Ph. D. Thesis

The role of inflammatory and immunological processes in the development of chronic apical periodontitis

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1. Introduction

Bacterial infection of the dental pulp ultimately leads to the development of chronic apical periodontitis. Because of the unfavorable anatomic surrounding, host defense mechanisms insufficient in most of the cases which results in pulpal destruction. Because of the rigid dentinal walls, inflammatory exudates do not have space, causing an increased pressure in the pulp chamber. These events tend to slow down the flow of blood, with a concomitant hypoxia which events finally cause the necrosis of the pulp tissues. The pulp chamber and root canals including the necrotic pulp tissue creates a suitable environment for bacterial colonization. Whereas the inflammatory immune responses may successfully eliminate the invading microorganisms in the region of the periapex, eradication of this flora in the root canal system cannot be completed. In this way the root canal system becomes a continuous source of microbes. The persisting infection and immuno-inflammatory reactions of the periapical tissues are accompanied by histological changes. These changes are characterized by the appearance of a cell-rich granulation tissue, infiltrated with macrophages, lymphocytes, plasma cells, neutrophils and fibrovascular elements in varying number. Periapical tissue damage and alveolar bone resorption takes place at the same time. The most frequent periapical lesions are periapical granuloma and radicular cysts.

Periapical lesions are suspected to compromise general health by local production of long range acting cytokines, which may elicit systemic inflammatory reactions, creating a chance for the development of a secondary disease of the host.

2. Objectives

In my work, I aimed to:

1. Assess the oral health status of patients, suffering in secondary focal diseases.
2. Determine the prevalence of chronic apical periodontitis lesions among our patient groups, compared to the results of other investigators.
3. Determine the ratio and in situ distribution of different cell types in granulomatous and granulofibromatous type of periapical granulomas.
4. Investigate the expression of IL-2Rα and major histocompatibility complex class II antigen (HLA-DR) in order to detect the activated state of the composing cells.
5. Determine the in situ distribution of three prototype chemokines, interleukin-8 (IL-8), monocyta chemoattractant protein-1 (MCP-1) and Rantes in chronic human periapical lesions by immunhistochemistry.
6. Quantify the levels of IL-6 and GM-CSF in chronic apical lesions, characterized by size, clinical symptoms and histological findings.

3. Materials and methods

1. Medical evaluation of the patients:

   After recording the case history, we performed an intraoral examination, including a cariological, prothetical and paradontal status. Sensitivity test, periapical radiography or orthopantomography were performed when needed. For electronic data recording we used the "DMS V. 3.4. eNET" program.

3. Tissue samples:

   Lesions were obtained by tooth extraction or apical surgery, with informed consent from patients. According to the criteria used, lesions were attached to the roots of the removed teeth, or were removed in one piece from the bony crypt during apical surgery. Specimens were dissected along their longitudinal axis into 2 approximately equal parts. One half was snap frozen and stored in liquid nitrogen and the other half was prepared for light microscope studies.

4. Characterization of inflammatory cell infiltrate and classification of dental periapical lesions:

   Specimens were fixed in 10% formaldehyde. Paraffin sections, 4-6μm thick were used for morphological examination, based on haematoxilin and eosin staining. Three sections per lesion were chosen for histological measurements. Two fields in each histopathological zone were examined per section. Samples were grouped according to the presence of epithelial and polymorphonuclear (PMN) cells. Lesions characterized by ≥ 20% neutrophil infiltration were classified polymorphonuclear cell-rich (PMN^+) lesions. Lesions with < 20% neutrophils were classified as polymorphonuclear cell-poor (PMN^-) tissues. Fields showing islets or arcades of epithelial cells were regarded as epithelial cell-containing tissues. No epithelial cells were found in epithelial cell-free lesions.

5. Histology and classification

   Criostat sections were cut at 4μm, dried, stabilized in acetone for 5 seconds and stored at -20°C covered with aluminum foil. Aspecific binding was blocked. As the first step of specific staining, we treated sections with polyclonal and monoclonal antibodies. Monoclonal antibodies lacking anti-human reactivity were used as controls. In our studies we used kits for the avidin-biotin –peroxidase complex (ABC) method and the

alkaline phosphatase anti-alkaline phosphatase (APAAP) technique following the user's directions of the manufacturers. After a counterstaining with hemalaun the sections were evaluated by light microscope. As part of the morphological studies, we appraised hematoxilin-eosin, Giemsa and van Gieson stained sections, as well. Ratios were determined from the combined-calculated data on positive cells obtained from 10-10 randomly selected microscopic fields of both the center and the peripheral portions of granulomas by using two representative immunostained serial sections for each antibody. The expression of IL-2Rα (CD25) and HLA-DR antigens of the surface of mononuclear cells were shown with the ABC method. In situ distribution of three prototype chemokines interleukin (IL)-8, monocyte chemo-attractant protein (MCP)-1 and Rantes was determined in chronic human periapical granulomas by immunohistochemistry using monoclonal antibodies and alkaline phosphatase antialkaline phoshpatase (APAAP) technique.

