Aim: To highlight the expression of vascular endothelial growth factor (VEGF) in human paraprosthesis gingival mucosa exposed to nickel and copper compounds using the immunohistochemical technique. The selected participants were wearers of fixed dentures made of nickel-based alloys and copper-based alloys. The gingival mucosa fragments were prelevated through excision after removing fixed denture and extraction one of its affected teeth. The gingival mucosa fragments were processed through the histological technique of paraffin inclusion. The paraffin-embedded tissue sections were usually stained with Hematoxylin–Eosin and processed by immunohistochemical technique with VEGF antibody. The gingival mucosa fragments from nickel-based alloys dentures wearers were diagnosed with papilloma and, also, gingival mucosa samples prelevated from copper-based alloys dentures wearers were diagnosed with condyloma acuminata. Immunohistochemical reaction for VEGF was different in the gingival mucosa fragments with papilloma compared with condyloma acuminata samples. In papillomatosis gingival mucosa fragments, VEGF was implicated in principal in vasodilatation and inflammation process, and secondary in angiogenesis. In gingival mucosa fragments with condyloma acuminata, the principal role of VEGF was in angiogenesis and secondary in inflammation.

Keywords: gingival mucosa, papilloma, condyloma acuminata, immunohistochemical reaction, VEGF, angiogenesis.

Introduction

The histological changes of the oral mucosa in contact with a metal alloy dentures is one of the current issues widely debated in the literature. Most dental fixed restorations are made of metal alloys. Numerous in vitro and in vivo studies demonstrated that metallic dentures or appliances release metal ions, in oral environment, mainly due to corrosion. Products released by these devices in the oral environment can spread locally and systemically and may have a role in the etiopathogenesis of oral and systemic pathological conditions. The quality and quantity of products released from dental metal alloys depend on the alloy type and the environmental parameters in which it is placed. In sufficient quantities, released metal ions—particularly Cu, Ni, Be, and abraded microparticles can also induce inflammation in the adjacent tissues of metal dentures [1]. In particular, Ni ions in solution recently have been shown to cause expression of inflammatory mediators, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and intercellular adhesion molecules (ICAMs) from keratinocytes, monocytes, and endothelial cells [2]. None or weakly toxic concentrations of Ni and Co chloride induced interleukin-6 (IL-6) and interleukin-8 (IL-8) release. The specific induction of IL-6 occurs at much lower concentrations of components released from dental alloys [3]. Some researchers suggest that metal ions are involved in proinflammatory activity at low toxicity and non-toxic levels. Increased IL-6 levels were observed in cell cultures exposed to copper (5–19-fold compared to untreated controls) and nickel (10-fold) [4]

It was highlighted a very complex relationships between VEGF, cellular adhesion molecules (CAMs) and pro-inflammatory markers [5] in many diseases: several types of cancer, diabetes, reproductive and immunoinflammatory disorders [6], and, also, in cardiovascular pathologies like ischemic heart disease, heart failure, stroke [5]. Furthermore, some studies have noted the link between angiogenesis and inflammation, highlighting a key role of TNF-α and CRP (C-reactive protein) [7–9].

VEGF is a highly conserved, disulfide-bonded dimeric glycoprotein of 34–45 kDa and it is produced by several cell types including fibroblasts, neutrophils, endothelial cells and peripheral blood mononuclear cells (PBMCs), macrophages [10] and, also, by activated T-cells and epidermal keratinocytes and binds to specific receptors expressed in endothelial cells [11, 12]. Six isoforms of human VEGF, which range from 121 to 206 amino acid residues, have been identified [13, 14].

VEGF was initially known as a vascular permeability factor due to its property to induce vascular hyperpermeability, which results in the leakage of plasma protein [15]. VEGF is also a highly potent angiogenic factor that can regulate and increase microvasculature by stimulating the endothelial cell mitosis and vascular permeability [11]. Other studies indicated an increasing of both IL-6 and VEGF circulating levels, in some pathological conditions

Abstract

The histological changes of the oral mucosa in contact with a metal alloy dentures is one of the current issues widely debated in the literature. Numerous in vitro and in vivo studies demonstrated that metallic dentures or appliances release metal ions, in oral environment, mainly due to corrosion. Products released by these devices in the oral environment can spread locally and systemically and may have a role in the etiopathogenesis of oral and systemic pathological conditions. The quality and quantity of products released from dental metal alloys depend on the alloy type and the environmental parameters in which it is placed. In sufficient quantities, released metal ions—particularly Cu, Ni, Be, and abraded microparticles can also induce inflammation in the adjacent tissues of metal dentures [1]. In particular, Ni ions in solution recently have been shown to cause expression of inflammatory mediators, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and intercellular adhesion molecules (ICAMs) from keratinocytes, monocytes, and endothelial cells [2]. None or weakly toxic concentrations of Ni and Co chloride induced interleukin-6 (IL-6) and interleukin-8 (IL-8) release. The specific induction of IL-6 occurs at much lower concentrations of components released from dental alloys [3]. Some researchers suggest that metal ions are involved in proinflammatory activity at low toxicity and non-toxic levels. Increased IL-6 levels were observed in cell cultures exposed to copper (5–19-fold compared to untreated controls) and nickel (10-fold) [4].

