Investigation of the role of neurohumoral factors, especially the vascular endothelial growth factor, in the gingival circulation

Doctorial theses

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1. INTRODUCTION

The vascular endothelial growth factor (VEGF) is known to exert its endothelium derived effects (e.g.: vascular permeability increase, endothelial cell proliferation) through its receptor type 2 (VEGFR2). Nevertheless, we have only limited information about its role in the gingival microcirculation, especially regarding the gingival venules. Several studies reported the vasodilator effect of VEGF, but all these experiments were performed usually on arteries, although the importance of venular system's physiology is well known. Leukocyte adherence, rolling, migration and the exudation responses take place in the postcapillary venules, where resistance and regulation of the microcirculation also occurs. Furthermore, the postcapillary venules seem to have a key role in postnatal angiogenesis, collaborating with the preformed microvasculature pericytes. All those experimental researches, which are dealing with the localization, function and interaction of VEGFR2 with other molecules (e.g.: nitric oxide (NO)), may open new frontiers in the therapy of tissue atrophy and/or periodontitis. On the other hand, our working group was unable to observe the deeper layers of the gingival tissue with the original protocol of vital microscopy, because of the optical features of the epithelium and connective tissue. It was also a problem, that because of the missing of one dimension, we could only make our measurements in two dimension. Therefore it was necessary to develop a new experimental method. The first step of this process was the development of a material, which due to its diffusion would clear the different tissues without significantly altering the biological mechanisms. There

was a further need to assemble an enhanced vital microscopic system, which would allow us to evaluate our data in real three dimension (3D).

2. AIMS

Our general goal was to investigate the regulatory role of VEGF and VEGFR2 in the diameter changes of gingival venules in healthy and pathological states.

Specifically:

1. We intended to investigate, if there is any effect of the application in itself, using saline.

2. To investigate the different concentrations of VEGF and their effect on the diameter of venules in healthy gingiva.

3. To investigate the amount and localization of VEGFR2 in healthy gingiva and to ascertain, if there is any effect of the selective VEGFR2 antagonist on its own on the diameter of gingival venules.

4. To investigate the effect of VEGF application after the selective blocking of its VEGFR2.

5. To investigate the regulatory role of VEGF after the blocking of NO synthase (NOS) as a pretreatment.

6. To investigate the amount and localization of VEGFR2 in gingivitis.

7. To map the localization and amount of VEGFR2, furthermore to observe the funtional changes after its selective inhibition in experimental diabetes.

8. To increase tissue transparency with the physiologic solutions developed by our team for this purpose and to establish the new protocol of real 3D vitalmicroscopy.

3. METHODS

3.1. Experimental animals

The experimental design and conditions of the animals were approved by the Animal Ethical Committee of Semmelweis University, Budapest, Hungary (22.1/4268/003/2009). In our experiments we used 93 Wistar male rats (300±37g). The assignment of the animals to the different groups happened randomly. We dropped 10 μL of every experimental solution onto the gingiva between the lower incisors to study their venular diameter regulatory role by vital microscopy.

The experimental groups were the following:

1. Control group (n=7, saline was used)

2. Different concentrations (0.1, 1, 10, 50 μg/mL; n=7 at each concentration) of VEGF (Recombinant Rat VEGF164, catalog #564-RV/CF, R&D Systems, Minneapolis, MN.) were applied on the gingiva, so as to set up our dose-effect curve.

3. In this group the selective VEGFR2 inhibitor 5-((7-benzyloxyquinazolin-4-yl)amino)-4-fluoro-2-methyl-phenol-hydrochloride (ZM323881; synthesized at Budapest University of Technology and Economics, Faculty of Chemical and Bioengineering, Hungary) was applied (20 μg/mL, n=7).

4. ZM323881 (20 μg/mL) was applied onto the gingiva of these rats at first, then VEGF after 15 minutes (50 μg/mL, n=7).

5. In this group all the experimental animals (n=14) received NOS blocker N^G-nitro-L-arginine-methyl-ester (L-NAME; CAS #51298-62-5, Sigma-Aldrich, St. Louis, MO.; 1 mg/mL in drinking water, *ad libitum*) for 1 week. After the premedication, we dropped VEGF (10 μg/mL) (n=7) or saline (n=7) on the gingiva.

6. Half of the rats (n=7) were premedicated for 1 week with ligature and composite block around their lower incisors in order to induce experimental gingivitis. The other half (n=7) received no premedication and served as control. During the experiment, animals of both subgroups received selective VEGFR2 inhibitor ZM323881 (20 μg/mL) on the gingiva.

7. Half of the animals (n=7) received pretreatment, namely we induced diabetes with iv. streptozotocin (85882 Fluka ≥98.0% (HPLC) N-(Methylnitrosocarbamoyl)-α-D-glucosamine, Sigma-Aldrich, St. Louis, MO, USA) 6 weeks before our experiments. Other half of the group (n=7) did not get any preparation and served as control. During the

experiment, animals of both subgroups received selective VEGFR2 inhibitor ZM323881 (20 µg/ml) on the gingiva.

