Lichen ruber planus- and leukoplakia-dependent impairment in relationship between Merkel cells and Aβ-nerve endings in the human oral mucosa

Inaugural-Dissertation zur Erlangung der zahnärztlichen Doktorwürde der Hohen Medizinischen Fakultät der Universität zu Köln

vorgelegt von Daniela Calderón Carrión aus Loja/Ecuador

Promoviert am
Dekan: Universitätsprofessor Dr. med. Dr. h.c. Th. Krieg
1. Berichterstatter: 
2. Berichterstatter: 

Erklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

Bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskriptes habe ich keine Unterstützungsleistungen erhalten.


Die Arbeit wurde von mir bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt und ist auch noch nicht veröffentlicht.

Köln, den
Die dieser Arbeit zugrunde liegenden Experimente sind nach entsprechender Anleitung durch Herrn Universitätsprofessor Dr. med. dent. W. Niedermeier von mir selbst durchgeführt worden.

Das humane Material, das in dieser Studie verwendet worden ist, wurde mit der Studiennummer 2980 von der Ethikkomission der Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf genehmigt.
ACKNOWLEDGEMENTS

First at all, I would like to express my thanks to Professor Dr. med. Dent. W. Niedermeyer for his attentive guidance and concern for this subject. He gave me much help in subject designing, throughout whole research work.

I am deeply grateful to Professor Dr. med. K. Addicks Institute I for Anatomy for his guidance and valuable advice in the whole process of this research.

I express my thanks to Professor W. Bloch Molecular sport medicine for his help and support.

I would also express my deeply gratitude to Dr. med. dent. Y. Korkmaz, for his valuable guidance. He helped me a lot with regard to every respect in my research work including subject designing, experiment arrangement. Without his help, my research work could hardly be accomplished.

I am grateful to Priv.-Doz. Dipl.-Math., Dr. rer. medic., Martin Hellmich for his help.

I am grateful to Dipl.-Biol. M. Kopp for her help, support and kindness.

I would like to thanks also Dr. med. dent. M. Di Gregorio for her help and guidance.

I would also express my gratitude to The Ecuadorian Republic for giving me the scholarship to my doctoral thesis.
I would like to dedicate this thesis to my beloved mother Gloria Maria de Lourdes Carrión Gordillo.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1</strong> Introduction</td>
<td>11</td>
</tr>
<tr>
<td><strong>2</strong> Literature Overview</td>
<td>13</td>
</tr>
<tr>
<td>2.1 The neuronal perception in the oral mucosa</td>
<td>13</td>
</tr>
<tr>
<td>2.1.1 Mechanoreceptors and mecanosensation</td>
<td>13</td>
</tr>
<tr>
<td>2.1.2 Modulation of oral mucosa epithelial cells by sensory neuropeptide</td>
<td>13</td>
</tr>
<tr>
<td>2.1.3 Merkel cells in the oral mucosa</td>
<td>15</td>
</tr>
<tr>
<td>2.2 The pre-malignant lesions in the oral mucosa</td>
<td>16</td>
</tr>
<tr>
<td>2.2.1 The etiology of the pre-malignant lesions in the oral mucosa</td>
<td>16</td>
</tr>
<tr>
<td>2.2.2 Tobacco and smoking</td>
<td>16</td>
</tr>
<tr>
<td>2.2.3 Alcohol consumption</td>
<td>17</td>
</tr>
<tr>
<td>2.2.4 Galvanic interactions between dental alloys</td>
<td>18</td>
</tr>
<tr>
<td>2.2.5 Lichen ruber planus and leukoplaikia in the oral mucosa</td>
<td>18</td>
</tr>
<tr>
<td>2.2.6 Lichen ruber planus in the oral mucosa</td>
<td>19</td>
</tr>
<tr>
<td>2.2.7 Leukoplaikia in the oral mucosa</td>
<td>20</td>
</tr>
<tr>
<td>3. Statement and aims of the study</td>
<td>22</td>
</tr>
<tr>
<td>3.1 Statement of the study</td>
<td>22</td>
</tr>
<tr>
<td>3.2 Aims of the study</td>
<td>24</td>
</tr>
<tr>
<td><strong>4</strong> Material and Methods</td>
<td>25</td>
</tr>
<tr>
<td>4.1 Ethical considerations and clinical anamnesis of patients</td>
<td>25</td>
</tr>
<tr>
<td>4.2 Measurements of the electric field strength</td>
<td>25</td>
</tr>
<tr>
<td>4.3 Tissue preparation</td>
<td>26</td>
</tr>
<tr>
<td>4.3.1 Fixation of tissues</td>
<td>26</td>
</tr>
<tr>
<td>4.3.2 Paraffin embedding and sectioning of samples</td>
<td>26</td>
</tr>
<tr>
<td>4.4 Hematoxylin and Eosin (H&amp;E) staining</td>
<td>27</td>
</tr>
<tr>
<td>4.5 Immunohistochemistry</td>
<td>27</td>
</tr>
<tr>
<td>4.5.1 The immuno-histochemical peroxidase method</td>
<td>28</td>
</tr>
<tr>
<td>4.5.2 Avidin-Biotin-Peroxidase Complex method</td>
<td>28</td>
</tr>
<tr>
<td>4.5.3 Immuno-histochemical controls for the Avidin-Biotin-Peroxidase Complex method</td>
<td>30</td>
</tr>
</tbody>
</table>
4.6 Immuno-fluorescence method .................................................. 30
4.6.1 Double immuno-fluorescence .................................................. 30
4.6.2 Confocal immuno-fluorescence analysis .................................. 31
4.6.3 The double confocal immuno-fluorescence controls .................. 32
4.7 Statistical analysis .................................................................. 32

5 Results .................................................................................. 33
5.1 Relationship of oral electric field strengths with lichen ruber planus and leukoplakia .......................................................... 33
5.2 Histologic findings ................................................................. 34
5.3 Immunohistochemistry ........................................................... 36
5.3.1 Avidin-Biotin-Peroxidase Complex method .............................. 36
5.3.2 Expression of pan-K and K8-18 in cells of the healthy oral mucosa and in oral mucosa diseased with the lichen ruber planus and leukoplakia .................. 36
5.3.3 Expression of K20 in Merkel cells and NF200 in nerve fibers of the healthy oral mucosa and in oral mucosa diseased with lichen ruber planus and leukoplakia ........................................ 38
5.3.4 Immunohistochemical controls of the Avidin-Biotin-Peroxidase Complex method ......................................................... 41
5.4 The double immunofluorescence method [confocal immunofluorescence method] ................................................................. 41
5.4.1 The confocal double immunofluorescence localization of K8-18 with K20 in cells of the healthy and lichen ruber planus and leukoplakia diseased human oral mucosa ........................................ 40
5.4.2 The confocal double immunofluorescence localization of K20 with NF200 in cells of the lamina propria of the healthy human oral mucosa .......... 42
5.4.3 The confocal double immunofluorescence localization of K20 with NF200 in cells of the healthy human oral mucosa ................. 43
5.4.4 The confocal double immunofluorescence localization of K20 with NF200 in cells of the human oral mucosa diseased with lichen ruber planus ................. 46
5.4.5 The confocal double immunofluorescence localization of K20 with NF200 in cells of the human oral mucosa diseased with leukoplakia .......... 47
5.4.6 Immunohistochemical controls of double immunofluorescence .... 48
5.5 Relationship between normal, lower and higher oral electric field strengths and impairment of the nerve endings .............................................49

6 Discussion ........................................................................................................51
6.1 Methodical considerations of the immuno-histochemical techniques ........52
6.2 Blocking of the endogenous peroxidase activity ........................................52
6.3 Antigen retrieval using proteinase K and Triton X-100 ...............................52
6.4 Blocking of free unspecific binding sites for immunohistochemical reagents .........................................................................................53
6.5 Blocking of free unspecific binding sites for secondary antibodies ..........53
6.6 Visualization of specific immunohistochemical binding sites with substrate chromogen reaction .................................................................54
6.7 There is a cell type dependent specific effect of oral electric field strength on the lichen ruber planus and leukoplakia ......................................54
6.8 Lichen ruber planus and leukoplakia-dependent loss of nerve endings and destruction of Merkel cells at the buccal mucosa epithelium .............57
6.9 Lichen ruber planus and leukoplakia-dependent loss of nerve endings at the buccal mucosa epithelial keratinocytes indicates impaired neuronal modulation of oral mucosa epithelial cells ........................................58
6.10 Lichen ruber planus and leukoplakia-dependent destruction of Merkel cells indicates impaired regulation and modulation of keratinocytes by Merkel cells .......................................................................................59
6.11 The close relationship between nerve endings and Merkel cells detected in healthy oral mucosa is not detectable in oral mucosa diseased with lichen ruber planus and leukoplakia .......................................................60
6.12 K20 positive Merkel cells are associated with the NF200 immunoreactive nerve fibers and nerve endings in the healthy oral mucosa ..........61
6.13 Lichen ruber planus induced an abolishment of nerve fibers and nerve endings associated with Merkel cells .........................................62
6.14 Leukoplakia induced an abolishment of nerve endings associated with Merkel cells .........................................................................................63
6.15 Clinical relevance of the results ................................................................64
6.16 Conclusion .......................................................................................................65
<table>
<thead>
<tr>
<th></th>
<th>Summary</th>
<th>Zusammenfassung</th>
<th>References</th>
<th>Curriculum vitae</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
List of tables

Table 1. The electric field strength values measured in patients diseased with the lichen ruber planus.................................................................82
Table 2. The electric field strength values measured in patients diseased with leukoplakia.................................................................81
Table 3. The electric field strength values measured in healthy patients.........83
Table 4. Healthy patients with electrical field strength values..................83
Table 5. Antibodies used in the immuno-histochemical incubation.............83
Table 6. Lichen ruber planus patients with lowest and highest electrical field strength values...............................................................84
Table 7. Leukoplakia patients with the lowest and highest electrical field strength values...............................................................84
Table 8. Statistical analysis of the galvanic electric field strength of healthy oral mucosa, lichen ruber planus and leukoplakia.........................84
Table 9. Statistical analysis of the galvanic electric field strength of healthy oral mucosa, lichen ruber planus and leukoplakia.........................85
Table 10. Patients with absence of nerves endings in higher and lower electric field strength.................................................................85
Table 11. Statistical analysis of relationship between oral electric field strengths and nerve endings.........................................................85
Introduction

The oral mucosa epithelium and lamina propria of the oral mucosa contain keratinocytes, melanocytes, dendritic cells (Langerhans cells) of different differentiation and maturation stages, blood vessels, nerve fibers and free nerve endings, encapsulated nerve endings such as Pacinian corpuscles, Meissner corpuscles and Merkel cells (Lumpkin and Caterina, 2007 [47]). Each cell of the oral mucosa is specialized in a certain function in a complex cell-extracellular matrix and cell-cell interaction. In homeostasis, the different types of cells in the oral mucosa epithelium and in the lamina propria regulate biological function/s of other cell/s in a paracrine and autocrine manner by release of cytokines, chemokines, neurotransmitters, neuropeptides or different humoral factors in physiological concentration/s under healthy conditions (Hilliges et al., 1994 [21]).

The oral mucosa epithelium represents the first line of defense against external environmental pathogens and noxious stimuli. In defense against noxious stimuli, a physiological healthy perception is a prerequisite for protection of oral mucosa epithelium and lamina propria (Squier and Brogden, 2011 [39]).

In the oral mucosa warming and cooling stimuli are perceived by nerve endings termed thermo-receptors. Stretch and pressure stimuli are perceived by touch receptors (mechanoreceptors) localized in the oral mucosa epithelium (Walker et al., 2000 [76]; Lumpkin and Caterina, 2007 [47]). Painful sensations are perceived by polymodal nociceptors activated by various types of noxious stimuli. Merkel cell-neurite complexes mediate a subset of slowly adapting responses to touch. In the basal layer, Merkel cells are associated in close relationship with myelinated Aβ-afferent nerve endings (Haebertle et al., 2004 [15]; Delmas et al., 2011 [10]).

In the oral mucosa epithelium, lichen ruber planus (Silverman, 2000 [65]; Sugarman et al., 2002 [67]) and especially leukoplakia (Liu et al., 2012 [44]) are diseases were considered as oral pre-malignant disorders. In the
etiology of lichen ruber planus and leukoplakia, the alcohol consumption (Andre, 1995 [2]), smoking (Johnson, 2001 [29]), parafunctions and dental treatment factors (Velly et al., 1998; Lockhardt et al., 1998) are described as responsible reasons for the pathological cellular changes in the oral mucosa epithelium. In experiments of our laboratory was also revealed that electric field strength involved in the pre-malignant alterations in keratinocytes cell line (Korraah et al., 2012 [39]). Therefore, in this study was investigated whether electric field strength may be related in the pathological changes of other cell populations such as nerve fibers, Merkel cells and neurite complex in the cases of pre-malignant lesions lichen ruber planus and leukoplakia in the oral mucosa.

The up- or down-regulation of cytokines, chemokines, neuropeptides or different factors released from different cells of the oral mucosa may alter normal function of cell/s under pathological conditions in the cases of lichen ruber planus and leukoplakia (Inovay et al., 1961 [27], Hou et al., 2011 [23]). It is unknown how the relationship between Merkel cells and Aβ-afferent terminals may be affected by lichen ruber planus and leukoplakia in the oral mucosa. Therefore, in the present study it was aimed to analyse if the sensitivity of the touch receptors, Merkel cell-neurite complex, could be influenced by lichen rubber planus and leukoplakia in the oral mucosa.
2 Literature Overview

2.1 Neuronal perception in the oral mucosa

The receptors of the oral mucosa are classified as thermoreceptors, mechanoreceptors (touch receptors) and nociceptors. Thermoreceptors are nerve endings perceiving warming and cooling stimuli (Lumpkin and Caterina, 2007 [47]). Mechanoreceptors are specialized receptors which perceive stretch, touch and pressure stimuli (Lumpkin and Caterina, 2007 [47]). Nociceptors are receptors which perceive nociception after various types of painful stimuli (Julius and Basbaum, 2001 [31]; Lumpkin and Caterina, 2007 [47]; Basbaum et al., 2009 [5]). Compared to free nerve endings in the oral mucosa, encapsulated nerve endings, such as Paccinian corpuscle, Meissner corpuscle and Merkel cells, are specialized mechanoreceptors.