It was highlighted a very complex relationships between VEGF, cellular adhesion molecules (CAMs) and pro-inflammatory markers [5] in many diseases: several types of cancer, diabetes, reproductive and immunoinflammatory disorders [6], and, also, in cardiovascular pathologies like ischemic heart disease, heart failure, stroke [5]. Furthermore, some studies have noted the link between angiogenesis and inflammation, highlighting a key role of TNF-α and CRP (C-reactive protein) [7–9].

VEGF is a highly conserved, disulfide-bonded dimeric glycoprotein of 34–45 kDa and it is produced by several cell types including fibroblasts, neutrophils, endothelial cells and peripheral blood mononuclear cells (PBMCs), macrophages [10] and, also, by activated T-cells and epidermal keratinocytes and binds to specific receptors expressed in endothelial cells [11, 12]. Six isoforms of human VEGF, which range from 121 to 206 amino acid residues, have been identified [13, 14].

VEGF was initially known as a vascular permeability factor due to its property to induce vascular hyperpermeability, which results in the leakage of plasma protein [15]. VEGF is also a highly potent angiogenic factor that can regulate and increase microvasculature by stimulating the endothelial cell mitosis and vascular permeability [11]. Other studies indicated an increasing of both IL-6 and VEGF circulating levels, in some pathological conditions

Keywords: gingival mucosa, papilloma, condyloma acuminata, immunohistochemical reaction, VEGF, angiogenesis.
as cancer and tumor progression influenced by visceral adipose tissue through VEGF mechanism [16, 17]. Moreover, VEGF is overexpressed in different types of human tumors and in dental and periodontal inflammatory diseases such as pulpitis, periodontitis and radicular cysts [18–20].

Gingival mucosa adjacent to the metallic alloy dentures can suffer various alterations due to exposure to nickel or copper compounds released from these. The purpose of this study was to highlight the variability immunohistochemical reaction for VEGF in human paraprosthetic gingival mucosa with different types of morphopathological lesions.

Materials and Methods

The study included both gender participants from urban and rural area, which came in the Prosthetic Dental Clinic within the Faculty of Dental Medicine, University of Medicine and Pharmacy of Craiova, Romania, during 2014.

The inclusion criteria: adults subjects, aged over 21 years, wearers of one or more fixed dentures more than five years.

The exclusion criteria: the smokers, those with associated diseases of oral mucosa, those who said they are likely to suffer from allergies and/or systemic diseases such as diabetes, vascular diseases and those who worked in a toxic environment.

The study was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova and all subjects gave written informed consent.

According to these criteria were selected eight participants aged between 51 and 64 years out of which five were wearers of fixed dentures made of nickel-based alloys and three were wearers of fixed dentures made of copper-based alloys. The selected participants have worn fixed dentures for a period between 7 and 13 years.

Sampling and preparation of gingival mucosa

The gingival mucosa samples were prelevated through excision after removing fixed denture and extraction one of its affected teeth. The excision of gingival mucosa was performed to smooth the edges of the extraction wound. The tissue fragments washed in saline physiologic solution were fixed for 24 hours in 4% buffered paraformaldehyde and then routinely processed for paraffin embedding. Paraffin blocks were cut at 3–4 μm. The paraffin-embedded tissue sections were dewaxed and usually stained with Hematoxylin–Eosin (HE) in order to evaluate the tissue morphology.

