosclerosis) and also responsible for the decrease of the blood flow in the small vessels.
THE MICROBIOLOGIC RESEARCH OF THE MICROBIAL FLORA IN PATIENTS WITH PERIODONTOPATHY AND DIABETES MELLITUS

In order to identify the anaerobic flora in patients with periodontopathy of diabetes mellitus I used the API 20A system that offers the possibility of fast identification with the help of 21 biochemical test.

The lab diagnosis of the anaerobic bacteria consists of several stages:

1. Sample collection
2. Bacteria isolation
3. Identification– the direct examination, isolation in pure culture necessary for identification, the identification of anaerobic bacteria using the API 20A kit.

In order to use the API 20A kit there are several test that must be performed, for example the culture and microscopic morphology, results that are obtained and useful for the complete confirmation or identification.

THE RESULTS OF THE MICROBIOLOGIC RESEARCH

In the case of most patients, the diabetic disease gives rise to the so-called belated complications. While some diabetics never reach such complications, others encounter them early. More or less, the belated complications manifest 15-20 years after the apparition of manifest hyperglycaemia. Some patients may present more simultaneous complications. In our research the greatest percentage of diabetic cases (57%) was the II type ones.

<table>
<thead>
<tr>
<th>Bacteria identified through the API 20A system</th>
<th>The percentage of patients identified in the pathologic lot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus intermedius</td>
<td>26% (10 patients)</td>
</tr>
<tr>
<td>Actinomyces istraelli</td>
<td>13% (5 patients) – G bacillus+</td>
</tr>
<tr>
<td>Streptococcus constelatus</td>
<td>13% (patients)</td>
</tr>
<tr>
<td>Gemella morbilorum</td>
<td>26% (10 patients)</td>
</tr>
<tr>
<td>Prevotella melaninogenica</td>
<td>11% (4 patients)- G bacillus -</td>
</tr>
<tr>
<td>Prevotella oralis</td>
<td>11% (4 patients)- G bacillus -</td>
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</table>
On the other hand, prospective studies have shown that PD in patients with diabetes mellitus may be related to a bad metabolic control of the diabetes mellitus and to several chronic diabetic complications [178, 174]. The suggestion was that the appropriate treatment for the periodontal disease in the case of the patients with diabetes mellitus may be effective for the decrease of diabetic complications [9, 107, 126].

Once confirmed the fact that the periodontal disease is widespread and more severe in the case of the persons with diabetes mellitus than in the case of the persons not suffering from such diseases, the scientific researchers searched for specific biologic mechanisms in order to explain the association of the two diseases. It is thought that the diabetes favours the apparition of periodontopathy through an excessive inflammatory response to the periodontal microflora.

Generally, the subgingival microflora in patients with periodontitis, that suffer from diabetes mellitus is equal to the one noticed in the case of the patients with periodontitis that do not suffer from diabetes [42, 90].

The inflammation, irrespective of the place where it appears is a local response of the host to the tissue injury as a reaction to microbial invasion and to the chemical and/or physical stimuli. Without taking account of the aggressor agent, the inflammation is characterized, in its severe form, by classic signs of redness, fever, sweat and pain. These tissue modifications that are noticeable in the case of the periodontium are the result of a series of complex events that may be microscopically and functionally detected and include:

- Vasodilatation with increased vascular permeability and blood flow;
- Liquid exudate (droplets);
- Leukocytes migration into extravascular spaces.

<table>
<thead>
<tr>
<th>Bacteria identified through API 20 A system</th>
<th>Description</th>
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<tbody>
<tr>
<td><em>Streptococcus intermedius</em></td>
<td>Gram-positive cocci, optional anaerobic</td>
</tr>
<tr>
<td><em>Actinomyces istraelli</em></td>
<td>Gram-positive bacilli, anaerobic</td>
</tr>
<tr>
<td><em>Streptococcus constelatus</em></td>
<td>Gram-positive cocci, optional anaerobic</td>
</tr>
<tr>
<td><em>Gemella morbilorum</em></td>
<td>Cocci related to streptococci</td>
</tr>
<tr>
<td><em>Prevotella melaninogenica</em></td>
<td>Gram-negative bacilli, anaerobic</td>
</tr>
<tr>
<td><em>Prevotella oralis</em></td>
<td>Gram-negative bacilli, anaerobic</td>
</tr>
</tbody>
</table>
THE HISTOLOGIC RESEARCH OF PERIODONTOPATHIES IN PATIENTS WITH DIABETES MELLITUS

The histologic material researched within this doctor's degree thesis was represented by fragments of periodontium collected from 46 patients clinical and paraclinical diagnosed with diabetes mellitus and with inflammatory disorders of periodontium. On the occasion of the execution of surgical treatment, we collected small fragments of periodontium, after previously informing the patients, fragments that were immediately brought to preparatory procedures for microscopic research. Therefore the collected biologic material was immediately placed in the neutral buffered formalin fixing solution 10% and processed for 24h using the classic histologic technique.