2.1.1 Mechanoreceptors and mechanosensation

On the basis of pharmacological and physiological functions, the peripheral nervous system is divided into the somatic and autonomic peripheral nervous system. The autonomic nervous system is divided into two functionally distinct parts, the sympathetic and the parasympathetic peripheral nervous system. The somatic nervous system is divided into motor and sensory parts of the peripheral nervous system. The sensory peripheral nervous system sends information to the central nervous system mainly from the internal organs and mainly from the external stimuli (Haeberle et al., 2004 [15]; Delmas et al., 2011 [10]).

The sensory peripheral nervous system perceives different external somatosensory stimuli, including pain, temperature, and touch. The afferent somatosensory nerve fibers innervate mucosa and the skin (Walker et al., 2000 [76]; Lumpkin and Caterina, 2007 [47]). These nerve fibers and nerve endings are divided into three groups based on conduction velocity: Aβ, Aδ, and C nerve fibers and nerve endings. The Aδ and C groups of nerve fibers
and nerve endings are nociceptors and temperature receptors (Lumpkin and Caterina, 2007 [47]).

Sensation of touch is mediated by Aβ fibers. Aβ fibers are subdivided into three groups: slowly adapting nerve fibers type I and type II and nerve fibers of rapidly adapting type. The slowly adapting nerve fibers type I innervate Merkel cell-neurite complexes (Haeberle et al., 2004 [16]; Lee and Caterina, 2005 [42]; Lumpkin and Caterina, 2007 [47]), while slowly adapting nerve fibers type II innervate Ruffini corpuscles (Lumpkin and Caterina, 2007 [47]). Nerve fibers of the rapidly adapting type innervate Meissner and Pacinian corpuscles (Lumpkin and Caterina, 2007 [47]).

Each of these nerve fiber types is important for perception of a specific form of touch stimuli (Lumpkin and Caterina, 2007[47]). Merkel cell-neurite complexes are composed of nerve endings, which are connected to Merkel cells found at the basal layer of the mucosal and skin epithelia first described in 1875 by Merkel (Halata et al., 2003 [17]). Merkel regarded the identified cells as having sensory touch function, and he named these same cells “Tastzellen” (touch cells) (Halata et al., 2003 [17]; Zimmermann et al., 2005[85]; Lumpkin and Caterina, 2007 [47]). Merkel cells have been proposed to be the sensory receptor cells of the Merkel cells-neurite complexes due to the fact that Merkel cells form synaptic contacts with somatosensory afferent nerve fibers and nerve endings (Haeberle et al., 2004 [16]; Lumpkin and Caterina, 2007 [47]; Wende et al., 2012 [80]).

2.1.2 Modulation of oral mucosa epithelial cells by sensory neuropeptide

In addition to the distribution in the lamina propria of the oral mucosa, Aβ-, Aδ- and C-nerve fibers are free intraepithelial nerve endings (Lundy and Linden, 2004 [49]). At the basal layer of the oral mucosa, Aδ- and C-nerve fibers are not associated with Merkel cells. It is known that these free nerve endings and Aβ-nerve endings release numerous sensory neuropeptides such as substance P, calcitonin gene-related peptide (CGRP), neuropeptide K (NPK), neurokinin A (NKA), neuropeptid galanin and somatostatin in
normal human buccal oral mucosa (Hilliges et al., 1994 [21]; Lundy and Linden, 2004 [49]).

The sensory free Aβ-, Aδ- and C-nerve endings release sensory neuropeptide substance P and CGRP (Julius and Basbaum, 2001 [31]; Basbaum et al., 2009 [5]) and they are involved in the terminal differentiation of keratinocytes (Scholzen et al., 1998 [64]; Yu et al., 2010 [83]), melanocytes (Hara et al., 1996 [19]) and in the maturation of the Langerhans cells (Hosoi et al., 1993 [22]; Asahina et al., 1995 [3]).

2.1.3 Merkel cells in the oral mucosa

Merkel cells are located in and distributed along the basal layer of the oral mucosa epithelium. At these localizations, Merkel cells reveal a connection with or close relationship to nerve fibers and nerve endings which are termed Merkel cells-neurite complexes (Wende et al., 2012 [80]; Haeberle et al., 2004 [16], Lumpkin and Caterina, 2007 [47]).

Merkel cells are characterized by small, electron-dense endocrine-like granules, intermediate filaments, and occasional desmosomes (Lucarz and Gerard, 2007[45]). The endocrine nature of Merkel cells is further proven by their immunoreactivity with various endocrine markers, especially chromogranin A and synaptophysin, which are the most frequently found (Moll et al., 2005 [55]; Boulais and Misery, 2007 [7]; Eispert et al., 2009[11]).

Merkel cells are bound to adjoining keratinocytes by desmosomes and contain intermediate filaments in their cytoplasm (Moll et al., 2005 [55]; Lucarz and Gerard, 2007 [45]; Boulais and Misery, 2007 [7]). The nucleus is lobed, and the cytoplasm is somewhat denser than that of melanocytes and Langerhans cells (Lucarz and Gerard, 2007 [45]; Boulais and Misery, 2007 [7]). In addition, Merkel cells express numerous cytokeratins (K), including K7, 8, 18, 19, and 20 (Moll et al., 1995 [54]; 2005 [55]; 2008 [57]). K20 is expressed mainly in Merkel cells (Moll et al., 1995 [54]).
Therefore, K20 is considered a reliable marker for Merkel cells (Moll et al., 1995 [54], 2005 [55]).

2.2 Pre-malignant lesions in the oral mucosa

In addition to erythroplakia, oral submucous fibrosis, actinic keratosis, discoid lupus erythematosus, dyskeratosis congenital and epidermolysis bullosa (Warnakulasuriya et al., 2007 [78]; van der Waal, 2009 [72]; Amagasa et al., 2011 [1]), lichen ruber planus (Silverman, 2000 [65]; Sugarman et al., 2002 [67]) and leukoplakia (Liu et al., 2012 [44]) are considered as oral pre-malignant disorders.

In oral pre-malignant lesions, age and gender distribution revealed a variety and proved to be in relationship with the risk of malignant transformation (Amagasa et al., 2011 [1]; Johnson et al., 2011 [39]). The pre-malignant lesion varies from site to site of the oral mucosa within the mouth and from population to population in dependence on different etiological factors (Reibel, 2003 [61]; Amagasa et al., 2011 [1]; Johnson et al., 2011 [29]).

2.2.1 The etiology of the pre-malignant lesions in the oral mucosa

In addition to different factors such as the alcohol consum (Andre, 1995 [2]), smoking (Johnson, 2001 [29]), parafunctions and dental treatment factors (Velly et al., 1998; Lockhardt et al., 1998), in vitro experiments of our laboratory revealed that electric field strength could be involved in the pre-malignant alterations in keratinocytes of the oral mucosa (Korraah et al., 2012 [39]). However, in in vivo studies the role of electric field strength in the development of the pre-malignant lesions lichen ruber planus and leukoplakia in the oral mucosa was not yet elucidated, since all studies measured galvanic potentials and not a specific electrostatic field strength.

2.2.3 Tobacco and smoking

Tobacco is considered a risk factor for the development of pre-malignant
disorders of the oral mucosa (Tomar et al., 1997 [70]; Johnson, 2001 [29]; Johnson et al., 2011 [30]). The occurrence of leukoplakia among individuals with tobacco users was found to be highly elevated (Bánóczy et al., 2001 [4]).

In the development of oral cancer, alcohol consumption with coincident use of tobacco strongly increases the occurrence of malignant changes in the oral mucosa (Blot et al., 1988 [6]; Johnson, 2001 [29]). Prevention and smoking cessation results in disappearance of a substantial number of leukoplakia (Tomar et al., 1997 [70]; Bánóczy et al., 2001 [4]).

Histopathologically, it was revealed that tobacco consumption causes alterations in terminal keratinization in keratinocytes of the hard palate epithelium (Bánóczy et al., 2001 [4]). It was reported that tobacco and alcohol consumption induced epigenetic alterations which are associated with development of oral malignant lesions (Choi and Myers, 2008 [8]).

2.2.4 Alcohol consumption

It was reported that alcohol consumption increased the risk of development of oral and oropharyngeal cancer by 30 times (Andre, 1995 [2]). Alcohol is hepatotoxic, reducing the effectiveness of enzyme systems essential for the detoxification of carcinogens, especially the glutathione-S-transferases and cytochrome P450 systems (Mari and Cederbaum, 2001 [50]). Alcohol induces oncogenesis by damage to phospholipids of the cell membrane and increases the cellular permeability. Among the components of alcoholic drinks N-nitrosodiethylamine and polycyclic aromatic hydrocarbons were found, which are considered carcinogenic (Ogden, 1998 [59]).

The major alcohol metabolizing enzymes are alcohol dehydrogenase, which oxidizes ethanol to acetaldehyde, and aldehyde dehydrogenase, which detoxifies acetaldehyde to acetate (Johnson, 2011 [30]). Acetaldehyde is responsible for the oral carcinogenic effect of ethanol, due to its multiple mutagenic effects on DNA. It was described that alcohol can impair DNA
repair mechanisms (Howie, 2001 [24]). In tumor-related genes of oral mucosa cells epigenetic changes were detected in the form of multiple methylation (Supic et al., 2011 [68]).

2.2.5 *Galvanic interactions between dental alloys*

Dental treatment with various metallic restorations results in different electric potentials between these various metallic restorations which induce various changes and damages of the oral mucosa epithelium cells (Inovay and Banoczy, 1961 [27]; Syrjänen et al., 1984 [69]; Schmalz and Garhammer, 2003 [63]). It is known that different dental metallic restorations can induce local cells' (Zalkind et al., 1998 [84]) and tissue responses including gingivitis, periodontitis and local oral mucosa changes such as lichen ruber planus (Schmalz, 1999 [62]; Geurtsen, 2002 [14]).

The differences in galvanic potentials in the oral cavity may be considered as an important reason for the development of oral lichen ruber planus (Geurtsen, 2002 [14]; Martin et al., 2003 [51]) and for the development of leukoplakia. In addition to various irritants and agents including tobacco, alcohol and chronic friction, galvanic electric potential reactions between different restorative dental metals may be involved (Schmalz, 1999 [62]; Geurtsen, 2002 [14]) in the etiology of leukoplakia.

2.2.6 *Lichen ruber planus and leukoplakia in the oral mucosa*

In the oral mucosa, lichen ruber planus (Silverman et al., 1984 [66]; Silverman, 2000 [65]; Yarom et al., 2009 [81]) and leukoplakia (van der Waal et al., 1997 [73]) are considered as pre-malignant lesions.

Lichen ruber planus is a chronic inflammatory autoimmune disease with T-cells-mediated inflammation in the oral mucosa (Jungell et al., 1989). Oral leukoplakia is characterized by epithelial hyperplasia, hyperkeratosis, and hyperparakeratosis, with or without epithelial dysplasia (van der Waal et al., 1997 [73]).
2.2.7 **Lichen ruber planus in the oral mucosa**

It is known that oral lichen ruber planus is a chronic inflammatory autoimmune disease of unknown etiology where T-cells are responsible for the inflammations. The lymphocytic infiltrate in lichen ruber planus is composed almost exclusively of T-cells, and the majority of T-cells within the epithelium and adjacent to damaged basal keratinocytes are activated CD8⁺ lymphocytes (Jungell et al., 1989).

Clinically lichen ruber planus presents as white striations, white papules, white plaques, erythema, erosions, or blisters affecting predominantly the buccal mucosa, tongue, and gingivae (Silverman et al., 2000 [65]). The lesions are usually bilateral, atrophic and erosive (Sugerman, 2002 [67]). Lichen ruber planus occurs in six clinical variants as reticular, popular, plaque-like, erosive, atrophic and bullous (Roopashree et al., 2010). Skin lesions can coincidentally appear in relationship with the oral lesions. The skin lesions are flat-topped violaceous papules, which appear in wrists, ankles, and genitalia (Sugerman, 2002 [67]).

Histologically lichen ruber planus present as a subepithelial infiltrate of lymphohistiocytes, and degeneration of basal keratinocytes. Parakeratosis, acanthosis, and saw-tooth rete peg formation may be seen (Khan et al., 2001).

Lichen ruber planus is considered as a pre-malignant lesion, although the malignant transformation is still under examination (van der Meij et al., 1999 [71]). It was described that squamous cell carcinoma may arise at the site of pre-existing lichen ruber planus lesion in less than five percent of study cases (Eisenberg, 2000 [23]). Dysplastic alterations in epithelial cells may be observed also in lichen ruber planus diseased oral mucosa epithelium (Krutckoff and Eisenberg, 1985). In addition, it was described that oral lichen planus is associated with an increased risk for chromosomal instability (Yarom et al., 2009[81]). These results indicate that oral lichen ruber planus possesses a malignant potential (Yarom et al., 2009 [81]).
2.2.8 Leukoplakia in the oral mucosa

Leukoplakia is a clinical term used to describe patches of keratosis. It must be distinguished from diseases that may cause similar white lesions, such as candidiasis or lichen ruber planus (van der Waal et al, 1997 [73]). The etiology of leukoplakia remains unknown. In addition to tobacco, alcohol, chronic friction and ultraviolet radiation, electrical galvanic reaction between unlike restorative metals in the oral cavity are involved in the etiology of leukoplakia (Schmalz, 1999 [62]; Geurtsen, 2002 [14]).