8. Animals of this experimental group (n=16) were treated with the physiologic clearing solutions developed by our working group, based on triethanolamin (TEA), diethanolamin (DEA) and monoethanolamin (MEA). We tested the clearing effects of these materials *in vitro* on different excised tissues (skeletal muscle, submandibular gland, small intestine), furthermore *in vivo* using our translucent real 3D vital microscopic system.

3.2. Preoperative arrangements; procedure of the experiment

Complete anesthesia was achieved by intraperitoneal pentobarbital sodium injection (Nembutal, 0.5 mL/kg, 6 m/m %, Sigma-Aldrich, St. Louis, MO, USA). Tracheostomy was performed to maintain the free airway. The lower lip was turned inside out and fixed to the cheek using sutures to provide adequate access to the lower labial gingival area of the mandible. The left femoral artery was cannulized to monitor the systemic blood pressure by a computerized data-acquisition system (Haemosys, Experimetria Ltd., Budapest, Hungary). Rectal temperature was recorded by a digital electronic thermometer, and a heating pad kept the value stable (38 ± 0.3°C) by means of a feedback mechanism. Vital microscopy (Nikon SMZ-1B, Tokyo, Japan) was used to observe the labial gingiva next to the lower incisors, described in details by *Gyurkovics et al. 2009*. Cold light illumination was applied to enhance visibility

(Intralux 5000, VOLPI AG, CH-8952 Schlieren, Schwitzerland). Direction of vascular blood flow was determined and a test postcapillary venule (ranged between 25-60 μm) next to the lower incisor was selected for measurements. We tried consistently to use the same venule leaving the superficial layer, but considering the variability in vascular morphology among animals, this seemed difficult to justify. This venule could be found halfway between the tip of the papilla incisiva and the fornix in approximately 60% of the rats. If we were not able to clearly identify the same venule, we used another one with comparable location and size across animals throughout the study. Experimental solutions at body temperature were dropped (10 μL) upon the labial gingiva next to the lower incisors using a Hamilton syringe (Hamilton syringe, Hamilton Company, Reno, NV, USA), which allowed precise dropping of the test substance onto the examined vessel. To avoid saliva contamination and dilution of the investigated materials, the examined area was set to be the highest part of the oral cavity. Diameter changes of the selected gingival venule were observed using a transmission light microscope (Nikon SMZ-1B, Tokyo, Japan, 72x zoom) combined with a digital camera (Nikon Coolpix 950, Tokyo, Japan, 39x optical zoom) and images were recorded before, and 1, 5, 15, 30 and 60 minutes after test substrate application. Desiccation of the investigated area was prevented by the usage of aluminum foil between the snapshots, which gave protection against the light as well, furthermore 1 minute before the given snapshot (except the snapshot taken in the 1st minute, before which the experimental solution itself had been applied at time 0.) a physiological solution at body temperature was dropped on the gingiva both in the experimental and the

adherent control group. The outer diameters of venules were evaluated with ImageTool software (UTHSCSA Image Tool for Windows v3.00, San Antonio, TX, USA). A 100 μm marking graticule was used to enable conversion from pixels to micrometers. Mean ± SEM were calculated after measuring the distance between the two given scales 10 times. The analysis was considered to be calibrated if SEM was < 0.5. The analyzing person (M. G.) was not aware of which experimental group the images being measured belonged to.

In case of animals in the gingivitis group (group 6, n=7) gingivitis was provoked by placing ligatures around and between the lower incisors next to the gingiva and coating the ligatures with light-cured resin composite (SureFil, Dentsply, York, PA, USA) to create plaque retention described in details by *Gyurkovics et al. 2012*. Care was taken to avoid tongue damage by the apparatus and not to alter the bite. Ligatures were fixed in place by stitching them around the mandible at the symphysis.
Seven days later selective VEGFR2 inhibitor ZM323881 was dropped (10 μL, 20 μg/mL) upon the labial gingiva of the rats (n=7) next to the lower incisors with the method described above.
By diabetic rats (group 7., n=7), 6 weeks after the iv. streptozotocin pretreatment we perfomed blood sugar test. Animals (n=7) above blood sugar level 20 mmol/L were kept for the experiment, and received ZM323881 upon their gingiva using the method described above. All the others (with blood sugar level under 20 mmol/L) were excluded from the study.