The biologic material, meaning the fragments of periodontium were forwardly processed using the classic histologic technique for paraffin inclusion, technique that allowed the execution of serial sections of 3-5µ thickness that can be optimally coloured an studied using the optic microscope.

THE RESULTS OF THE HISTOLOGIC RESEARCH

As known, the cover periodontium is a variety of the oral mucosa compound of a platy pavimentous epithelium which is a malpighian type without keratinization and a papillary chorion rich in collagen fibres linked by a basal membrane.

In regard of the investigated patients, there were noticed histologic modifications both on the gingival epithelium and on the chorion. The histologic modifications did not correlate to the values of glycaemia but rather to the hygienic state of oral mucosa.

The necrosis of the covering epithelium was accompanied by the destruction of the basal membrane and of the superficial vessels that led to the occurrence of micro-haemorrhages therefore the underlying connective tissue was exposed to the oral environment. The necrosis of the covering epithelium may be the consequence of the aggravation of the microbial flora of the oral cavity because it is well known the fact that in the case of the diabetic patients that hardly respond to the treatment, the glucose excess allows the exaggerated development of the microbial flora.
In regard of the chorion we signalised deep changes, larger than in the case of the epithelium, changes that were present in patients with diabetes mellitus. The periodontal connective tissue was the headquarters of a chronic inflammatory process of variable intensity from one case to another and even from one area to another but in the same case.

We consider that the periodontal inflammatory process was caused by the suffusion of the microbial flora into the oral cavity on the level of the periodontium as a result of the particular conditions created by the excess of glucose or by the metabolites resulted from its incomplete metabolization.

Among the cells belonging to the immune defence system, the lymphocytes were the most frequent. These cells were identified in regard of the cellular characters. They are spherical cells, without specific granulation in cytoplasm. The nucleus is voluminous, oval, hyperchromic and unsegmented, occupying most of the cell, intensely basophilic, relatively homogenous coloured (like an ink stain) due to the large content of heterochromatin. Within the nucleus sometimes there can be noticed the presence of a nucleolus. The cytoplasm is intensely basophilic, diminished, having the shape of a ring or of a half-moon situated at the periphery of the nucleus.

The periodontium chronic inflammatory process, although meant to restore the structural and functional local integrity, to preserve the homeostasis of the dento-maxillary apparatus by means of its intensity and through mediators that are locally set free, especially the metalloproteinases that might become an aggravating process of the periodontal injury.

DISCUSSIONS

There were reported a series of injuries of the oral mucosa in the case of patients with diabetes mellitus, including lichen planus and recurrent aphtous stomatitis. Not all the results of the research proved this relation although these disorders are frequent and often noticed in the case of patients that do not suffer from diabetes. Opposed to, the oral candidiasis was consistent in the case of patients with diabetes [65]. The oral candidiasis is a manifestation of an immunocompromised status and of a decrease of the salivary flow.

Moreover the immune response is scarce in the case of these patients [162], registering an increase of interleukins, as IL-6 and IL-1β, along with the tumour necrosis alpha factor (TNF) and prostaglandin E2 (PGE2). Some authors consider that these factors might act by stimulating the periodontal inflammatory response. [150]
Other authors proved that within the sub gingival bacterial plaque of patients with chronic periodontopathies there were identified over 24 species of bacteria that are responsible for the occurrence and sustenance of the periodontal disease. [36]

It is proven that during the evolution of the periodontal disease, the bacteria invade the epithelial covering of the gum and afterwards the connective tissue. This invasion was proven within the aggravated ulcerative-necrotic gum diseases, localised juvenile periodontitis, the gradual progressive periodontitis of adults and children suffering from Papillon-Lefevre syndrome related to periodontopathies. Using the morphologic criteria or colouring with fluorescent antibodies, there were identified within these tissues the following types of germs: spirochetes, actinobacillus actinomicetes, bacteroides gingivalis, bacteroides intermedius, actinomices species, fusobacterium nucleatum and mycoplasma. Recent optic and electronic microscopic studies have proven the invasion and presence of bacteria in cement and in the radicular dentin of the teeth with periodontal disease. The bacteria may be present in the dentin and even in the dental pulp. [2].

THE IMMUNOHISTOCHEMICAL RESEARCH OF PERIODONTOPATHIES IN PATIENTS WITH DIABETES MELLITUS

The immunohistochemistry represents an opportunity to complete the histopathologic diagnosis but it does not represent a diagnosis itself. During the last years, all around the world the immunohistochemical techniques have been widely used in order to put a positive and differential diagnosis of some injuries to establish the aetiology and histogenesis of some cells in the diagnosis of the metastasis of primary unknown origins in the embryology and forensic medicine studies, etc.