The most common sites in which leukoplakia occur are the buccal mucosa, alveolar mucosa, and lower lip; however, lesions in the floor of the mouth, lateral tongue, and lower lip are most likely to show dysplastic or malignant changes (Lumerman, 1995 [46]). There are two clinical variants of leukoplakia: the homogeneous and the non-homogeneous type. In the homogeneous type the lesions are white and uniformly flat, with thin appearance that exhibit shallow cracks and have smooth, wrinkled or corrugated surface with a constant texture throughout, whereas the lesions in the non-homogeneous type are white or white-red lesion that are irregularly flat, nodular or exophytic (Kumar et al., 2012 [41]).

Oral leukoplakia shows characteristic histologic findings such as epithelial hyperplasia, hyperkeratosis, and hyperparakeratosis, with or without epithelial dysplasia or carcinoma (Feller, 2011 [13]). The presence of dysplasia has been associated with a risk of progression to cancer (Warnakulasuriya, 2008 [79]). In leukoplakia diseased oral mucosa epithelium, basal cells lose cellular polarity and occur in more than one layer with basaloid appearance. The nuclear-cytoplasmic ratio is increased especially in basal epithelial cells. The epithelium reveals drop-shaped rete pegs with irregular epithelial stratification. In addition to few abnormal mitoses in the superficial half of the epithelium, epithelial cells reveal pleomorphism and nuclear hyperchromatism with enlarged nucleoli. In
stratum spinosum single cells reveal keratinization (Kramer et al., 1978 [40]; van der Waal et al., 1997 [73]).

**Leukoplakia** is one of the most common precancerous lesions of the oral cavity with a high risk of malignant transformation (Reibel, 2003 [61]; Epstein et al., 2003 [12]; Amagasa et al., 2011 [1]; Johnson et al., 2011 [28]). Dependent on dysplasia grade, epithelial dysplasia lesions are divided into mild, moderate and severe degree (van der Waal et al., 1997 [73]). It was described that epithelial dysplasia may be correlated with the age of the patient (Vesper et al., 1992 [74]). In comparison to leukoplakia without dysplasia features, leukoplakia with moderate or severe dysplasia in epithelial cells may have a greater potential for malignant transformation (van der Waal et al., 1997 [73]; Reibel 2003 [61]). However, there are reports, which described the malignant transformation of leukoplakia without dysplastic features (Waldron and Shafer, 1975 [75]; Silverman et al., 1984 [66]; Hansen et al., 1985 [39]).
3 Statement and aims of the study

In the oral cavity, the human oral mucosa epithelium represents the first line of defence against external environmental pathogens and noxious stimuli and the primary function of oral mucosa epithelium is the protection of the underlying tissue. Harmful environmental agents that may be involved in diseases of the oral mucosa epithelium include microbial toxins and enzymes (bacterial and viral infections), antigens and carcinogens such as food, smoking and alcohol. In addition, trauma, chemical substances and ultraviolet irradiation may be involved in the lesions of the oral mucosa.

3.1 Statement of the study

Different noxious stimuli alone or in a combination of more than two factors may exert pathological effects on the biological functions of different cell types in the oral mucosa. In addition to the various reasons, it is known that galvanic electric potential is involved in the lesions of the oral mucosa. In *in vitro* experiments, a relationship between leukoplakia and electric field strength was described [39]. The relationship between the electric field strength and clinical signs of leukoplakia has been recently demonstrated by a clinical study group at the Department of Prosthetic Dentistry of the University of Cologne Germany [82].

The different noxious stimuli change normal biological functions of cells. The relationship between galvanic electric potential and occurrence of the lichen ruber planus is unknown. The relationships between galvanic electric potential in lichen ruber planus and in leukoplakia were also not compared. In addition, it is also not known, whether there is a relationship of galvanic electric potential effect on the perception of the oral mucosa epithelium cells, such as on Merkel cells-neurile complex, influencing Aβ-nerve fibers which are responsible for mechanosensation.

In the oral mucosa epithelium, a physiological healthy perception is a prerequisite for protection of the oral mucosa from harmful stretch and
pressure stimuli. To avoid non-physiological pressure places, in the design of a prosthetic treatment the healthy perception and sensitivity of the touch receptors in the oral mucosa are of major importance. Merkel cells are mechanosensory cells that transduce touch and then communicate with afferents by synaptic transmission. However, the effects of the lichen ruber planus and leukoplakia on the relationship between Merkel cells and nerve endings in the oral mucosa are not known.

It was described that increased keratinization of the oral mucosa in patients with complete dentures was considered as a protective mechanism [69]. Therefore, a physiological thickness of the oral mucosa epithelium is necessary. Thus, proliferation of keratinocytes by neuronal modulation at the basal membrane and the differentiation of the keratinocytes in oral mucosa epithelial layers are important.

The healthy oral mucosa epithelium is innervated by Aβ-, Aδ- and C-nerve fibers and nerve endings [49]. In addition to the keratinocytes, the oral mucosa epithelium consists of Langerhans cells and melanocytes. Keratinocytes, melanocytes and Langerhans cells are regulated by several neuropeptides (galanin, NPY, VIP, substance P, CGRP) which are released by Aβ-, Aδ- and C-nerve fibers and nerve endings. The sensory neuropeptide substance P and CGRP released by Aβ-, Aδ- and C-nerve fibers [31, 5] are involved in the terminal differentiation of keratinocytes [64, 83], melanocytes [19] and in the maturation of the Langerhans cells [39, 3]. However, the effects of lichen ruber planus and leukoplakia on the nerve fibers and nerve endings which release the various neuropeptides such as substance P and CGRP are not known.

In the epithelium, the sensory neuropeptides (substance P, CGRP) released by Aβ-, Aδ- and C-nerve fibers and their receptors are also expressed in keratinocytes [43]. The sensory neuropeptides substance P and CGRP are involved in the modulation of nociception and inflammatory pain in the oral mucosa epithelium [49]. But the effects of lichen ruber planus and
leukoplakia on nerve fibers and nerve endings, which release sensory neuropeptides in the oral mucosa are unknown.

3.2 Aims of the study

In comparison to the known role in leukoplakia, it is unclear, whether galvanic electric potential is involved in the pathogenesis of lichen ruber planus in the oral mucosa. Therefore in this study was investigated, whether galvanic electric potential may be considered as a direct reason for lichen ruber planus and leukoplakia. In this study was also aimed to compare the magnitude of the galvanic electric potentials in the lichen ruber planus and leukoplakia.

In the healthy oral mucosa, there is a relationship between Merkel cells and nerve endings. The effect of galvanic electric potential on the development of lichen ruber planus and leukoplakia and thus on the relationship of nerve endings and Merkel cells was also not cleared. In this study was examined, whether the relationship between Merkel cells and nerve endings was changed directly by galvanic electric potential.

In the oral mucosa epithelium, the mechanosensation mediated by Merkel cells and nerve endings in a close neuronal relationship under pathological conditions was not yet investigated. Lichen ruber planus and leukoplakia are lesions were observed often in the oral mucosa. In the course of long time, these lesions may be considered as precancerous lesions. Therefore the nociception and pain symptomatic is important for a treatment of these lesions.

The main aim of this study is therefore to investigate, whether relationship between Merkel cells and nerve endings may be changed in lichen ruber planus and leukoplakia.
4 Material and Methods

4.1 Ethical considerations and clinical anamnesis of patients

The clinical anamneses and clinical investigations were performed for a specific diagnosis of lichen ruber planus and leukoplakia. In the cases of lichen ruber planus and leukoplakia, the electric field strength measurements were performed only in patients, which exhibited different prosthetic materials in their recent prosthodontics treatments.

The clinical study was performed at the Departments of Prosthetic Dentistry and for Oral and Maxillofacial Surgery at the University of Cologne. These results were then confirmed with histo-pathological findings, which were obtained in the Institute of Pathology at the University of Cologne.

For all tissues patients agreed to have the tissues examined for research purposes. Procurement of human oral mucosa samples at surgery was approved by the Human Ethics Committee of the Heinrich-Heine-University, Düsseldorf.

4.2 Measurements of the electric field strength.

The electric field strength in healthy patients as well as in patients with leukoplakia and with lichen ruber planus was measured by a clinical study group of the Department of Prosthetic Dentistry at the University of Cologne [82].

The patients with different dental metal restorations were evaluated due to suspected diagnose on lichen ruber planus and leukoplakia. In addition to the detailed examination of the oral mucosa, electric field strength of the oral mucosa of twenty-one patients were measured due to suspected diagnosis to the lichen ruber planus (n=10; Table 1) and leukoplakia (n=11; Table 2).
The patients showing no oral mucosa alterations were taken as a control (n=10; Table 3), four of them (n=4; Table 4) gave consent to a biopsy of the oral mucosa.

The electric field strength was measured through a digital multimeter with DC voltage range (direct current) from 100nV to 1kV and a DC resistance (direct current breaking) from 100 using measuring electrodes with low polarisation affinity [83].

4.3 Tissue preparation

Tissues were prepared and then collected in a fixative (4% paraformaldehyde in 0.1 M PBS, pH 7.4) for histo-pathological confirmations of suspected diagnosis of lichen ruber planus and leukoplakia.

To compare the changes in cellular composition and in cell structures of the different cell types in the oral mucosa epithelium (keratinocytes, melanocytes, Langerhans cells, nerve endings, Merkel cells) and in the Lamina propria (blood vessels, nerve fibers, extracellular matrix such as collagens, inflammatory cells) of the oral mucosa, healthy oral mucosa samples were collected from patients which were treated by plastic surgery.

4.3.1 Fixation of tissues

The samples were fixed in 4% PFA paraformaldehyde ± 0.2% picric acid solution at 4°C for 72 hrs. Then tissues were washed in 0.1 M PBS pH 7.4 at 4°C. The samples were embedded in paraffin.

4.3.2 Paraffin embedding and sections of samples

The collected samples were dehydrated in ascending series of isopropanol at 50% (2x), 70% (2x), 96 % (2x), 100%(2x), 5 min for each step.
Then tissues were cleared in XEM-200 I and XEM-200 II and embedded in Paraplast Plus I and Paraplast Plus II. All steps of paraffin embedding were performed at vacuum.

Using a Microtome (Microm HM 355, Walldorf, Germany) samples were cut at 10 μm each.

4.4 Hematoxylin and Eosin (H&E) Staining

The paraffin sections (n-54) were first deparaffinized in 2 changes of xylene for 10 minutes each and rehydrated in 2 changes of absolute ethanol for 5 minutes each and ethanol 95%, 90%, 80%, 70% for 2 minutes each.

Then the sections were washed briefly in distilled water. Subsequently, the sections were immersed into Mayer’s hematoxylin solution for 8 min, washed in running tap water, rinsed in distilled water for 2 minutes, and then immersed in eosin solution for 1 minute and washed again.

The sections were dehydrated through 95% ethanol and absolute ethanol (2 changes for 5 minutes each), cleared in 2 changes of xylene for 5 minutes each and mounted with xylene based mounting medium Entellan (Merck KGaA, Darmstadt, Germany).

4.5 Immunohistochemistry

In the present study two immuno-histochemical methods were performed: peroxidase and immunofluorescence methods.

In the peroxidase immuno-histochemical methods, it is known that Avidin-Biotin-Peroxidase Complex Method is very sensible. Therefore peroxidase incubations were performed in serial sections of all patients using the Avidin-Biotin-Peroxidase Method.
In the immunofluorescence method, confocal-double immuno-histochemical method was performed. Using confocal double immuno-histochemical incubations, two different proteins were characterized in the same cells. (Table. 5). In addition, in the same cells the subcellular localization of two different proteins were detected and characterized. Using a cell chromosomal marker (DRAQ5), nuclei of cells were demonstrated for double confocal immunofluorescence.

To compare effects of the electric field strength on the relationship between nerve endings and Merkel cells, double immunofluorescence incubations were performed in sections of 4 normal tissues and in sections of 3 patients (Tables 4, 6, 7) with higher electric field strength measurements and in sections of 3 patients with lower electric field strength measurements of lichen ruber planus and leukoplakia.

4.5.1 The immuno-histochemical peroxidase method

4.5.2 Avidin-Biotin-Peroxidase Complex Method

The sections (n=98) were applied in two changes of xylene for 15 minutes each and rehydrated in 1 change of 100% ethanol for 5 minutes, 90%, 70% and 50% ethanol for 2 minutes each. The deparaffinized sections were rinsed in distilled water for 5 minutes.

Subsequently, the sections were rinsed in 2 changes of 0.05 M TBS for 15 minutes each. The antigen retrieval in the samples was performed using Proteinase K (DAKO, Hamburg, Germany) for 3 minutes and subsequently samples were rinsed in two changes of TBS for 15 minutes each.

The endogenous peroxidase was inhibited by 0.05 M TBS containing 0.3% H₂O₂ for 20 minutes at room temperature and subsequently samples were rinsed in 0.05 M TBS pH 7.6 for 5 minutes. Then, the sections were treated with 0.25%, Triton X-100 + 0.05 M TBS pH 7.6 for 30 minutes and later rinsed in 0.05 M TBS pH 7.6 for 5 minutes.
The unspecific binding sites for antibodies were blocked with 2% Bovine Serum Albumin (BSA) (Bovine Serum Albumin Fraction V, PAA, Pasching, Austria) + 5% Normal Goat Serum (NGS) (Vector Laboratories, Burlingame, CA) for 30 minutes. Subsequently, the sections were incubated for 48 hours at 4°C in 0.05 M TBS pH 7.6 with the following antibodies: anti-cytokeratin 8-18 (mouse monoclonal [5D3] to cytokeratin 8-18, 1:50; Abcam, Cambridge, UK), and anti-pan cytokeratin (mouse monoclonal anti-pan cytokeratin, 1:100; Sigma, St. Louis, MO).

Then the sections were rinsed in two changes in 0.05 M TBS pH 7.6 for 15 minutes each. The sections were incubated in biotinylated secondary antibody anti-mouse IgG (1:500, 1:50; Vector) in 0.05 M TBS pH 7.6 for 1 hour at room temperature.