6. Quantitative analysis of cytokines in human periapical lesion extracts using an enzyme-linked immunosorbent assay (ELISA):

Tissue samples stored in liquid nitrogen were thawed on ice. After adding a protease inhibitor cocktail, lesions were cut into small pieces with scissors, then homogenized ultrasonically, and centrifuged. Supernatants were harvested, divided into aliquots and stored at -80°C until assayed. Samples were assayed for protein content. IL-6 and GM-CSF concentrations of the samples were determined using an ELISA kit (R&D Systems, Minneapolis, MN). The optical densities were determined using a microplate reader (Molecular Devices, Menlo Park, CA). Results were calculated using the standard curves created in each assay. Concentrations were than normalized for the protein content of the samples and given as pg/μg protein. Data collected were first examined for normality by the Kolmogorov-Smirnov test. Data sets with normal

distribution were analyzed using Student's t-test, according to requirements of the standard deviation. In other cases we used the non-parametric Mann-Whitney test. P values below 0.05 were considered statistically significant.

4. Results

1. Occurrence of chronic persisting apical periodontitis in our patients groups

The relationship between dental foci and diseases of remote organs of the host is a controversial issue of the medicine in the last few decades. Patients, suffering from suspected focal diseases were investigated for the presence of persisting chronic infammatory disorders in the oral cavity. During 1997 and 1998, we examined 261 patients. In 83% of the patients dental foci could be revealed. In 17% of the cases we could not find any chronic inflammatory disorders. The most commonly diagnosed disorder (36%) was pulp necrosis. We found chronic apical periodontitis in 21% of the cases, parodontitis complicata in 20% of the patients, incomplete root canal filling in 10%, impacted teeth and radix relicta in 9% and failed root canal filling in 4% of the patients. These diseases could be detected in 100% of the patients suffering in uveitis, 91% among the patients with cardiac or vascular disorders, 89% of the patients with vasculitis, 74% of the patients suffering of autoimmune diseases, 73% of the patients with ekzema, 72% among the patients with renal disease and 71% of the patients suffering in different dermatological diseases.

2. Types and prevalence of different chronical periapical periodontitis lesions

Between 1992 and 1997, 299 human periapical lesions were removed and analyzed. All of the examined tissues proved to be one of the chronical periodontitis types. Of the lesions assessed 195 (65.2%) were found histopathologically to be cystic, 96

(32.1%) were diagnosed as chronic apical periodontitis and 8 (2.7%) were observed as “other lesions”.

3. Characterisation of inflammatory cell infiltrate in dental periapical lesions

Granulomatous and granulofibromatous type of periapical granuloma were selected to determine the ratio and in situ distribution of different cell types. These lesions consisted of lymphocytes, neutrophil granulocytes, macrophage, plasma cells and fibrovascular elements. Accumulations of foam cells, Malassez’s epithelial rests, and Russel’s bodies were observed in some cases. Mononuclear cells and polymorphonuclear leukocytes formed classical granulomatous tissue, without significant segregation of the cellular components. Signs of epithelial proliferation and perivasculitis were seen in some cases. The activated epithelial cells formed arcades or islets within the granulomatous tissue. Bundles of collagen fibers tended to form a fibrous capsule around the central tissue. This fibrous capsule was poor in cells, contained only a few osteoclast cells and clusters of 10-70 mast cells. Mast cells were seen near the border line between the granulomatous and fibrotic zones. The central granulation tissue was invariably devoid of mast cells. In situ distribution of CD2+, CD3+, CD4+, CD8+, CD56+ and CD68+ cells were determined by series of immunhistocemical studies using monoclonal antibodies. Labeled cells exhibited circumferential brown staining of moderate to strong intensity. About the half of the cells (55%) reacted with the used pan T markers. CD2 and CD3 labeling showed about the same results. The number of CD2+ or CD3+ T cells corresponded well with the total number of CD4+ and CD8+ cell. CD4+ and CD8+ lymphocytes were invariably present and evenly distributed within the granulation tissue. The ratios of CD8+ cells (34%) slightly outnumbered CD4+ cells (22%). NK cells were evenly distributed however their ratio was lower than the proportion of T cells. CD14+ macrophages