Immunohistochemistry

Serial sections were dewaxed, rehydrated and then incubated with 0.3% hydrogen peroxide in methanol in order to inhibit endogenous peroxidase and with the requested normal serum diluted 1:75 in order to block non-specific binding. Afterwards sections were incubated overnight at 4°C in a moist chamber with a rabbit polyclonal antibody recognizing human VEGF, 1:200 diluted (Boehringer Mannheim). All the dilutions were made in phosphate-buffered saline (PBS). For the negative control, we used PBS solution instead of each primary antibody. After excess reagent removing, sections were further incubated with the specific biotinilated secondary antibody (1:200) for 30 minutes in a moist chamber at room temperature. After washing with PBS, further incubation was carried out with the Avidin–Biotin–Peroxidase complex (Vector Laboratories) for 60 minutes. Peroxidase activity was revealed with 3,3’-diaminobenzidine (Sigma Chemical Co.), 1:75, in Tris buffer with 1% hydrogen peroxide. Sections were counterstained with Mayer’s Hematoxylin, mounted with Eukit and evaluated with a Nikon microscope.

Results

The examination of microscopic preparations usually stained with HE indicated papilloma lesions in gingival mucosa fragments from nickel-based alloys dentures wearers and condyloma acuminata lesions in gingival mucosa fragments from copper-based alloys dentures wearers. It was observed numerous proinflammatory cells in lamina propria of samples with papilloma and an increasing number of neoangiogenesis blood vessels in superficial connective tissue of papillae of condyloma acuminata lesions (Figure 1).

Immunohistochemical reaction for VEGF was positive in epithelium and lamina propria for both types of samples, but it was observed different aspects of the immunoreaction for VEGF in the gingival fragments with papilloma compared with the gingival mucosa fragments with condyloma acuminata (Tables 1 and 2).
**Table 1 – Immunohistochemical expression of VEGF in gingival mucosa fragments with papilloma**

<table>
<thead>
<tr>
<th>No.</th>
<th>Histological localization</th>
<th>Highlighted cells</th>
<th>Results</th>
</tr>
</thead>
</table>
| 1.  | Gingival epithelium      | Epithelial cells  | • absent reaction in the superficial layer;  
|     |                          |                   | • constant positive reaction in upper area of intermediate layer;  
|     |                          |                   | • inconstant positive reaction basal layer. |
| 2.  | Lamina propria           | Endothelial cells  | • weak positive reaction in connective papillae and in superficial area of lamina propria;  
|     |                          | Pericytes         | • intense positive reaction in deep area of lamina propria. |
|     |                          | Fibroblasts       | Fibrocytes |

The microscopic analysis of sections obtained by immunohistochemical processing of papillomatosis gingival mucosa fragments showed a zonal reaction of VEGF. The superficial epithelial layer was negative for VEGF and the upper zone of intermediate epithelial layer presented a constant cytoplasmic positive reaction. A weak and inconstant positive reaction was observed in basal epithelial layer (Figure 2).

Dilated blood vessels with VEGF-positive endothelial cells and pericytes were highlighted in the superficial connective tissue of lamina propria. In the same area, positive fibroblasts for VEGF were also found. VEGF-positive reaction was present in the deep area of connective tissue from papillomatosis samples in endothelial cells and pericytes and, also, in perivascular inflammatory cell infiltrate (Figure 3).

**Table 2 – Immunohistochemical expression of VEGF in gingival mucosa fragments with condyloma acuminata**

<table>
<thead>
<tr>
<th>No.</th>
<th>Histological localization</th>
<th>Highlighted cells</th>
<th>Results</th>
</tr>
</thead>
</table>
| 1.  | Gingival epithelium      | Epithelial cells  | • absent reaction in the superficial layer;  
|     |                          |                   | • intense positive reaction in upper area of intermediate layer;  
|     |                          |                   | • constant positive reaction in basal layer. |
| 2.  | Lamina propria           | Endothelial cells  | • intense positive reaction in connective papillae and in superficial area of lamina propria;  
|     |                          | Pericytes         | • weak positive reaction and even the absence of reaction in deep lamina propria. |
|     |                          | Fibroblasts       | Fibrocytes |

The microscopic examination of sections obtained from gingival mucosa fragments with condyloma acuminata indicated zonal variability of immunohistochemical reaction of VEGF. By examining the overview pictures was found the same absence of VEGF reaction in the superficial layer of epithelial like the papiloma but, unlike the papiloma, it was noticed an intense VEGF-positive reaction in the upper zone of intermediate layer of the epithelial (Figure 4).

In the basolayer of the epithelium was observed a constant VEGF-positive reaction. Also, cells with typical mitosis and VEGF-positive reaction were found in the...
basal epithelial layer. Epithelial cells with VEGF-positive reaction were observed in basal cell layer surrounding the connective tissue axis, which hosts new angiogenesis vessels. Numerous endothelial cells, pericytes and fibroblasts with intense VEGF-positive reaction were observed in the connective papillae and in the adjacent layer of lamina propria; these structures had a remarkable trend of organizing themselves around a vascular lumen (Figure 5).