The sections were washed in 0.05 M TBS pH 7.6, two changes for 15 minutes each. Then, the sections were incubated in avidin-biotin-peroxidase complex (1:100; Vector) for one hour.

The sections were rinsed in 0.05M TBS pH 7.6 (two changes for 15 minutes each) and finally, the binding sites of antigen-antibodies signals were visualized with 0.5% 3,3% diaminobenzidine tetrahydrochloride (Sigma) in 0.05M Tris-HCl buffer, pH 7.6, containing 0.01% H₂O₂ and 0.01% nickel ammonium sulfate.

The sections were rinsed in distilled water (two changes for 15 minutes each), and dehydrated with 50%, 70%, 60%, 90%, 95% Ethanol (each step for 2 minutes) and 100% I and 100% II Ethanol 2 minutes successively.

Afterwards the sections were mounted using Entellan (Merek KGaA, Darmstadt, Germany).
4.5.3 **Immuno-histochemical controls for the Avidin-Biotin-Peroxidase Complex Method**

Immuno-histochemical incubations without primary but with secondary antibodies (affinity purified biotinylated goat anti-mouse IgG (Vector) and affinity purified biotinylated goat anti-rabbit IgG (Vector)) were carried out as negative controls.

4.6 **Immuno-fluorescence method**

4.6.1 **Double Immuno-fluorescence**

The sections (n=11) were deparaffinized in two changes of xylene for 15 minutes each and rehydrated in two changes of 100% ethanol for 5 minutes and 90%, 70%, 50% ethanol for 2 minutes each. Then the sections were rinsed in distilled water for 5 minutes, and washed in 0.05 M TBS pH 7.6 in 2 changes for 15 minutes each.

The antigen retrieval in the samples was performed using Proteinase K (DAKO) for 3 minutes and the samples were rinsed in 2 changes of 0.05 M TBS pH 7.6 for 15 minutes each.

The unspecific sites for the antibodies were blocked with blocking solution containing 2% BSA + 5% NGS for 30 minutes. Subsequently, the sections were incubated with the following first primary antibodies: mouse monoclonal [5D3] to cytokeratin 8-18 (K8-18) (1:50; Abcam), and mouse monoclonal anti-NF200 (1:2000; Sigma, Taufkirchen, Germany) over night at 4°C in 0.05M TBS pH 7.6.

Then the sections were rinsed in two changes of 0.05 M TBS pH 7.6 for 15 minutes each and incubated with secondary antibody (488-DyeLight conjugated goat anti-mouse IgG; Pierce Biotechnology, Rockford, IL) against first antibodies at 1:500 for K8-18 and NF200 in 0.05 M TBS pH 7.6
dilutions. From this step on, all the following procedures that followed were
done avoiding light.

The sections were rinsed in two changes of 0.05 M TBS for 15 minutes each
and the unspecific binding sites for the antibodies were blocked with 2% BSA
(PAA) + 5% NGS (Vector) for 30 minutes. Subsequently, the sections
were incubated with second antibody, rabbit polyclonal against cytokeatin
20 (K20) (Abcam) at 1:250 dilution in 0.05 M TBS over night at 4°C.

The sections were rinsed in 2 changes of 0.05 M TBS pH 7.6 for 15 minutes
each and incubated with second secondary antibody 549-DyeLight
conjugated goat anti-rabbit IgG (Pierce Biotechnology) at a dilution of
1:500 for one hour.

For cell nucleus identification, the sections were incubated with DRAQ5
(Axxora, Lörrach, Germany) for 15 minutes. Then, the sections were rinsed
in two changes of 0.05 M TBS pH 7.6 for 15 minutes each. Finally, the
sections were mountain using Aqua Poly/Mount (Polysciences Inc,
Warrington, PA, USA).

4.6.2 Confocal Immuno-fluorescence analysis

Three colour fluorescent images were acquired on an LSM 510 META
confocal microscope (Carl Zeiss, Oberkochen, Germany).

The 488 nm excitation beam of an argon-krypton laser and a 505-530 nm
band-pass emission filter were used for selective viewing of the green
(identification of K8-18 and NF200) fluorochrome. The 543 nm excitation
beam and 560-613 nm band-pass emission filter were used for selective
viewing of the red (identification of K20) fluorochrome. The 633 nm
excitation beam and 649-702 nm band-pass emission filter were used for
selective viewing of the far-red fluorochrome (identification of DRAQ5)
[34, 38].

31
4.6.3 The double confocal immuno-fluorescence controls

In confocal double immunohistochemical incubations, controls were performed without primary antibodies but with DRAQ5 for DNA staining and with secondary antibodies (488-conjugated goat anti-mouse IgG and 549-conjugated anti-rabbit IgG).

4.7 Statistical analysis

The statistical differences in electric field strength values between healthy (control) oral mucosa and lichen ruber planus and leukoplakia diseased oral mucosa, were analysed using the two-tailed Student’s *t*-test implemented in the software package SPSS for Windows, Version 10.0. One-way ANOVA with Bonferroni *post hoc* test was used to compare multiple means. Data are presented as mean ± SD. Statistical significance was considered at a *p* value ≤0.05.

To compare the relationship between the diagnose, the higher and lower electric potential with absence of the nerve endings of the Merkel cells, the oral electric field strengths of healthy (*n*=4; Table 4), lichen ruber planus (*n*=3 higher/*n*=3 lower; Table 6) and leukoplakia (*n*=3 higher/*n*=3 lower; Table 7), were statistically analysed using the same statistical method.
5 Results

5.1 Relationship of oral electric field strengths with lichen ruber planus and leukoplakia

Statistical analysis (Tables 8, 9) revealed that oral electric field strengths of healthy oral mucosa specimens (01.80 ± 0.91; n=10) was significantly (p=0.009) lower in comparison to the lichen ruber planus (12.62 ± 0.674; n=10) (Fig. 1). The oral electric field strength of the leukoplakia (18.18 ± 10.93; n=10) was significantly (p=0.000) higher detected in comparison to the oral electric field strengths of the healthy samples (01.80 ± 0.91; n=10) (Fig. 1).

Moreover, statistical results (Table 8, 9) showed that oral electric field strength of the lichen ruber planus (12.62±06.74; n=10) was insignificantly (p=0.319) lower measured in comparison to the results of leukoplakia (18.18±10.93; n=10) (Fig. 1).

![Fig.1 Statistical relationship of oral electric field strengths (V/m) with healthy, lichen ruber planus and leukoplakia. Statistical comparison of electric field strength (V/m) of healthy oral mucosa (01.80±0.91 V/m), lichen ruber planus (12.62±6.74 V/m) and leukoplakia (18.18±10.93 V/m) indicating significant differences between lichen ruber planus (p=0.009) and leukoplakia (p=0.000) each versus healthy controls.](image-url)
5.2 Histologic findings

In the present study, a relationship between oral electric field strengths and lichen ruber planus and leukoplakia was detected in a general and cell type-independent manner.

In the healthy palatal oral mucosa cytokeratin 20- (K20) positive Merkel cells are closely associated with NF200 stained nerve fibers and nerve endings. In oral mucosa diseased by lichen ruber planus and leukoplakia, neurofilament 200- (NF200) positive nerve fibers and nerve endings were not observed around K20 positive Merkel cells (Fig. 11, 12, 13). Localization of K20 in Merkel cells was confirmed also by staining of pan-cytokeratin (pan-K) and K8-18 in control incubations (Fig. 3 E, F, H, I).

In H&E stained sections of the palatal healthy oral mucosa (n=4), epithelial cells formed an intact epithelial barrier. Regular rete pegs were observed. Keratinocytes were detected almost in same sizes with oval cell nuclei. At the basal epithelial layer, basal membrane was observed intact. In the lamina propria, collagen fibers, blood vessels, nerve fibers and stroma cells were distributed in a physiological cellular architecture (Fig. 2 A, B).

In lichen ruber planus sections of all patients (n=10), the epithelium is thin and rete pegs could not be detected. In comparison to the leukoplakia, keratinocytes were observed almost in the same size. An orderly basal membrane structure was not detectable. In the basal layer and in the lamina propria, numerous inflammatory cells occurred. In grades of inflammation-dependent manner, there are structural changes in the epithelium. In higher inflammation, epithelium was thin. Inflammation was detected in all cases of lichen ruber planus (Fig. 2 C, D).

Depending on localization sites of keratinocytes, there are differences in the sizes of cells from leukoplakia sections (n=11; Table 2). In comparison to the other differentiation layers of keratinocytes, in the basal layer, larger cells were detected than in other cell layers. Cell nuclei of the keratinocytes
at the basal layer were greater than in other differentiation layers. In addition, at the basal cell layer, the classical rete pegs were not observed. Instead, there were numerous oval invaginations of the oval-shaped epithelial cells at the basal cell layer with dysplastic changes. In leukoplakia, inflammation was not frequently observed. Only in some cases, inflammation was observed in the lamina propria (Fig. 2 E, F).
Figure 2. H&E staining in cells of healthy oral mucosa and in oral mucosa diseased on the lichen ruber planus and leukoplakia. In the healthy oral mucosa epithelium (A, B), regular rete pegs were observed. Keratinocytes were detected almost in the same sizes as oval cell nuclei. The basal membrane was detected intact. In Lichen ruber planus (C, D), the epithelium was thin and rete pegs were not identifiable. Keratinocytes were observed almost in the same sizes. Basal membrane was not intact. There were numerous lymphocytes with chronic infiltration in the lamina propria. In leukoplakia (E, F), cells with their nuclei at the basal layer were detected larger than in other cell layers. The rete pegs were not observed. There were numerous oval invaginations of the oval-shaped epithelial cells at the basal cell layer with dysplastic changes. Bar: A-F=50 μm.
5.3 Immunohistochemistry

5.3.1 Avidin-Biotin-Peroxidase Complex Method

5.3.2 Expression of pan-K and K8-18 in cells of the healthy oral mucosa and in oral mucosa diseased with lichen ruber planus and leukoplakia

In keratinocytes of the healthy oral mucosa epithelium and in Merkel cells at the basal layer, pan-K was detected. Merkel cells were strongly positive for K8-18. Keratinocytes of the basal layer revealed a moderate staining for K8-18, while keratinocytes of the other epithelial cell layers were negative for staining of K8-18 (Fig. 3 A, B, C).

In the oral mucosa epithelium with lichen ruber planus lesions, pan-K was detected in keratinocytes of all layers of the oral mucosa epithelium with different staining intensities. In Merkel cells at the basal layer, pan-K, K8-18 was detected (Fig. 3 D, E, F).

In keratinocytes of all layers (stratum basale, spinosum, granulosum, lucidum and corneum) of the oral mucosa epithelium, a staining for pan-K was detected with different staining intensities, while K8-18 was not identifiable in keratinocytes of the epithelium diseased with leukoplakia. In numerous Merkel cells at the basal layer, pan-K and K8-18 were detected (Fig. 3 G, H, I).
Figure 3. Expression of pan-K and K8-18 in cells of healthy oral mucosa and in oral mucosa diseased with Lichen ruber planus and Leukoplakia. H&E staining (A, D, G), pan-K (B, E, H) and K8-18 (C, F, I) in keratinocytes and in Merkel cells of oral mucosa from two different patients (P1 and P2). Depending on individual variance, K8-18 is expressed with strong (C) and weakly (F) staining intensities for keratinocytes and in numerous (C, asterisk) and in a small number (F, asterisk) of Merkel cells. e: epithelium of oral mucosa, bl: the basal layer, lp: lamina propria. Bar: A-F 50 μm, G-I 20 μm
5.3.3 Expression of K20 in Merkel cells and NF200 in nerve fibers of the healthy oral mucosa and in oral mucosa diseased with lichen ruber planus and leukoplakia

NF200 positive nerve fibers and nerve endings are detected in the basal layer of the oral mucosa epithelium. Intraepithelial nerve endings, which were positive for NF200, were identified. In healthy oral mucosa epithelium, nerve fibers are distributed in the lamina propria as free nerve fibers or around blood vessels (Fig. 4 A, B).

In lichen ruber planus and in leukoplakia, K20 was detected in Merkel cells at the basal layer of the oral mucosa epithelium. In both cases, NF200 was only weakly detectable in some nerve endings in the lamina propria of the oral mucosa epithelium. In the oral mucosa epithelium diseased with lichen ruber planus and in leukoplakia, NF200 was not detectable (Fig. 4 C, D, E, F).
Figure 4. Expression of K20 and NF200 in cells of oral healthy mucosa and oral mucosa diseased with Lichen ruber planus and Leukoplakia. K20 is expressed with strong staining intensities only in Merkel cells (A, C, E asterisk) at the basal layer of the oral mucosa epithelium. NF200 positive nerve fibers are distributed in lamina propria (B asterisk) at the basal layer and intraepithelial of the oral mucosa epithelium. Bar: A-F= 50 μm.
5.3.4 Immunohistochemical controls of the Avidin-Biotin-Peroxidase Complex Method

Immunohistochemical control incubations without primary (mouse anti-pan K, mouse anti-K8-18, mouse anti-NF200, rabbit anti-K20) but with secondary antibodies (biotinylated goat anti-mouse IgG, biotinylated goat anti-rabbit IgG) resulted in the disappearance of the specific reaction product for avidin-biotin-peroxidase complex (Fig. 5).

Figure 5. Immunohistochemical control incubations for ABC method. In incubations without primary antibodies but with secondary antibodies (goat biotinylated anti-rabbit IgG and goat biotinylated anti-mouse IgG), a specific immunohistochemical signal was not detectable. Bar: A-D=50μm.
5.4 Double Immunofluorescence Method [Confocal Immunofluorescence Method]

5.4.1 The confocal double immunofluorescence localization of K8-18 with K20 in cells of healthy and lichen ruber planus and leukoplakia diseased human oral mucosa

In healthy human palatal mucosa epithelium, basal keratinocytes were stained by K8-18 (Fig. 6 B, F). Merkel cells at basal layer of the oral mucosa epithelium revealed a strong immunoreaction for K8-18 in the cell membrane as well as in cytoplasm (Fig. 6 B, F). With the exception of Merkel cells, all epithelial cells and cells of the lamina propria were negative for staining of K20 (Fig. 6 C, G). In Merkel cells, the Merkel cell marker K20 revealed a co-localization with K8-18 (Fig. 6 D, H).