were distributed all over the periapical area, but their proportion was lower than that of T-lymphocytes (22%). The proportion of macrophages was significantly higher labeling with CD68 monoclonal antibody (55%). In the granulomatous zone of the lesion predominantly round or polygonal macrophages were found, whereas dendriteshaped macrophages were characteristic for the peripheral areas. The ratio of major histocompatibility complex class II- positive cells (HLA-DR+) was in between the frequency of T lymphocytes and macrophages. Both small lymphocyte-like mononuclear cells and bigger ones resembling macrophages displayed a mild to strong circumferential staining with the anti-HLA-DR antibody. The majority of lymphocytes expressed IL-2Rα, as indicated by the moderate to strong cell membrane staining with the applied monoclonal antibody (48%). The majority of mononuclear cells expressed IL-2Rα were morphologically small sized lymphocytes.

4. In situ distribution of three prototype chemokines in human chronic periapical granuloma

   IL-8, MCP-1 and Rantes exhibited a characteristic differential localization pattern in each of the investigated samples. Large polygonal cells, in the islets of Malassez's epithelial rests, displayed strong cytoplasmatic staining with the anti-IL-8 monoclonal antibody. MCP-1 staining was confined to the negatively charged molecules of the exracellular matrix. No cellular staining was observed for Rantes, however each investigated chemokine, including Rantes, exhibited a finely dispersed staining pattern within the extracellular matrix of the granulation tissue.

5. Quantitative analysis of interleukin-6 and granulocyte-macrophage colony-stimulating factor in apical periodontitis

   Lesions contained 236.2± 62.0 pg/µg IL-6 and 206.9± 52.7 pg/µg GM-CSF, whereas controls contained 9.3 ± 1.9 pg/µg 1.9 pg/µg IL-6 and 199.9± 94.5 pg/µg GM-CSF.

Although granuloma homogenates contained higher levels IL-6 and GM-CSF than control samples, differences were not significant. as the groups did not follow a normal distribution statistically. However, in samples coincidentally possessing symptomatic and epithelialized features (group1), compared to those characterized by the combination of assymptomatic, small, polymorphonuclear cell-poor (PMN^-) and non-epithelialized features (group2), we found significantly higher levels of IL-6 and GM-CSF. Moreover, both group 1 (n=8) and group 2 (n=13) showed significantly higher levels of IL-6 than the group of negative controls. For GM-CSF, group 1 showed significantly higher cytokinelevels than the negative control group, while group 2 showed no significant difference when compared to the control group.

5.Discussion

1. There has been a renewed interest in the influence that foci of infection within the oral cavity may compromise the general health of the body. The aim of our study was to identify those diseases in which dental foci can be diagnosed at the highest rate. We also intended to identify the most frequent lesions and to determine the frequencies of the existing dental foci in our patient groups. In 83% of the patients we could reveal persisting chronic inflammatory disorder in the oral cavity. The most commonly diagnosed disorders were pulp necrosis, chronic apical periodontitis and periodontitis complicata respectively. We found high incidence of these disorders in all of the patient groups. These diseases could be detected most frequently among chronic cardiac disorder patients, patients with uveitis and dermatological diseases. In order to determine whether there is a causual relationship between the diseases and systemic diseases prospective study is needed on the basis. Further research is required to determine the changes of different inflammatory blood parameters parallel the

elimination of dental foci of these patients. This sort of investigation may provide a better understanding of the damaging mechanisms of dental foci to remote organs.

2. In our previous study we found chronic apical periodontitis as the second most frequently occurring dental focus. Chronic apical periodontitis is an inflammation at the region of the tooth apex of a long standing nature and characterized by the presence of a granulomatous tissue. The granulomatous tissue is infiltrated by immuno-inflammatory cells also called apical granuloma. Apical granuloma is predominantly infiltrated with lymphocytes, plasma cells and macrophages and may be epithelialized or non-epithelialized. Another chronic apical periodontitis form is periapical cyst that is characterized by the same granulomatous tissue with a distinct epithelium-lined pathological cavity. In the periapical true cyst the cavity is completely enclosed in an epithelial lining so that no communications to the root canal exist. This form seems to be a self-sustaining lesion that is no longer dependent on the source of irritation from the canal system. The periapical pocket cyst presents as a saclike, epithelium-lined cavity that is open to and continuous with the root canal. Pocket cyst have a high healing potential. Any alteration in the canal contents may have an effect on the cavity or lumen thus on the surrounding epithelium. The two types of cysts can be differentiated only by an apical foramen related study using serial or step-serial sectioning technique.