In order to begin the immunohistochemical sections the sections have been previously deparaffinized in three consecutive xylene baths (for 15 minutes each) and then rehydrated through washing in decreasent concentration alcohols (100%, 70%, 50%, 15 minutes each). In the end the sections were put into distilled water to remove any trace of alcohol from the histological section.

The immunohistochemical technique itself includes a standard algorithm with some variations depending on the antibodies that are being used.

RESULTS
On our formulations coloured with classical colouring we put forward the presence of some round mononuclear cells of lymphocyte, plasma cells and macrophage type and rarely of granulocytic type. This microscopic aspect confirms the fact that within the periodontopathies related to the diabetes mellitus there exist a chronic inflammatory process with or without flare.

In order to differentiate the cells from the inflammatory infiltrate of the periodontium we used more specific antibodies, namely:

- CD3, to emphasize the T Lymphocytes,
- CD20, to emphasize the B Lymphocytes,
- CD68, to emphasize the microphage cell distribution.

The CD3 antibody used to emphasize the T Lymphocytes reacts to the epsilon chain of the CD3 antigen on the surface of the T Lymphocytes.

Our research allowed us to emphasize within the covering gum epithelium the presence of some CD68 positive cells that may be dendritic cells or more precisely Langerhans cells. These cells also belong to the monocyte -macrophage cell system originating from the red bone marrow but having the ability to penetrate the basal membrane of the covering epithelia and to be localised among the epithelial cells.

These intraepithelial macrophages act to capture the antigens from the surface of the covering epithelium and to present it to the immune defence system cells, mainly the T Lymphocytes in order to induce an adequate immune response.

The immunohistochemical study of vascular changes from the periodontium was achieved using the CD34 antibody that marks purposely to endothelial cells and angioblastic cells.

The anti-CD34 antibody acknowledges a transmembrane protein with a molecular mass of 110 kD which is intensely phosphorylated. This protein seems to play an important role in the cellular adhesion acting as a ligand for different lectin classes.

This way CD34 and its hematopoietic predecessors would adhere to the cells that express lectins to the level of the bone marrow.

The antigen is present in approximately 1% of the mononuclear cells of the bone marrow including the hematopoietic predecessors/stem cell.

Within physiological conditions, the angiogenesis process in the adult person is very low, the endothelial cells form the normal vessels being neproliferative and the mitotic index is lower than 0,01%.
The occurrence of local aggressions that perturb the tissue and vascular homeostasis is followed by an occurrence of the inflammatory process and by the vascular recovery process. The vascular recovery reproduces the histological stages of the normal organogenesis in particular conditions related to the nature, degree and localisation of the tissue or of the organ that has been hit by the initial destructive process.

As seen on our formulations, during the first stages, the angiogenesis process is an exclusively capillary process. The new blood vessels result from the pre-existing ones by the transformation of the endothelial cells into angioplastic ones, cells that capable of rapid mitosis and generation of "vascular buds".

**IV. DISCUSSIONS**

In our research, by using specific antibodies in the immunohistochemical study, we have determined that among the cells of the immune defence system the ones that are best represented were the T Lymphocytes. The T Lymphocytes were present in the gum epithelium and in the chorion.

The T Lymphocytes accomplish within the immune response many functions: dissolution of the cells that express the non-self molecules on their surface, adjust the immune response mediates the reactions of delayed hypersensitivity etc. These functions are the result of functional heterogeneity and are the result of the presence of some distinct T Lymphocytes subpopulations:

- Tc Lymphocytes (Tc, cytotoxic or cytolitic) that define on their surface the T8 (CD8) marker T8;
- Th Lymphocytes (helper) that carry on their cellular surface the CD4 marker;
- Ts Lymphocytes (suppressor), bearer of the CD4 marker;
- TD or TDH Lymphocytes (delayed hypersensitivity) that express the CD8 marker.

Th-2 (Th-b) cells secrete cytokines of type 2: IL-4, IL-5, IL-6 and IL-10 (but does not produce by secretion IL-2) and stimulates the activity of the memory B lymphocytes. The cytokines of type 2 (IL-4, IL-5) stimulate the humoral immune response towards the extra cellular parasites (stimulates the differentiation of B lymphocytes towards the plasmocytes) and the laying of the allergic state through their capacity to induce the IgE synthesis and to stimulate the mastocytes.