Figure 6. Confocal double immunofluorescence localization of K8-18 with K20 in cells of human healthy oral mucosa. DRAQ5 is localized in nuclei of oral mucosa epithelium (A, E (a higher magnification of A)). Colocalization of K8-18 (B, F (a higher magnification of B)) with K20 (C, G (a higher magnification of C)) is detected only in Merkel cells (D, H (a higher magnification of D)). Basal keratinocytes are only positive for K8-18 (D, H). Bar: A-D=50μm, E-H=20μm.

In oral mucosa epithelium diseased by lichen ruber planus, Merkel cells at the basal layer were positive for K8-18 (Fig. 7 B, F). Merkel cell marker K20 was co-localized with K8-18. Merkel cells were destructed (Fig. 7 D, H). In the lamina propria, numerous inflamed cells were positive for DRAQ5. In all lichen ruber planus cases, inflammation was observed in the oral mucosa epithelium (Fig. 7 A, E).
Figure 7. Confocal double immunofluorescence localization of K8-18 with K20 in cells of human oral mucosa diseased with lichen ruber planus. DRAQ5 was localized in nuclei of oral mucosa epithelium and of numerous inflammatory cells at lamina propria (A, E (a higher magnification of A)). Colocalization of K8-18 (B, F (a higher magnification of B)) with K20 (C, G (a higher magnification of C)) was detected in Merkel cells (D, H (a higher magnification of D)). Bar: A-D=50μm, E-H=20 μm

In numerous Merkel cells of leukoplakia diseased epithelium, K8-18 (Fig. 8 B, F) and K20 (Fig. 8 C, G) were co-localized subcellular in the membrane as well as in the cytoplasm of Merkel cells (Fig. 8 D, H). In sections of leukoplakia, Merkel cells were destructed. Rete pegs were not identified.

Figure 8. Confocal double immunofluorescence localization of K8-18 with K20 in cells of human oral mucosa diseased with leukoplakia. In leukoplakia diseased oral mucosa epithelium, DRAQ5 was identified in numerous nuclei with large and round form especially at the basal layer (A, E (a higher magnification of A)). In numerous Merkel cells of Leukoplakia diseased epithelium, K8-18 ((B, F (a higher magnification of B)) and K20 (C, G (a higher magnification of C)) were co-localized (D, H (a higher magnification of D)). Bar: A-D=50 μm, E-H=20 μm
5.4.2 Confocal double immunofluorescence localization of K20 with NF200 in cells of the lamina propria of healthy human oral mucosa.

NF200 was detected in numerous nerve fiber bundles, in nerve fibers and in nerve endings in the lamina propria of healthy palatal mucosa (Fig. 9 B, F). Nerve fibers were frequently detected around blood vessels distributed in the lamina propria. In some cases there was no relationship between nerve fibers and blood vessels. In different cells of the lamina propria of healthy palatal mucosa, Merkel cell marker K20 was not identified (Fig. 9 C, G).

![Images of Confocal double immunofluorescence localization of NF200 with K20 in cells of the lamina propria of human healthy oral mucosa.](image)

Figure 9. Confocal double immunofluorescence localization of NF200 with K20 in cells of the lamina propria of human healthy oral mucosa. DRAQ5 was localized in nuclei of cells of the lamina propria (A, E). Nerve fibers of the lamina propria were positive for NF200 (B, F). K20 was not detectable in cells of the lamina propria of healthy human oral mucosa (C, G). Bar: A-H=20μm.

5.4.3 Confocal double immunofluorescence localization of K20 with NF200 in cells of healthy human oral mucosa

The myelinated A-nerve fibers marker NF200 was detected in nerve fibers of the lamina propria (Fig. 10 B). Merkel cell marker K20 was identified in Merkel cells located in the basal layer of palatal healthy oral mucosa epithelium (Fig. 10 C). NF200 positive myelinated nerve fibers and nerve endings were detected with close relationship to Merkel cells (Fig. 10 D). The three fluorochrome images (DRAQ5 (blue), nerve fibers (green) and Merkel cell (red)) of the 6 μm depth z-series were merged at 1 μm interval into a single image shown in Figure 11 from A to F.
Figure 10. Confocal double immunofluorescence localization of NF200 with K 20 in cells of the healthy human oral mucosa. DRAQ5 was localized in nuclei of cells of the oral mucosa (A, D). Nerve fibers distributed in the lamina propria of the oral mucosa were positive for NF200 (B, D). K20 was localized in Merkel cells which were closely associated with NF200-positive nerve fibers and nerve endings at the basal layer of the healthy human oral mucosa (C, D). Bar: A-D=50 μm.

Figure 11. Confocal double immunofluorescence localization of NF200 in nerve fibers and nerve endings with a relationship to the K 20 positive Merkel cells of healthy human oral mucosa. DRAQ5 (blue) was localized in nuclei (A-F) of cells of the oral mucosa. Nerve fibers (green) were positive for NF200 (A-F). K20 (red) was localized in Merkel cells, which were closely associated with NF200 immunoreactive nerve fibers and nerve endings. The 6 (A-F) confocal images in a region of Fig. 9 at 1 μm intervals (z-step) throughout the depth (6 μm of the 30 μm thick section) of the section were collected for each fluorochrome (DRAQ5, blue; NF200, green; K20, red) at each z-step. Bar: A-F=20 μm.
In close relationship to the several Merkel cells, nerve fibers and nerve endings were detected by NF200 (Fig. 12 D). The three fluorochrome images (DRAQ5 (chromosome staining, blue), nerve fibers and nerve endings staining (green) and Merkel cell staining (red) of the confocal microscopy were merged at 1 μm interval into a single image at the 6 μm depth z-series shown from A to F in Figure 13.

![Figure 12. Confocal double immunofluorescence localization of NF200 in thick nerve fibers with a close relationship to the K 20 positive Merkel cells of healthy human oral mucosa. DRAQ5 was localized in cell nuclei of the oral mucosa epithelium and lamina propria (A, D). The NF200 (green) positive thick nerve fibers (B, D) were distributed in the lamina propria and with close relationship to the numerous K20 (red) positive Merkel cells (C, D) at the basal layer of the epithelium. Bar: A-D=50 μm.](image-url)
Figure 13. Confocal double immunofluorescence localization of NF200 in thick nerve endings with a relationship to the K20 positive Merkel cells of healthy human oral mucosa. DRAQ5 (blue) was localized in cell nuclei (A-F) of the healthy human oral mucosa. The thick nerve fibers (green) were positive for NF200 (A-F) with a close relationship to the numerous K20 (red) positive Merkel cells at the basal layer of the oral mucosa. The 6 (A-F) confocal images in a region of Fig. 10 at 1 μm intervals (z-step) throughout the depth (6 μm of the 30 μm thick section) of the section were collected for each fluorochrome (DRAQ5, blue; NF200, green; K20, red) at each z-step. Bar: A-F=20 μm.

5.4.4 Confocal double immunofluorescence localization of K20 with NF200 in cells of human oral mucosa diseased with the lichen ruber planus

In all cases of lichen ruber planus, chronic inflammatory cells were abundantly observed. Depending on the degree of inflammation, there were destructive changes in epithelial cells at the stratum basalis. In Merkel cells at the basal layer, K20 was detected (Fig. 14 C, G). In sections of all lichen ruber planus samples, K20 was detected in destructed and degraded Merkel cells with different sizes and different forms. In one single case of oral mucosa epithelium diseased with lichen ruber planus, NF200 was detected only in a few nerve endings and nerve fibers (Fig. 14 B, F). These nerve fibers revealed no relationship to the K20 positive Merkel cells at the basal layer of the oral mucosa epithelium (Fig. 14 D, H).
Figure 14. Double immunofluorescence localization of NF200 with K 20 in cells of the human oral mucosa diseased with lichen ruber planus. DRAQ5 was localized in nuclei of epithelial cells and in inflammatory cells (A, E (a higher magnification of A)). NF200 was detected only in the single cross-sectioned nerve fibers of the lichen ruber planus diseased oral mucosa (B, F (a higher magnification of B)). There was no relationship between K20 positive Merkel cells (C, G (a higher magnification of C) and a few of NF200 positive nerve fibers (D, H (a higher magnification of D). Bar: A-H=50µm.

5.4.5  Confocal double immunofluorescence localization of K 20 with NF200 in cells of human oral mucosa diseased with the leukoplakia

In the case of leukoplakia diseased oral mucosa epithelium, nerve fibers or nerve endings could not be detected in all sections (Table 10) by NF200 staining (Fig. 15 B, F). There were only few nerve fibers which were detected by a weak staining intensity only in the lamina propria.

In oral mucosa epithelium diseased with leukoplakia, especially at the basal layer of the oral mucosa epithelium, DRAQ5 positive nuclei of keratinocytes were identified in large and round form (Fig. 15 A, E) compared to the ovale form of the nuclei form of healthy oral mucosa epithelium. Similarly to the keratinocytes, Merkel cells were also detected with large and round forms of nuclei (Fig. 15 C, G). Merkel cells revealed dysplasia related changes in different sizes and in higher degradation levels. In comparison to lichen ruber planus, in leukoplakia, loss of nerve fibers in the oral mucosa epithelium was higher (Fig. 15 B, F).
Figure 15. Double immunofluorescence analysis of NF200 with K20 in cells of the human oral mucosa diseased with leukoplakia. DRAQ5 was localized in nuclei of oral mucosa epithelium (A, E (a higher magnification of A)). Note the presence of large cell nuclei in the basal epithelial keratinocytes (A, E). There was no staining for NF200 in nerve fibers (B, F (a higher magnification of B)). K20 was detected in Merkel cells (C, G (a higher magnification of C)). Note the absence of nerve fibers around Merkel cells (D, H (a higher magnification of D)). Bar: A-D=20 μm.

5.4.6 Immunohistochemical controls of double immunofluorescence

In confocal double immunohistochemical control incubations, cells were detected by staining of DRAQ5 in nuclei of all cells (Fig. 16 A, E). In control sections, immunohistochemical control incubations revealed no staining by secondary antibodies (DyeLight 488-coupled anti-mouse IgG and DyeLight 549-coupled anti-rabbit IgG) directed against the first and second primary antibodies, when incubations were performed without first (mouse anti-CK8-18, mouse anti-NF200) or second (rabbit anti-CK20) primary antibodies (Fig. 16 B, C, F, G).
Figure 16. Double immunofluorescence control incubations. In a section of a lichen ruber planus sample, DRAQ5 was localized in nuclei of oral mucosa epithelial keratinocytes but also in numerous inflammatory cells at the lamina propria (A, E (a higher magnification of A)). In incubations without primary antibodies but with DyeLight 488-conjugated anti-mouse IgG (B, F (a higher magnification of B)) and DyeLight 549-conjugated anti-rabbit IgG) (C, G (a higher magnification of C)), an immuno-histochemical signal was not detected. In merge, only DRAQ5 positive cell nuclei were detected (D, H (a higher magnification of D)). Bar:A-H=50μm.

5.5 Relationship between oral electric field strengths and impairment of nerve endings

Statistical analysis (Fisher’s exact test) revealed significant associations between the clinical diagnosis of leukoplakia/lichen ruber planus and the absence of free nerve endings (p=0.005). Furthermore, t-test showed significant associations for the absence of free nerve endings in leukoplakia and controls (p=0.005) as well as for lichen ruber planus and controls (p=0.048). There was no statistical difference between the occurrence of free nerve endings in cases of leukoplakia versus lichen ruber planus (p=1.00) (Table 11).

To determine, whether oral electric field strengths in normal (n=4), lichen ruber planus (n=6) and in leukoplakia (n=6) have effects on the occurrence and relationship between nerve endings and Merkel cells, we performed double immunofluorescence experiments using K20 (marker for Merkel cells) and NF200 (marker for nerve endings) (Fig. 10, 11, 12, 13, 14, 15).
In all 4 normal sections, nerve endings were detected with a close relationship to the Merkel cells (Fig. 10, 11, 12, 13). In five sections of lichen ruber planus, Merkel cells were detected without any relationship to nerve endings. In lichen ruber planus sections, only one nerve ending was detected intraepithelially but without a relationship to the Merkel cells (Fig.14). Single nerve fibers were detected in lamina propria in lichen ruber planus (Fig.17).

In all leukoplakia sections, NF200 immunoreactive intraepithelial nerve endings were not identified (Fig.15). In all sections of lichen ruber planus and leukoplakia, Merkel cells were detected by K20 immunoreactivity at the basal layer of oral mucosa (Fig.17).

![Figure 17. Statistical analysis of relationship between oral electric field strengths and impairment of the nerve endings. Box-plot of electric field strength by diagnosis with overlaid individual values (box represents 25th, 50th and 75th percentiles, whiskers extend to minimum and maximum). The association of electric field strength and absence of free nerve endings is positive (i.e. higher field strength goes along with absent free nerve endings), however not statistically significant (odds ratio 1.06, 95% confidence interval 0.94 to 1.19, p=0.362, logistic regression).](image-url)
6 Discussion

In the present study the following new results were found: (i) in general, there is a relationship between oral electric field strength and Lichen ruber planus and especially Leukoplakia, but these effects specially could not be observed in nerve fibers and in nerve endings related with Merkel cells and were abolished in cases of lichen ruber planus and in leukoplakia; (ii) there was a strong expression of NF200 (a marker for myelinated Aβ- and Aδ-fibers) in numerous nerve fibers and nerve endings of healthy palatal oral mucosa, while lichen ruber planus and leukoplakia induced a complete loss or a strong decrease in expression of NF200 in nerve fibers of the oral mucosa; (iii) K20 (a marker for Merkel cells) immuno-reactive Merkel cells revealed a close relationship with or connection to the NF200 positive nerve endings in healthy palatal oral mucosa; (iv) lichen ruber planus induced an abolishment of nerve fibers and nerve endings in the oral mucosa and around Merkel cells and (v) leukoplakia induced an abolishment of the nerve fibers and nerve endings connected to Merkel cells.