   In the past four decades many histopathologic studies were performed to determine the frequency of incidence of different forms of apical periodontitis. The reported incidence of radicular cyst among human periapical lesions varies from 6% to 54%. The incidence of periapical granuloma varies from 34% to 94%. The controversial results can be explained by the wide variety of sampling, tissue processing methods and different classification-criteria used by the authors of the publications. We

reported a high incidence of radicular cyst (65.2%) and a lower incidence of periapical granulomas among periapical lesions in our material similarly to other authors. Our results coincide with the assumption of other investigators that true cysts cannot be cured with conservative therapy, so these lesions have to be removed surgically.

3. Human periapical granuloma is considered as one of the most frequent dental foci. In the periapical lesion, complex events of tissue destruction, repair and long range acting effects are taking place. These processes cannot be understood without knowing the quantitative cell composition of the lesion. According to our study the lesions consisted of lymphocytes, neutrophil and eosinophil granulocytes, macrophages, plasma cells and fibrovascular elements. Lymphocytes were the predominant cell type (50%); their number corresponded well with the total number of CD4+ and CD8+ cells. Studies are divided regarding the relative concentrations of CD4+ and CD8+ subsets. Several reports demonstrated that T-suppressor (CD8+) cells are outnumber T-helper (CD4+) cells in chronic lesions, with a resultant CD4/CD8 ratio of ≅1.0 or less, compared with a CD4/CD8 ratio of ≅ 2.0 in peripheral blood. Kinetic studies of Stashenko et al.suggest that, after the first several days, there are few differences in the cell infiltrate with time after pulp exposure. A notable exception is that CD8+ cells increase with increasing lesion chronicity. In our study we could repeatedly confirm the existence of an altered CD4/CD8 balance in favour of CD8+ lymphocytes. We found also a remarkably high proportion of CD14+ macrophage cells and less CD56+ natural killer (NK) cells in chronic periapical lesions. To have a better understanding of the ability of these lesions to adversely effect remote organs, we demonstrated the expression of IL-2Rα and HLA-DR antigens in human periapical granuloma samples. The ratio of HLA-DR+ and IL-2Rα expressing lymphocytes was similar to that of CD2+ lymphocytes, suggesting that some of the T cells within the lesion might indeed

be in an activated state. A paracrine loop of stimulation between CD4+ IL-2-producing T-helper-1 cells and IL-2Rα expressing CD8+ cytotoxic T-lymphocyte precursors that do not produce significant amounts of IL-2 may explain the progressive change in CD4/CD8 ratio described in developing animal periapical granulomas as well as the relative excess of CD8+ T-lymphocytes in the chronic human lesion. The relatively high proportion of CD68+ cells suggests that most of the macrophag cells present in the lesions, are in an activated state.

4. IL-8, MCP-1 and Rantes represent basic functional prototypes of the chemokine family of regulatory proteins characterized by well-established effects on the migration and activation of inflammatory cells. In addition to its well-known effect on driving neutrophil emigration from blood, IL-8 induces epithelial cell proliferation. Thus IL-8 may be responsible for three features characteristic of periapical granuloma lesions: neutrophil recruitment, expansion of the Malassez's epithelial cell rests by an autocrine mechanism and the angiogenic growth of blood vessels. The recruitment of monocytes, eosinophils and memory T cells into granuloma lesions may due to the presence of MCP-1 and Rantes, respectively. The differential appearance of MCP-1, but not the other tested chemokines, on the endothelial cells of small venules suggests that only this chemokine is involved in continuous leukocyte recruitment into the granulomatous lesions at the periapical area, while IL-8 and Rantes may still reach the vessels and act leukocyte recruitment but only periodically. The observation, that all three chemokines present were captured by the extra cellular matrix suggests their potential role in the distribution of different leukocyte subpopulations within the lesions.
5. Our earlier observations suggested that patients with apical periodontitis have elevated acute-phase protein serum levels that decrease to normal following surgical removal of the lesions. Similarly, spontaneous and mannozyme-induced whole-blood chemiluminescence