The inflammatory cytokines as IL-1, IL-6 şi TNF-α, all of them, important mediators of periodontal inflammation, play an important role in the glucid and lipid metabolism. The plasmatic concentrations of IL-6 and TNF-α may grow in obese persons and type 2 diabetic
patients. We must notice the fact that IL-6 and TNF-α act as adipokines that serve to promote the catabolism and the loss of weight and are therefore involved in the insulin resistance. There was suggested the fact that the inflamed periodontium might act as an endocrine source of inflammatory mediators as TNF-α, IL-1 and IL-6, that determine the consecutive growth of the insulin resistance [143].

Although some authors underlined the importance of polymorphonuclear neutrophils in the evolution of periodontopathies, we have rarely found few polymorphonuclear neutrophils. These aspects make us believe that in the chronic periodontopathies, the infiltration with neutrophils is reduced. Nevertheless some authors [68] consider that the neutrophil is the first line of defence against the periodontal agents. Other authors [106, 21] noticed function and recruitment shortage of neutrophils in diabetic patients. Bissada and his collaborators proved that the neutrophils from the peripheral blood have a diminished chemotactic activity in the type 1 diabetic patients suffering from severe periodontitis compared to the diabetic patients suffering from moderate periodontitis and non-diabetic patients but suffering from severe periodontitis. Moreover they showed that the phagocytosis activity of the neutrophils from the peripheral blood of type 1 diabetic patients suffering from localised periodontitis was lower than in the case of non-diabetic patients suffering from localised periodontitis.

In the matter of the reaction of the monocyte macrophage cells in patients with chronic periodontopathy and diabetes mellitus, we have ascertained that collectively the number of this type of cells has been diminished compared to the number of lymphocytes and their arrangement was inhomogeneous.

We believe that in the local limited repairing processes like chronic periodontopathy, the angioblasts derive from the endothelial cells of the normal blood vessels that are not affected by the pathologic process but which are stimulated by numerous biochemical factors set free by various cells (lymphocytes, granulocytes, macrophages) within the inflammatory centre. Under the influence of mitotic factors, the endothelial cells of the blood vessels accumulate nuclear and cytoplasmic material and proliferates intensely generating cell cords.
CONCLUSIONS

The API kit proved to be a sensitive and useful system for the detection of anaerobic bacteria, although rather cumbersome.

The anaerobic bacteria encountered in our research are various but rarely met (Gemella) as compared to the ones encountered in non-diabetic patients within the specialty literature.

The histologic changes registered on the gum epithelium in patients with diabetes mellitus and periodontopathies went from total necrosis of the covering epithelium to hyperplasia and hyperkeratosis.

The connective periodontal tissue was the headquarters of the chronic inflammatory process prevailing on lymphocytes, plasmocytes and macrophages of variable intensity from one case to another and even from one area to another at the same patient.

We consider that the periodontal inflammatory process has been triggered by the penetration of microbial microflora from the oral cavity at the level of periodontium, as a consequence of the particular conditions created by the diabetes mellitus.

Out of the cells of the immune system, the most numerous have been the lymphocytes. The presence of the lymphocytes in the periodontal inflammatory breeding ground is owed to the fact that they are mobile cells with very active locomotion movements. They are able to distinguish between the protean structure incident to the organism and the foreign protean structures secrete in opposition to which the B lymphocytes produce by secretion antibodies (immunoglobulins) and the T lymphocytes produce by secretion lymphokines.

The occurrence of the vascular congestion is due to the fact that within the periodontal chorion the presence of the antigens stimulated the induction by the immune defence cells of some biochemical mediators having a vasodilative effect. The vascular congestion is an essential constituent of the inflammatory process and is responsible for the erythema and oedema of the periodontium.

The immunohistochemical quantitative and qualitative analyses of the immune defence system cells that are present in the periodontal inflammatory infiltrate allowed us to ascertain that the T Lymphocytes were the best represented cells of the immune system in the periodontopathies related to diabetes mellitus. They were inhomogeneous in the connective structure of the periodontium, most of the T Lymphocytes being identified under the covering
epithelium and around the blood vessels. Rather frequently T Lymphocytes were identified within the structure of the covering epithelium.

The macrophages were rare and diffusely spread in the connective tissue of the inflammatory periodontium, fact that suggests that the intensity of the inflammatory reaction in periodontopathies is variable from one patient to another according to the quantity of antigens present in the periodontium but also according to the tissue destruction that call for macrophages.

Another vascular change observed in our patients was the angiogenesis, characterized by the formation of new vessels beginning with the pre-existing venation. We appreciate that the occurrence of the angiogenesis process characterizes the regenerating potential of the periodontium. The mechanisms that characterize the angiogenesis are extremely dynamic processes; modulated by the intracellular interactions and by the extracellular matrix cells in the presence of the growth and morphogenesis factors set free by the inflammatory infiltrate cells.
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