In addition to other factors, such as tobacco, smoking and alcohol, which are responsible for the development of a lichen ruber planus and leukoplakia, the results of the present study indicated that electric field strength should be considered as an important factor for lichen ruber planus and especially for leukoplakia in oral mucosa. However, in the cases of lichen ruber planus and leukoplakia, for the loss of relationship between nerve endings and Merkel cells, the galvanic electric potential could not be detected as a direct reason.

In the abolishment of a relationship between nerve endings and Merkel cells, inflammation in the case of lichen ruber planus and dysplasia in the case of leukoplakia could be involved in degradation of nerve fibers and nerve endings which are connected to Merkel cells in healthy oral mucosa.
6.1 Methodical considerations of the immuno-histochemical techniques

The immuno-histochemical localization of pan-K, K8-18, K20 in Merkel cells and NF200 in nerve fibers and nerve endings of oral mucosa indicates that epitopes recognized by this antibody are resistant to paraffin embedding of tissue sections. However, for the performed incubations in the present study, blocking of the endogenous peroxidase, antigen retrieval, blocking of unspecific binding sites and optimal concentration of the chromogen substrate (DAB) required experiences which must be used in the immuno-histochemical incubations for the detection of the specific antigen-antibody binding sites.

6.2 Blocking of endogenous peroxidase activity

In tissue sections, cells contain high endogenous peroxidase activity. Antigen-primary antibody-secondary antibody bind avidin-biotin-peroxidase-complex and this peroxidase is visualized using 3,3' diaminobenzidine tetrahydrochloride (DAB) [34]. Therefore, in addition to the avidin-biotin-peroxidase complex, it is possible that DAB binds to normal cell endogenous peroxidase and reveals unspecific staining. For elimination of tissue endogenous peroxidase activity, sections were first incubated with a solution containing 0.3 % H$_2$O$_2$ in 0.1 M TBS in start step of the immuno-histochemical incubations [37].

6.3 Antigen retrieval using Proteinase K and Triton X-100

Tissues were immersion-fixed using 4% paraformaldehyde. Although paraformaldehyde facilitates a very good tissue fixation with preservation of tissue and cell architecture, paraformaldehyde can also influence the structure of proteins (antigens) masking the epitope/s where antibodies bind. To set these antibody binding sites (epitope/s) free, tissue sections should be treated with a proteolytic enzyme. Thus, the binding sites for an antibody may be reconstituted (retrieved). Therefore, in the present study, Proteinase K was used for proteolytic induced epitope retrieval.
In addition, to improve antibody penetration, Triton X-100 was used. The paraffin sections revealed better results by using Triton X-100 as a permeabilization reagent. In Triton X-100 treated sections a reduced background was observed [37]. Triton X-100 treated sections revealed different cells in an oral mucosa cell architecture using light microscopy and confocal double immuno-fluorescence [34, 37].

6.4. **Blocking of free unspecific binding sites for immunohistochemical reagents**

In tissue sections, there are free bindings sites which must be saturated to prevent the unspecific binding of immuno-histochemical reagents including antibodies and avidin-biotin-peroxidase complex solution [33, 36,35,38]. In the present study, the unspecific binding sites in the sections for immuno-histochemical reagents were blocked by 2% bovine serum albumine (BSA)[33, 36]. Due to saturation of the unspecific binding sites in the tissue section using 2% BSA, in immuno-histochemical results a strongly reduced background and a specific stained immuno-histochemical reaction was observed [35, 38].

In matrix albumin were found in high concentrations. This matrix may have effects on the binding of an antibody to the specific binding sites. There are albumin sensitive antibodies which may require albumine for their binding to their specific epitope/s. In addition to the saturation of unspecific binding sites in the matrix, albumine also facilitates simultaneously a strong binding of an antibody to its specific epitope/s.

6.5 **Blocking of free unspecific binding sites for secondary antibodies**

In the immuno-histochemical incubations of the present study, secondary antibodies were used which were made in goat. The secondary antibodies against primary antibodies are polyclonal antibodies and can recognize various epitopes possibly occurring in the tissue sections. Therefore, in all
immuno-histochemical incubations normal goat serum was used as blocking solution to block possible unspecific binding sites of the secondary antibody [33, 36, 35, 38].

6.6 Visualization of specific immuno-histochemical binding sites with substrate chromogen reaction

The staining of the antigen-antibody-peroxidase complex was developed by 10 mg of DAB chromogen substrate solution dissolved in 50 ml 0.05 M Tris-HCl pH 7.6. The antigen-antibody reaction sites were visualized by 3,3'-diaminobenzidine tetrahydrochloride with a brown staining. The intensity of the 3,3'-diaminobenzidine tetrahydrochloride staining was enhanced by use of 0.01% nickel ammonium sulfate resulting in a dark brown staining. These DAB-substrate concentrations was found with numerous experiments and were considered optimal due to strongly reduced background with clearly stained specific binding sites for the antibodies which were quantitative [33, 36, 35, 38].

6.7 There is a cell type dependent specific effect of oral electric field strength on the lichen ruber planus and leukoplakia

The present study showed that oral electric field strengths of oral mucosa lesion with lichen ruber planus (12.62 ± 06.74; n=10) was significantly higher (p=0.009) in comparison to the healthy oral mucosa electric field strengths (01.80 ± 0.91; n=10) (5.1, 5.5). This finding revealed that higher electric field strengths are involved in the pathogenesis of lichen ruber planus, corresponding with the results of Korraah et al. [39], Yotova a., 2013 [82].

In comparison to the oral electric field strengths of the healthy samples (01.80 ± 0.91; n=10), the oral electric field strengths of the leukoplakia (18.18 ± 10.93; n=10) was significantly higher (p=0.000) (s 5.1). These data indicated that higher electric field strengths were strongly associated with the pathogenesis of the precancerous lesion leukoplakia [39, 82].

55
In addition, the statistical data of the present study revealed that oral electric field strengths of the lichen ruber planus (12.62 ± 06.74; n=10) was lower measured in comparison to the measurements of leukoplakia (18.18 ± 10.93; n=10). However, this value was found not significant (p=0.319) (s 5.1, 5.5).

There is an association between absence of nerve endings and diagnosis of lichen ruber planus and leukoplakia (p=0.005, Fisher’s exact test; leukoplakia vs. healthy p=0.005, lichen ruber planus vs. healthy p=0.048, leukoplakia vs. lichen ruber planus =1) (Fig.17; s 5.5). However, existence of intraepithelial nerve endings was observed in one case of lichen ruber planus. Therefore, it was assumed that the small sample of immunohistological-evaluated patients (i.e. 6 leukoplakia, 6 lichen ruber planus, 4 healthy) does not differ in a systematic way from the complete collective.

In statistical analysis of the present data, an association of electric field strength and absence of free nerve endings was positive (Fig.17). In one case nerve endings were detected intraepithelially at the basal layer. Tendency for an association between electric field strengths and absence of nerve endings was not found with a statistical significance (odds ratio 1.06, 95% confidence interval 0.94 to 1.19, p=0.362, logistic regression)(Fig.17; 5.5), presumably due to the small sample size.

In the present study from one patient single nerve endings were detected intraepithelially (Fig.14, 17; s 5.5). Due to small sample sizes investigated cannot be concluded that there is an association between higher oral electric field strengths and absence of nerve endings distributed around Merkel cells. Therefore, additional investigations are required for the suggestion whether electric field strengths exert its effects in a non-cell-specific or in a cell-specific manner. This should be clarified in future experiments.

The data of the present study confirmed early results that higher electric field strengths were associated with cellular changes in keratinocytes-like
cells [39]. In these data it was determined specifically in in vitro experiments that higher electric field strengths induced cell proliferation in MSK-LEUK1 cell lines, which have characteristics of premalignant keratinocytes [39]. This study showed that these changes are associated with lesions like oral squamous cell carcinoma [39]. Similarly, in lichen ruber planus and leukoplakia an important role of higher oral electric field strengths was demonstrated with in vivo experiments with histological changes of keratinocytes [82]. In the present study, the effect/s of electric fields on the other cell population such as axons and Merkel cells were investigated (a neuroendocrine cell) for the first time with in vivo experiments using immunohistochemical methods. In comparison to the healthy samples, in lower (7.9, 5.83 V/m) as well as in higher (31.00, 33.00 V/m) strengths of electric fields was detected that in lichen ruber planus and leukoplakia the physiological relationship between nerve endings and Merkel cells is destroyed (Fig. 17). It is possible that oral electric field strength may have different effects on the different cell populations of oral mucosa in a cell type specific manner.

The oral mucosa contains different cells such as keratinocytes, melanocytes, Langerhans cells, nerve fibers, blood vessels, fibroblasts and stroma cells [39]. Between these cells, there are numerous cell-cell and cell-matrix interactions. In earlier data was detected that cells like premalignant keratinocytes are influenced by oral electric field strength [39]. It is possible that higher oral electric field strengths primarily induced changes in specific cell types such as keratinocytes [39] but not direct on the nerve fibers and on the Merkel cells which considered as a neuroendocrine cell. Thus it is possible that a physiological interaction between keratinocytes and Merkel cells was destroyed due to effects of electric field strengths on the keratinocytes indirectly. It is possible that degraded or changed keratinocytes cannot influence Merkel cells or cannot respond to the effects of Merkel cells. Due to missing cell-cell contacts, Merkel cells may be also changed pathologically. This should be investigated in in vivo and in vitro future experiments.
It should be emphasized that due to pathological changes in the biological behaviour of cells of the oral mucosa epithelium associated with higher electric field strengths, reasons responsible for different galvanic electric field strengths in the oral mucosa must be considered during dental treatment.

6.8 Lichen ruber planus and leukoplakia-dependent loss of nerve endings and destruction of Merkel cells at the buccal mucosa epithelium

Merkel cells are required for touch sensitivity [17, 47, 80]. There are evidences [26, 53,39], which support the notion that Merkel cells-neurite complex unit is required for an intact mechano-sensory touch signaling. It is therefore possible that deletion of a component (Merkel cell or nerve endings) from the Merkel cells-neurite complex unit results in loss of touch signaling perception in oral mucosa epithelium. For example, by enzymatic treatment, photoablation or knockout mouse model, Merkel cells were removed. The removal of Merkel cells induces an abolishment of the responses of slowly adapting afferents [26, 53, 32]. These results support the notion that Merkel cells are required for the Merkel cell-neurite complex mediated mechano-sensation in the oral mucosa.

In comparison to the results of healthy oral mucosa epithelium, the absence of Aβ- and Aδ-nerve fibers and nerve endings (NF200 is a marker of the myelinated Aβ- and Aδ-nerve fibers) in oral mucosa diseased with lichen ruber planus and leukoplakia (Fig 14, 15; s 5.4.4, 5.4.5), is compatible with the notion that the differentiation of keratinocytes, Langerhans cells and melanocytes can not be regulated and modulated by neuropeptides substance P (substance P) and calcitonin gene-related peptide (CGRP) in the oral mucosa diseased with lichen ruber planus and leukoplakia.

In the in vivo findings of the present study, deletion of nerve endings in the Merkel cells-neurite complex unit in lichen ruber planus and leukoplakia may result in a loss of Merkel cells-neurite complex-dependent keratinocytes proliferation and differentiation and loss of Merkel cells-
neurite complex-dependent perception of the touch signaling in the oral mucosa epithelium (Fig. 4, 14, 15), (s. 5.3.3, 5.4.4, 5.4.5).

6.9 Lichen ruber planus and leukoplakia-dependent loss of nerve endings at the buccal mucosa epithelial keratinocytes indicates impaired neuronal modulation of oral mucosa epithelial cells.

It is known that thickness of keratinocytes in the epidermis is modulated by a balance between growth and differentiation of keratinocytes [25]. Keratinocytes are regulated by proliferation and differentiation factors including retinoic acid, calcium, and various trophic factors, such as transforming growth factors [25]. Increased keratinization of the oral mucosa in patients with complete dentures was considered as a protective mechanism against mechanical irritation [69]. For a healthy placement of total prosthesis on the oral mucosa epithelium, a physiological thickness of the oral mucosa epithelium is necessary. Therefore, proliferation of keratinocytes at the basal membrane and differentiation of the keratinocytes in oral mucosa epithelial layers are important.

NF200 is a marker for myelinated sensory Aβ- and Aδ-nerve fibers. In the present study, the Aβ- and Aδ-nerve fibers and nerve endings were not detected in the oral mucosa epithelium diseased with lichen ruber planus and leukoplakia (Fig. 4)(s. 5.3.3). In normal palatal mucosa epithelium, Aβ- and Aδ-nerve fibers and nerve endings were detected intraepithelial with a close relationship to keratinocytes. It is known that keratinocyte proliferation is modulated by sensory nerve fibers and nerve endings [25]. This is compatible with the suggestion, that lichen ruber planus and leukoplakia-dependent loss of sensory nerve fibers or nerve endings at buccal mucosa keratinocytes can induce impairment of the neuronal modulation of oral keratinocytes.

In skin it was revealed that sensory nerve endings have close contact with keratinocytes [25]. In addition to the noxious stimuli transduction of the external stimuli to the central nervous system, there is evidence which
suggests that sensory nerve fibers influence keratinocytes (Kruger and Halata, 1996) and Langerhans cells [22, 3] by releasing sensory neuropeptides. In the oral mucosa, sensory nerve fibers contain sensory neuropeptides substance P and CGRP [49]. It is possible that the thickness of buccal mucosa epithelium could be regulated in differentiation of keratinocytes by sensory neuropeptides substance P, CGRP.