In deep area of lamina propria, the VEGF reaction was weakly positive mainly in grouped or isolated fibroblasts and fibrocytes, and no proinflammatory cells were noted. Extended acellular area of connective tissue of deep lamina propria were negative for VEGF staining (Figure 6).
Discussion

Papilloma inflammatory lesions of gingival mucosa were observed in study participants who wore nickel-based alloys dentures and condyloma acuminata lesions of gingival mucosa were highlighted in copper-based alloys dentures wearers.

Immunohistochemical analyses of VEGF protein was similar in epithelial layer of both gingival mucosa fragments, but VEGF reaction was different in their lamina propria. VEGF is a multifunctional cytokine that shows distinguished functions in angiogenesis, lymphangiogenesis, vascular permeability, and hematopoiesis [15]. Also, the production of VEGF primarily by inflammatory and epithelial cells, has been demonstrated in nasal polyps by immunohistochemical methods and in vitro studies using cultured nasal epithelial cells [21–23]. Other studies demonstrated that VEGF is expressed by oral and epidermal keratinocytes and is up-regulated during wound healing [24]. It was also analyzed the level of VEGF released in the culture media of human gingival epithelial cells from patients with generalized chronic periodontitis. The authors have shown that the meaningful amounts of VEGF released suggest potential for promoting wound healing and tissue regeneration after grafting [25]. Other authors, also, argue that high expression of VEGF in periodontal disease may predict a greater regeneration capacity of gingival tissue [26].

The presence of positive VEGF cells in the epithelial layer of gingival mucosa fragments examined in this study may suggest a high healing and regeneration capacity of mucosa. Also, epithelial cells with VEGF-positive reaction surrounding the connective tissue with new blood vessels were highlighted in gingival mucosa fragments with condyloma acuminata. Initiating the healing of gingival mucosa could be the consequence of trauma due to release of metal ions and corrosion products of dental metal alloys. The toxic, immunologic and carcinogenic effects have been documented for some Ni compounds. Even NiTi alloys pose a risk of promoting an inflammatory response in soft tissues by activating monocytes [2]. Nickel accumulation was found to be higher in dental plaque samples of patients receiving orthodontic therapy in comparison with untreated subjects [27]. Inflammation response to nickel is considered as type IV hypersensitivity and is manifested as nickel allergic contact stomatitis [28]. Also, copper-based dental alloy or elements released from these are involved in some allergic reactions such as hypersensitivity reactions or in local chronic toxic reactions [29]. These reactions are most often located in the area of contact with the toxic agent [30].

The epithelial cells with typical mitosis had a VEGF positive reaction in basal layer of gingival mucosa fragments with condyloma acuminata. VEGF is known as a specific and potent mitogen as well as a survival factor for vascular endothelial cells both in vitro and in vivo [15]. Immunohistochemical analyses of VEGF highlighted in epithelial cells with and without typical mitoses may suggest the involvement of VEGF in the process of healing, regeneration and epithelial homeostasis. Some studies suggest that the involvement and high expression of VEGF, CD44 and CD133 in periodontal disease may predict a greater regeneration capacity of gingival tissue [26]. These properties indicate that gingival epithelium can be used as treatment in wound healing and tissue regeneration. It has been demonstrated that human cultured epithelial sheets prepared by tissue engineering techniques provide useful graft material for wound healing and tissue regeneration [25].

On the other hand, other authors suggest that VEGF is an important factor in the pathogenesis of the aggressive and chronic forms of periodontitis. Therefore, it is considered that the influence of angiogenesis in the development, progression, and healing of periodontal lesions is currently under investigation [31]. It is useful to mention the role of constant exposure of oral and gingival mucosa to salivary growth factors, which play a critical role in mucosal homeostasis and tissue repair. VEGF was also found in normal salivary glands, having a pleiotropic role in tissue repair via neovascularization, reepithelialization, and regulation of extracellular matrix [32].

It is known that VEGF increases vascular permeability, which leads to extravascular exudation and edema [33]. In superficial lamina propria of fragments with papilloma was observed dilated blood vessels and endothelial cells and pericytes with positive VEGF reaction. In deep lamina propria were also noted perivascular inflammatory cell infiltrate and positive VEGF fibroblasts. It is also known that VEGF is produced by endothelial cells of blood vessels, infiltrating inflammatory cells, and fibroblasts [21]. These features indicate the association of VEGF in chronic inflammation. Also, all these aspects suggest a bidirectional relationship between endothelial cells, VEGF protein and inflammatory cells.

However, the factors or cellular components involved in the development of mucosal edema in chronic inflammation remain to be clarified [34].