in patients with apical lesions is elevated prior to elimination of the lesion, demonstrating an activated state of neutrophil leukocytes in these patients. Cytokines that functionally activate neutrophil leukocytes are granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Synergistically, IL-6 induces or augments neutrophil cytotoxic activity as well as stimulates hematopoiesis with subsequent leukocytosis. Macrophages drawn to the site by endogenous growth factors such as TNF-α, IL-1β and IL-6. The positive loops of stimulation may lead to an exacerbation of the periodontal inflammation. GM-CSF is also produced by other cells. The clinical and pathologic role of these cells in acute exacerbation is still unclear. We are unaware of other investigations having demonstrated the production of GM-CSF in periradicular lesions. In our study, we fund an elevated level of IL-6 and GM-CSF in the lesions characterized by symptomatic and epithelial-cell containing features (group1) compared to the control tissues and the lesions characterized by the asymptomatic, non-epithelialized, small sized and PMN cell poor features (group2). These findings suggest that local production of IL-6 and GM-CSF may influence the composition of the cell population residing in apical periodontitis and may play a role in activating these cells and therefore in advancing the progression of the local process. Beside the local effects, IL-6 and GM-CSF with their long-range actions are suspected to compromise general health.

6. New findings

1. We determined in a retrospective study that most of the patient groups (83%) suffered from persisting chronic inflammatory disorder in the oral cavity. The most commonly diagnosed disorders were pulp necrosis, chronic apical periodontitis and periodontitis respectively.
2. We reported a relatively high incidence of radicular cyst (65.2%) and a lower incidence of periapical granulomas among periapical lesions of an extraction or periapical surgery origin. This result coincides with the assumption that true cysts cannot be cured with conservative therapy, so these lesions have to be removed finally.
3. We examined the quantitative cell composition of the periapical lesions applying immunhistochemical method. We reported among the first authors that the predominant cell types in granulomatous and granulofibrotic periapical lesions are T-lymphocytes and macrophages. Regarding the T lymphocyte subsets, we found that T suppressor (CD8+) cells outnumbered T helper (CD4+) lymphocytes. We also determined the expression of IL-2Rα and HLA-DR antigens on the same specimens using immunhistocemical method, in order to detect the activated state of the predominant cells. We described among the first ones that HLA-DR antigens are expressed on the mononuclear cells of these lesions. We were the first who reported the presence of IL-2 receptor α chain (IL-2 α or CD25) on the surface of T lymphocytes of the lesions.
4. We described among the first ones the *in situ* distribution of three prototype chemokines interleukin (IL-8), monocyte chemoattractant protein (MCP-1) and Rantes

in chronic human periapical granulomas as they may play a role in establishing the cellular composition of chronic apical periodontitis, thus augmenting the intensity of local inflammation and tissue damage. The presence of Rantes was not examined in human periapical granuloma before, not even in animal periapical granuloma model studies. We determined that the chemokines we examined using immunohistological method showed characteristic and distinct patterns on the surfaces of the composing cells and in the extra cellular matrix.

5. We quantified for the first time the levels of interleukin-6 (IL-6) and granulocyte-macrophage colony-stimulating factor (GM-CSF) in apical periodontitis from supernatants of homogenised tissue samples. In our study, we found an elevated level of IL-6 and GM-CSF in the lesions characterized by symptomatic and epithelial-cell containing features (group1) compared to the control tissues and the lesions characterized by the asymptomatic, non-epithelialized, small sized and PMN cell poor features (group2).

7. Utilization of results

1. We are planning to use the results we obtained on screening of chronic inflammatory dental disorders in focal diseases, in our future follow-up studies. Parallel with the elimination of the dental disorders we will conduct to monitor blood and serum parameters in order to obtain data of the systemic effect of the persisting chronic dental disorders.
2. The result of our histological study reminds us that 10 to 15% of these lesions can not heal after a correctly performed conservative therapy. Therefore we have to put even more stress on recall examinations of our endodontic patients.
3. The results of determining the ratio and in situ distribution of different and activated cell types of periapical granulomas, basically influenced our endodontical therapeutical approach. Our results supported the notion that periapical lesions are consecutive disorders. Therefore the aim of the endodontic therapy has to be the nearly perfect elimination of the infection from the root canal(s) and then performing a root canal filling with a good apical seal in order to prevent the periapical region from reinfection.

   Revealing the systemic effects of apical periodontitis also lays stress upon regular controlling of the endodontically treated teeth. We also have to consider the patient's general health upon making a treatment plan. Conservative therapy is recommended for patients with good general condition. Patients with altered immunity or suffering in certain organic diseases may also be treated via conservative therapy but constant consultation is needed with the patient's general practitioner.

Publication related to the thesis:

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