Lichen ruber planus and leukoplakia induce loss of nerve fibers and nerve endings in epithelium of the oral mucosa. This results in missing of the neuronal factors and neuropeptide in cells of the oral mucosa epithelium. It is therefore speculated that the proliferation and differentiation of the oral mucosa epithelial cells is impaired in cases of lichen ruber planus and leukoplakia. Loss of the sensory nerve endings at keratinocytes of the buccal mucosa diseased with lichen ruber planus and leukoplakia is compatible with the notion that neuropeptide dependent differentiation of the buccal oral mucosa keratinocytes and Langerhans cells is impaired. Because the oral mucosa epithelium represents the first line of defence against external environmental pathogens and noxious stimuli, in defence against noxious stimuli, a neuronal regulation of healthy cellular proliferation and differentiation (keratinocytes) and differentiation of various cells (melanocytes and Langerhans cells) in the oral mucosa epithelium is a prerequisite for protection of oral mucosa epithelium.

6.10 Lichen ruber planus and leukoplakia-dependent destruction of Merkel cells indicates impaired regulation and modulation of keratinocytes by Merkel cells.

In the present study, lichen ruber planus-dependent cellular changes in Merkel cells were observed. In comparison to results in healthy subjects, in lichen ruber planus, Merkel cells in different sizes were detected and some cells were observed in degraded non-physiologic architecture (Fig. 4). Some degraded cells were detected intra-epithelially. It is possible that Merkel cells were influenced by inflammatory mediators in the case of lichen ruber planus. In leukoplakia, Merkel cells revealed different sizes and nuclei of
cells were large compared to healthy Merkel cells. Therefore, in the present study it was speculated that leukoplakia-dependent dysplasia in keratinocytes may influence Merkel cells. However, this suggestion must be supported by further experiments.

In lichen ruber planus, loss of epithelial wide indicated that keratinocytes are destroyed in their terminal differentiation in the oral mucosa epithelium. In the thin epithelium of the lichen ruber planus, basal membrane destruction may have an important consequence. In all lichen ruber planus oral mucosa sections, the basal membrane was destructed and could not be observed. As a result of degradation of basal membrane by inflammatory cytokines, chemokines and other inflammatory mediators in the case of lichen ruber planus, the stem cells cannot proliferate. Therefore the terminal differentiation of keratinocytes cannot occur. The oral mucosa epithelium becomes thinner and thus it cannot function as a protective barrier against harmful irritants due to loss of normal physiological keratinization from basal layer up to stratum corneum of the oral mucosa epithelium.

In addition to the sensory transduction role of slowly adapting afferents, it was proposed that Merkel cell-neurite complexes are involved in innervation of the epithelial cells during development of mouse epidermis [60]. In the results of healthy oral mucosa epithelium, Merkel cell-neurite complex revealed a close relationship to the basal layer keratinocytes which may be involved in the regulation of keratinocytes at the basal layer of the oral mucosa epithelium. Changes in the Merkel cell phenotypes were detected in lichen ruber planus and leukoplakia and supported the suggestion that epithelial cells at the basal layer of the oral mucosa epithelium could be influenced by lichen ruber planus- and leukoplakia-dependent alterations in Merkel cells.
6.11 The close relationship between nerve endings and Merkel cells detected in healthy oral mucosa is not detectable in oral mucosa diseased with lichen ruber planus and leukoplakia.

It was shown in *in vitro* (Suzuki et al., 2005) and *in vivo* [47] experiments that there is a close relationship between nerve endings and Merkel cells. In the present study, a close relationship between NF200 (a marker for myelinated A-nerve fibers) positive nerve endings and K20 (a marker for Merkel cells) positive Merkel cells was demonstrated using double immunohistochemistry by confocal microscopy (Fig. 10, 11, 12, 13) (s. 5.4.3). Thus, a physiological touch perception exists in the oral mucosa epithelium of the healthy oral mucosa.

The effects of lichen ruber planus and leukoplakia on the relationship between nerve endings and Merkel cells are unknown. The results of the present study revealed that in the oral mucosa diseased with lichen ruber planus the relationship between nerve endings and Merkel cells is destroyed by loss of NF200-positive nerve fibers and nerve endings. In the case of leukoplakia the mechano-sensation by Merkel cells-neurite complex in diseased oral mucosa is impaired due to loss of nerve fibers and nerve endings. In the oral mucosa epithelium, a healthy perception is a prerequisite for protection of oral mucosa from harmful stretch and pressure stimuli. The results of the present study revealed that the protective role of oral mechano-sensation is impaired by lichen ruber planus and leukoplakia.

6.12 K20 positive Merkel cells are associated with the NF200 immunoreactive nerve fibers and nerve endings in healthy oral mucosa.

The mechano-sensory stimulus touch is mediated by mechano-sensory cells which transduce pressure or stretch into electrical signals [15, 80]. The Merkel cell-neurite complex is a touch receptor and is considered as a model for studying mechano-sensory signaling [15, 80]. The Merkel cell-neurite complex mediates slowly adapting type I responses [39] and the
somatosensory nerve endings contact Merkel cells that activate sensory afferent endings [15].

In the healthy oral palatal mucosa results of the present study, a close relationship between Merkel cells and nerve endings supported the idea that Merkel cells are mechano-sensory cells and activate Aβ-sensory afferent endings (NF200 is a marker for myelinated Aβ- and Aδ-sensory nerve fibers). The results of healthy oral mucosa epithelium revealed that touch stimuli were perceived by Merkel cell-neurite complex in the healthy oral mucosa epithelium.

6.13 Lichen ruber planus induced an abolishment of nerve fibers and nerve endings associated with Merkel cells.

In the present study it was found that lichen ruber planus induced loss in relationship between Merkel cells and afferent myelinated Aβ-nerve fiber ending (Fig. 4, 7). Thus, a physiological mechano-sensation by Merkel cells is abolished due to T-cell dependent inflammation by lichen ruber planus. These changes are associated with loss of NF200 positive nerve fibers and nerve endings (Aβ-nerve fibers) related with Merkel cells, in lamina propria, in the basal layer and intraepithelial. Due to loss of mechano-sensation, the oral mucosa epithelium cannot be considered as a protective barrier against unphysiological touch stimuli which may induce tissue damages.

The mechanism of impairments in mechano-sensation, inflammation, injury and in diseases was reported [39], however, the mechanism/s responsible for loss of nerve fibers in lichen ruber planus is unknown. It is possible that the nerve fibers and nerve endings may be degraded by inflammatory mediators released by different cells in lichen ruber planus associated T-cell infiltration.

The loss of neuronal sensation in oral mucosa epithelium may also be associated with the destruction of neurotransmission modulating normal cellular interactions. The neuronal sensation is required for different cell
functions such as cell proliferation and cell differentiation in the oral mucosa. In the present study the oral mucosa epithelium was detected as thin in comparison to the healthy epithelium thickness. This is compatible with the suggestion that the terminal differentiation of keratinocytes in oral mucosa epithelium diseased with lichen ruber planus is impaired. Lichen ruber planus induces defects in the terminal differentiation of keratinocytes. Therefore, in the case of lichen ruber planus, the oral mucosa epithelium cannot be considered as a protective barrier against harmful stimuli.

6.14 Leukoplakia induced an abolishment of nerve endings associated with Merkel cells.

There are several results of studies which supported the notion that Merkel cells are sensory receptor cells transmitting touch signals through synaptic contacts to nerve endings of somatosensory neurons. For example, Merkel cells contain dense-core vesicles that resemble neurosecretory vesicles [20]. In addition, Merkel cell-neurite complexes have membrane densities at synaptic active zones [52]. It was found that Merkel cells express the molecular tools to send excitatory as well as modulatory signals to sensory neurons [15]. In Merkel cells, expressions of neuronal transcriptional factors, voltage-gated ion channels (voltage-gated K+-channel subunits) and synaptic proteins were also demonstrated [15].

Leukoplakia may destroy the relationship between Merkel cells and afferent myelinated Aβ-nerve fibers. In comparison to lichen ruber planus, this disorder can occur especially in leukoplakia (Fig. 4, 15). In leukoplakia, dysplasia occurs in cells of the basal layer of the oral mucosa epithelium. The pathological changes in keratinocytes and Merkel cells are associated with loss of NF200 positive nerve fibers and nerve endings around Merkel cells. In Merkel cell sizes and forms there are differences which suggest that Merkel cells may be influenced by dysplastic cell changes at the basal cell layer.
Loss of nerve fibers and nerve endings can be triggered by leukoplakia-dependent-dysplasia changes in the oral mucosa epithelium. In dysplasia of the oral mucosa epithelium, it is possible, that the degradation of nerve fibers may take place by autophagy and neuro-degeneration. It is also known that autophagy is associated with axonal dystrophy, axonopathy [9] and axonal degeneration[77]. However, in the oral mucosa epithelium, future experiments are required to understand the molecular mechanisms of nerve fibers degradation in leukoplakia.

In normal palatal oral mucosa epithelium, Merkel cells are distributed in an orderly fashion at the basal layer of the oral mucosa epithelium. In oral mucosa of leukoplakia, Merkel cells are distributed intra-epithelially with different sizes in a disorderly manner. In some cases leukoplakia was associated with inflammation in the lamina propria and in the basal layer of the epithelium. It is possible that some Merkel cells may be degraded by dysplasia and inflammation. In addition to the dysplasia-dependent nuclear changes in keratinocytes and Merkel cells, inflammatory mediators could exert additional effects on the cell population of the oral mucosa especially at the basal layer. Loss of neuronal mechano-sensation in oral mucosa epithelium may be associated with destruction of neuro-transmission which is required for neuronal regulation of different cell functions such as cell proliferation and cell differentiation.

6.15 Clinical relevance of the results

In lichen ruber planus and especially in leukoplakia, the pathological changes in cells of the oral mucosa epithelium are associated with higher electrical field strength. Oral mucosa of patients wearing prosthesis or fillings with different dental metals, should be frequently checked, whether in the oral mucosa of this patient, especially in the buccal mucosa regions a lichen ruber planus or leukoplakia occurs. In these cases, in addition to the other possible reasons, it should be elicited, whether different dental metals of prosthesis and fillings are possible for lichen ruber planus and leukoplakia lesions. Therefore, reasons responsible for electrical field
strength in the oral mucosa should be considered during dental treatment. In the cases where different metals are used in prosthetic and filling treatment of teeth, new prosthetic and filling treatment should be planned and performed.

In addition to the keratinocytes with dysplasia appearances in the basal layer of the oral mucosa in leukoplakia, Merkel cells revealed also atypical changes in cell nuclei and cell membranes in different sizes and with intraepithelial distribution patterns in the basal layer of the human oral mucosa which could be associated with a malignant progress, such as Merkel cell carcinoma. Therefore, it should be also elicited by histopathological examination whether Merkel cells of the oral mucosa from leukoplakia patients may reveal malign progress properties, which may be specific for a Merkel cell carcinoma.

The regulation of the thickness of the oral mucosa epithelium by modulation of sensory nerve fibers and nerve endings in a balance between growth and differentiation of keratinocytes is very important. In the oral mucosa, the placement and the physiological occlusion of denture during mastication, a physiological thickness of oral mucosa epithelium modulated by an intact innervation is a requirement for a complete or partial denture in the oral mucosa epithelium.

In the oral mucosa epithelium, a healthy perception is a prerequisite for protection of the oral mucosa from harmful stretch and pressure stimuli which may be exerted by prosthetic treatment of teeth. To avoid non-physiological pressure places, in the design of a prosthetic treatment the healthy perception and sensitivity of the touch receptors in the oral mucosa are of major importance. Lichen ruber planus and leukoplakia induce loss in connection between Merkel cells and afferent myelinated Aβ-nerve fiber endings. Thus a mechano-sensation regulated by Merkel cells is abolished due to lichen ruber planus and leukoplakia effects. It is therefore important for the dental clinic that a prosthetic treatment should only be planned and performed after treatment of the lichen ruber planus and leukoplakia.
6.16 Conclusion

The oral mucosa epithelium protects different sub-epithelial cells against harmful chemical, thermal and mechanical irritants. Under effects of these harmful irritants, cells of the oral mucosa epithelium reveal different changes in dependence of inflammation, degeneration or hyperplasia. The neuronal perception is strongly influenced by lichen ruber planus and leukoplakia. These inflammation- (lichen ruber planus) and dysplasia (leukoplakia)-dependent cellular changes and tissue damages induce loss of physiological protection of oral mucosa epithelium against injurious irritants which may benefit pre-malign or malign lesions.

In the present study, it was established that there is a relationship between oral electric field strength with lichen ruber planus and especially with leukoplakia. The lichen ruber planus and leukoplakia induce abolishment of a relationship between Merkel cells and afferent myelinated Aβ-nerve endings inducing a deletion of mechano-sensation in the oral mucosa epithelium.

It was concluded that innervation-dependent regulation of the epithelial thickness may be impaired by loss of nerve fibers and nerve endings. Because the perception of the touch receptors is also impaired in lichen ruber planus and leukoplakia, in the prosthetic treatment of teeth, the whole oral cavity, clinically, especially buccal mucosa must be observed and examined. In existence of lichen ruber planus and leukoplakia, these lesions must be treated beforehand. Then a prosthetic treatment should be planned and performed, since a healthy innervation and mechano-sensation is a prerequisite for protection of the oral mucosa epithelium in its physiological thickness by terminal differentiation of keratinocytes against the harmful stretch and pressure stimuli which may be exerted by dental therapies.
Summary

The healthy oral mucosa epithelium is innervated by Aβ, Aδ, and C nerve fibers and nerve endings. The sensation of touch is mediated by Aβ fibers. Aβ fibers are subdivided into three groups: slowly adapting nerve fibers type I, and type II and a rapidly adapting nerve fibers type. Merkel cell-neurite complexes are somatosensory receptors that initiate the perception of touch and mediate slowly adapting type I responses. In addition, the Merkel cell-neurite complex is involved in regulation of proliferation and differentiation of keratinocytes. The sensory neuropeptide substance P and CGRP released by Aβ-, Aδ- and C-nerve fibers are also involved in the modulation of terminal differentiation of keratinocytes, melanocytes and in the maturation of the Langerhans cells.