Numerous neoangiogenesis blood vessels with endothelial cells and pericytes VEGF-positive were observed in lamina propria of analyzed gingival mucosa fragments with condyloma acuminata. VEGF, the key angiogenic growth factor, stimulates proliferation, migration, and tube formation in endothelial cells [35]. During angiogenic stimulation, several cell types like inflammatory, tumor and mural cells produce VEGF-A, which increases vascular permeability and proliferation and sprouting of endothelial cells [36]. In the presence of a proangiogenic stimulus, local endothelial cells change their shape, degrade and invade the extracellular matrix. During this process, endothelial cells proliferate, forming tubular structures that coalesce with other newly forming vessels. These new structures are ultimately covered by pericytes or vascular smooth muscle cells (VSMC), giving rise to mature and stable blood vessels that enable adequate blood flow and prevent further sprouting. A decrease in pericyte coverage of blood vessels has been associated with increased vessel permeability and tumor metastasis [37]. These hypotheses are confirmed also by studying VEGF and other cytokines ratio in gingiva with chronic periodontitis. The results of the study confirm an augmented proliferative fraction of the endothelium in gingiva with chronic periodontitis [38]. Considering all these aspects, VEGF has an important role in neoangiogenesis, inflammation, repair and, probably, in oral mucosa homeostasis [39].
Regarding immunorepression of VEGF protein in gingival fibroblasts were noticed some aspects. First of all, in superficial area of papillomatosis gingival mucosa were observed VEGF-positive fibroblasts. In deep area of lamina propria of these gingival mucosa fragments were highlighted fibroblasts and fibrocytes with intense positive VEGF reaction. It is known that fibroblasts are a heterogeneous group of cells with distinct properties and functions [40]. The fibroblasts present in connective tissues, generally participate in inflammation and immunomodulation. They are also key cells in many physiological and pathological processes, including wound healing, inflammation, fibrosis and cancer [41]. Fibrocytes may be present in gingival connective tissue, especially during inflammation and wound healing [42]. These facts suggest the idea that the fibroblasts and fibrocytes were implicated in wound healing and regeneration processes only in gingival mucosa fragments with papilloma.

The absence of VEGF reaction and of the inflammatory cells were observed in deep lamina propria of gingival mucosa fragments with condyloma acuminata. These aspects suggest that the gingival fibroblasts can also have immunosuppressive functions that may relate to processes important for wound healing and tissue regeneration. These include suppression of peripheral blood monocyte proliferation and differentiation into dendritic cells in vitro. The effect on dendritic cell differentiation is mediated by IL-6 and VEGF secreted by gingival fibroblasts and may lead to increased immune tolerance [43, 44]. These facts suggest that in lamina propria, fibroblasts and fibrocytes are related to wound healing and tissue regeneration by participating more to angiogenesis and immune tolerance than inflammation process [44].

**Conclusions**

In papillomatosis gingival mucosa fragments, VEGF protein was implicated mainly in vasodilation and inflammation process, and secondary in angiogenesis. In gingival mucosa fragments with condyloma acuminata, the principal role of VEGF was in angiogenesis and secretory inflammation. In these fragments, it is possible that VEGF may have a role in immune tolerance to the compounds released from copper-based dental alloys dentures. In gingival mucosa with inflammation lesions, the vasodilation, inflammation, angiogenesis and the tissue healing are interconnected processes and, also, under VEGF modulation.

**Conflict of interests**

The authors declare that they have no conflict of interests.

**References**


[20] Artese L, Rubini C, Ferrero G, Fioroni M, Santinelli A, Piattelli A. VEGF protein was implicated mainly in vasodilation and inflammation process, and secondary in angiogenesis. In gingival mucosa fragments with condyloma acuminata, the principal role of VEGF was in angiogenesis and secretory inflammation. In these fragments, it is possible that VEGF may have a role in immune tolerance to the compounds released from copper-based dental alloys dentures. In gingival mucosa with inflammation lesions, the vasodilation, inflammation, angiogenesis and the tissue healing are interconnected processes and, also, under VEGF modulation.
Immunoexpression of vascular endothelial growth factor in gingival mucosa with papilloma and condyloma acuminata

Corresponding author
Monica Scrieciu, Associate Professor, MD PhD, Department of Prosthetic Dentistry, University of Medicine and Pharmacy of Craiova, 2–4 Petru Rareș Street, 200349 Craiova, Dolj County, Romania; Phone +40723–516 539, e-mail: scrieciu_monica@yahoo.com

Received: February 6, 2015
Accepted: October 20, 2015