Lichen ruber planus is a chronic inflammatory autoimmune disease with T-cells-mediated inflammation in the oral mucosa, while oral leukoplakia is characterized with epithelial hyperplasia, hyperkeratosis, and hyperparakeratosis, with or without epithelial dysplasia. The effects of lichen ruber planus and leukoplakia on the Merkel cells, Merkel cells-neurite complexes, and nerve endings which release several neuropeptides are not known.

In the present study, the relationship between oral electrical field strength with lichen ruber planus (n= 10) and with leukoplakia (n=12) was measured and compared in patients groups. Merkel cell-neurite complex was characterized and compared in healthy and diseased oral mucosa sections by immuno-histochemical avidin-biotin-peroxidase complex and double confocal immunofluorescence methods using K20 (a marker for Merkel cells) and NF200 (a marker for myelinated Aβ- and Aδ-nerve fibers) antibodies. The results were tested and confirmed by incubations using pan-K and K8-18 antibodies.

The data revealed that there is a cell type-independent relationship between oral electric potentials and lichen ruber planus and leukoplakia. In healthy
oral mucosa, K20 positive Merkel cells were detected with a close relationship to the NF200 immuno-reactive nerve endings. Intraepithelial nerve endings were positive for NF200. In diseased sections, only Merkel cells were detected, while nerve endings were not identifiable. Lichen ruber planus and leukoplakia are associated with an impairment of intraepithelial free nerve endings.

The oral galvanic potential should be considered as a reason for lichen ruber planus and especially for leukoplakia. The regulation of keratinocyte proliferation and differentiation by Merkel cell-neurite complex is impaired in lichen ruber planus and leukoplakia. The differentiation of keratinocytes, melanocytes and maturation of the Langerhans cells by neuropeptide is deleted due to loss of intraepithelial free nerve endings in lichen ruber planus and leukoplakia. Finally, it was concluded that the disappearance of nerve endings to the Merkel cells results in loss of touch signaling perception in oral mucosa epithelium diseased with lichen ruber planus and leukoplakia.
Zusammenfassung


Lichen ruber planus ist eine chronische inflammatorische Autoimmunerkrankung mit T-Zell-mediierter Entzündung der oralen Mukosa, während Leukoplakie durch epitheliale Hyperplasie, Hyperkeratose und Hyperparakeratose charakterisiert ist. Die Effekte von Lichen ruber planus und Leukoplakie auf die Merkelzellen, Merkelzell-Axon-Komplexe und auf die freien Nervenendigungen, die mehrere sensorische Neuropeptide ausschütten, sind nicht bekannt.


References


10 Curriculum vitae

Persönliche Daten

Name: Daniela Janeth Calderón Carrión
Adresse: Lauro Guerrero 0857 y 10 de Agosto Loja-Ecuador
Telefon: 01742773727
e-mail-Adresse: danny_jcc@hotmail.com
Familienstand: ledig
Staatsangehörigkeit: ecuadorianisch
Geburtsdaten: 22. Oktober 1984 in Loja/Ecuador

Beruf

seit 07/2007 Zahnärztin

Schulische Ausbildung/Studium

2002 – 2007 Studium in der Zahnmedizin (Note 9/10)
Universidad Nacional de Loja; Loja-Ecuador
Titel der Diplomarbeit:
Zahnärztin

1996 – 2007 Abitur (Note 18 sehr gut)
Militärschule Oberstlt. Lauro Guerrero Loja Ecuador

Berufliche Erfahrungen

03.2009 - 11.2009 Zahnärztin beim Ecuadorianischen Institut für Sozialversicherung. Instituto Ecuatoriano de seguridad social (IESS).


Weiterbildung

01.2009 – 11.2009 Zahn Implantation San Francisco Universität Quito-Ecuador
Tables

Table 1. The electric field strength values measured in patients diseased with the lichen ruber planus.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease</th>
<th>V/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>lichen ruber planus</td>
<td>8,33</td>
</tr>
<tr>
<td>2</td>
<td>lichen ruber planus</td>
<td>11,00</td>
</tr>
<tr>
<td>3</td>
<td>lichen ruber planus</td>
<td>8,33</td>
</tr>
<tr>
<td>4</td>
<td>lichen ruber planus</td>
<td>12,33</td>
</tr>
<tr>
<td>5</td>
<td>lichen ruber planus</td>
<td>11,00</td>
</tr>
<tr>
<td>6</td>
<td>lichen ruber planus</td>
<td>11,00</td>
</tr>
<tr>
<td>7</td>
<td>lichen ruber planus</td>
<td>11,00</td>
</tr>
<tr>
<td>8</td>
<td>lichen ruber planus</td>
<td>14,33</td>
</tr>
<tr>
<td>9</td>
<td>lichen ruber planus</td>
<td>7,97</td>
</tr>
<tr>
<td>10</td>
<td>lichen ruber planus</td>
<td>31,00</td>
</tr>
</tbody>
</table>

Table 2. The electric field strength values measured in patients diseased with leukoplakia.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease</th>
<th>V/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>leukoplakia</td>
<td>8,33</td>
</tr>
<tr>
<td>2</td>
<td>leukoplakia</td>
<td>10,67</td>
</tr>
<tr>
<td>3</td>
<td>leukoplakia</td>
<td>18,67</td>
</tr>
<tr>
<td>4</td>
<td>leukoplakia</td>
<td>27,00</td>
</tr>
<tr>
<td>5</td>
<td>leukoplakia</td>
<td>30,00</td>
</tr>
<tr>
<td>6</td>
<td>leukoplakia</td>
<td>30,67</td>
</tr>
<tr>
<td>7</td>
<td>leukoplakia</td>
<td>33,00</td>
</tr>
<tr>
<td>8</td>
<td>leukoplakia</td>
<td>5,83</td>
</tr>
<tr>
<td>9</td>
<td>leukoplakia</td>
<td>5,83</td>
</tr>
<tr>
<td>10</td>
<td>leukoplakia</td>
<td>10,00</td>
</tr>
<tr>
<td>11</td>
<td>leukoplakia</td>
<td>7,73</td>
</tr>
</tbody>
</table>
Table 3. The electric field strength values measured in healthy patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Healthy</th>
<th>V/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>healthy</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>healthy</td>
<td>1.00</td>
</tr>
<tr>
<td>3</td>
<td>healthy</td>
<td>2.00</td>
</tr>
<tr>
<td>4</td>
<td>healthy</td>
<td>1.00</td>
</tr>
<tr>
<td>5</td>
<td>healthy</td>
<td>1.00</td>
</tr>
<tr>
<td>6</td>
<td>healthy</td>
<td>1.00</td>
</tr>
<tr>
<td>7</td>
<td>healthy</td>
<td>3.00</td>
</tr>
<tr>
<td>8</td>
<td>healthy</td>
<td>3.00</td>
</tr>
<tr>
<td>9</td>
<td>healthy</td>
<td>3.00</td>
</tr>
<tr>
<td>10</td>
<td>healthy</td>
<td>2.00</td>
</tr>
</tbody>
</table>

Table 4. Healthy patients with electrical field strength values. To compare effects of the electric field strength on the relationship between nerve endings and Merkel cells, double immunofluorescence incubations were performed in sections of following four normal tissue samples.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Healthy</th>
<th>V/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Healthy</td>
<td>3</td>
</tr>
<tr>
<td>P2</td>
<td>Healthy</td>
<td>3</td>
</tr>
<tr>
<td>P3</td>
<td>Healthy</td>
<td>4</td>
</tr>
<tr>
<td>P4</td>
<td>Healthy</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 5. Antibodies used in the immuno-histochemical incubations.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description</th>
<th>Firma</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-pan cytokeratin</td>
<td>Mouse, monoclonal</td>
<td>Vector, Saint Louis U.S.A</td>
<td>(1:400)</td>
</tr>
<tr>
<td>Anti-cytokeratin 8-18</td>
<td>Mouse, monoclonal</td>
<td>Abcam, Cambridge, UK</td>
<td>(1:50)</td>
</tr>
<tr>
<td>Anti-cytokeratin 20</td>
<td>Rabbit, monoclonal</td>
<td>Abcam, Cambridge, UK</td>
<td>(1:250)</td>
</tr>
<tr>
<td>Anti-200 kD neurofilament</td>
<td>Mouse, monoclonal</td>
<td>Sigma, Taukirchen, Germany</td>
<td>(1:1000)</td>
</tr>
</tbody>
</table>
Table 6. Lichen ruber planus patients with lowest and highest electrical field strength values. The double immunofluorescence incubations were performed in the listed sections of 3 patients with higher electric field strength measurements and in the listed sections of 3 patients with lower electric field strength measurements.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease</th>
<th>V/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>lichen ruber</td>
<td>7,97</td>
</tr>
<tr>
<td>P2</td>
<td>lichen ruber</td>
<td>8,33</td>
</tr>
<tr>
<td>P3</td>
<td>lichen ruber</td>
<td>8,33</td>
</tr>
<tr>
<td>P4</td>
<td>lichen ruber</td>
<td>12,33</td>
</tr>
<tr>
<td>P5</td>
<td>lichen ruber</td>
<td>14,33</td>
</tr>
<tr>
<td>P6</td>
<td>lichen ruber</td>
<td>31</td>
</tr>
</tbody>
</table>

Table 7. Leukoplakia patients with the lowest and highest electrical field strength values. The double immunofluorescence incubations were performed in the listed sections of 3 patients with higher electric field strength measurements and in the listed sections of 3 patients with lower electric field strength measurements.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease</th>
<th>V/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>leukoplakia</td>
<td>2,67</td>
</tr>
<tr>
<td>P2</td>
<td>leukoplakia</td>
<td>5,83</td>
</tr>
<tr>
<td>P3</td>
<td>leukoplakia</td>
<td>7,73</td>
</tr>
<tr>
<td>P4</td>
<td>leukoplakia</td>
<td>30</td>
</tr>
<tr>
<td>P5</td>
<td>leukoplakia</td>
<td>30,67</td>
</tr>
<tr>
<td>P6</td>
<td>leukoplakia</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 8. Statistical analysis of the galvanic electric field strength of healthy oral mucosa, lichen ruber planus and leukoplakia. Statistical differences in galvanic electric field strength values between healthy oral mucosa, lichen ruber planus, and leukoplakia, were analyzed using the two-tailed Student's t-test. One-way ANOVA with Bonferroni post hoc test was used to compare multiple means. Data are presented as mean ± SD. Statistical significance was considered at a p value ≤0.05

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Standard deviation</th>
<th>Standard error of the mean</th>
<th>95% Confidence interval mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>healthy</td>
<td>10</td>
<td>0,91894</td>
<td>0,29059</td>
<td>1,426</td>
</tr>
<tr>
<td>lichen ruber planus</td>
<td>10</td>
<td>6,74751</td>
<td>2,13375</td>
<td>7,8021</td>
</tr>
<tr>
<td>leukoplakia</td>
<td>10</td>
<td>10,93429</td>
<td>3,45773</td>
<td>10,3651</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>9,96955</td>
<td>1,82018</td>
<td>7,1493</td>
</tr>
</tbody>
</table>
Table 9. Statistical analysis of the galvanic electric field strength of healthy oral mucosa, lichen ruber planus and leukoplakia. The statistical differences in galvanic electric field strength values between healthy oral mucosa (n=10) and lichen ruber planus (n=10) and leukoplakia (n=10), were analyzed using the two-tailed Student's t-test. One-way ANOVA with Bonferroni post hoc test was used to compare multiple means. Data are presented as mean ± SD. Statistical significance was considered at a p value ≤0.05

<table>
<thead>
<tr>
<th>(J) Group</th>
<th>(J) Group</th>
<th>Standard deviation</th>
<th>Standard error</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lichen ruber planus</td>
<td>-10,8290*</td>
<td>3,32598</td>
<td>0.009</td>
</tr>
<tr>
<td>healthy</td>
<td>leukoplakia</td>
<td>-16,3870*</td>
<td>3,32598</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>healthy</td>
<td>10,8290*</td>
<td>3,32598</td>
<td>0.009</td>
</tr>
<tr>
<td>lichen ruber planus</td>
<td>leukoplakia</td>
<td>-5,55800</td>
<td>3,32598</td>
<td>0.319</td>
</tr>
<tr>
<td></td>
<td>healthy</td>
<td>16,3870*</td>
<td>3,32598</td>
<td>0.000</td>
</tr>
<tr>
<td>leukoplakia</td>
<td>Lichen ruber planus</td>
<td>5,55800</td>
<td>3,32598</td>
<td>0.319</td>
</tr>
</tbody>
</table>

Table 10. Patients with absence of nerves endings in higher and lower electric field strength.

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Lichen Ruber planus</th>
<th>Leukoplakia</th>
</tr>
</thead>
<tbody>
<tr>
<td>positiv</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>negativ</td>
<td>0</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 11. Statistical analysis of relationship between oral electric field strengths and nerve endings. The statistical association of diagnosis and absence of free nerve endings is highly significant (p=0.005, Fisher’s exact test; Lp vs. He p=0.005, Lrp vs. He p=0.048, Lp vs. Lrp=1).

<table>
<thead>
<tr>
<th>Free nerve endings * Diagnosis Crosstabulation</th>
<th>Diagnosis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lp</td>
<td>Lrp</td>
</tr>
<tr>
<td>Count</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>%within Diagnosis</td>
<td>100,0%</td>
<td>83,3%</td>
</tr>
<tr>
<td>Count</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>%within Diagnosis</td>
<td>0,0%</td>
<td>16,7%</td>
</tr>
<tr>
<td>Count</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>%within Diagnosis</td>
<td>100,0%</td>
<td>100,0%</td>
</tr>
</tbody>
</table>