3.3. Immunohistochemistry

By some of the animals in the 1st (control, n=4), 6th (experimental gingivitis, n=4) and 7th (experimental diabetes, n=4) groups, the papilla incisiva was excised and tissue samples were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h and embedded in paraffin. Several series of 12 μm sections were cut and mounted on gelatin-coated glass slides. The sections were then rehydrated and processed for preembedding immunocytochemistry. The slides were reacted overnight at room temperature with a VEGFR2 primary antibody raised in rabbit [dilution 1:20 in 0.01 M (PBS), Cell Signaling, Danvers, MA, USA]. Following brief rinses in PBS, the sections were exposed to a secondary antiserum (1:50, Immpress, Vector, Burlingame, CA, USA) and the immunoprecipitate was visualized by the brown color of diaminobenzidine (Sigma-Aldrich). As controls, the primary antibody or the secondary antiserum were omitted to verify specificity. The slides were then counterstained with hematoxylin and covered in mounting medium (DePeX, Electron Microscopy Sciences, Fort Washington, PA, USA). Photomicrographs were taken using a transmission light microscope (Olympus Vanox, Tokyo, Japan).

3.4. Western blot analysis

For the western blot analysis we used n=3 rats from the 1st, n=3 from the 6th and n=3 from the 7th group. Excised gingiva specimens were dropped into liquid nitrogen. The frozen rat gingiva tissues were then homogenized with glass homogenizer in an ice-cold buffer containing 25 mM Tris (pH 7.4), 1% NP-40, 100 mM NaCl, 4 mM EDTA, 1 mM NaVO4, 10 mM NaF, 1 mM DTT (all chemicals were purchased from Sigma) and supplemented with 50xPIC protease inhibitor cocktail (BD Biosciences Pharmingen, San Diego, CA, USA). Protein concentrations were determined by using the bicinchoninic acid method (Pierce, Rockford, IL, USA). Tissue lysates were mixed 1:1 with 29 Laemmli sample buffer and boiled for 5 minutes at 95°C. Equal amounts of protein for each sample were loaded onto 8% polyacrylamide gels (Biorad, Hercules, CA, USA), separated by SDS-PAGE, and transferred to nitrocellulose membranes (Biorad). The membranes were blocked with 5% non-fat dry milk and incubated with anti-VEGFR2 rabbit monoclonal antibody (Cell Signaling, Danvers, MA, USA) and anti-rabbit IgG-HRP secondary antibody (Cell Signaling). Specific bands were visualized with the Amersham enhanced chemiluminescence system (GE Healthcare, Buckinghamshire, UK) using the GeneGnome Chemiluminescent detection system (GeneGnome Chemiluminescent detection system, Syngene, Frederik, MD, USA).

3.5. Statistical analysis

Venule diameter changes in the experimental groups were compared both to baseline and to values of the control group. At pre-analysis, the raw data was evaluated and described as mean ± SEM, and their normality was checked by Shapiro–Wilk's W test. Further statistical analysis was performed using two-way (treatment & time) repeated measures analysis of variance (ANOVA) with contrast values, followed by Fisher LSD, Tukey's HSD and Bonferroni post hoc test according to the adequate group, when a significant difference among means was detected. Differences were considered statistically significant at $p<0.05$. Each analysis was performed using statistical software package (SAS/STAT, Software Release 9.1.3., SAS Institute Inc., Cary, NC, USA).

4. Results

Neither the VEGF, nor the ZM323881 solutions changed the systemic blood pressure significantly (baseline: 125/85 ± 8 mm Hg). There was no significant diameter change in either the 1st group (saline) or the VEGF (at 0.1 μg/mL and at 1 μg/mL concentration) treated 2nd group. The VEGF concentrations of 10 μg/mL and 50 μg/mL (2nd group) caused significant venodilation in the 5th and 15th minutes after administration which gradually decreased till the 45th minute. ZM323881 on its own (3rd group) did not induce any significant diameter changes in the gingival venules. When the ZM323881 pretreatment was followed by VEGF application (4th group), there was

no significant change either. The pretreatment with L-NAME (5th group) increased the baseline systemic blood pressure significantly (155/120 ± 12 mm Hg), and decreased the baseline diameter of gingival venules as compared to the control (35 ± 2 μm vs. 44 ± 2 μm, respectively). VEGF dropped after L-NAME pretreatment did not alter the systemic blood pressure. The L-NAME pretreatment inhibited the venodilatory effect of the 10 μg/mL VEGF (5th group).

In the wall of the gingival arterioles and venules we detected only a weak and scattered immunreaction against the VEGFR2 under healthy conditions. Immunoreactive precipitate was apparent in the endothelium, representing the innermost lining layer of the vascular wall where the cell nuclei tend to be parallel to the long axis of the vessel. Similarly, a weak punctate immunoreactivity was observed in the second vascular layer, composed of smooth muscle cells (SMCs) encircling the internal layer, with the puncta representing the cross-sectioned muscle cells. Vascular pericytes, located adjacent to the muscular layer, also occasionally exhibited VEGFR2 reactivity.

In case of experimental gingivitis (6th group) and diabetes (7th group) neither the disease, nor local ZM323881 altered the systemic blood pressure (baseline: 125/85 ± 8 mm Hg), but baseline diameters of gingival venules in both groupswere significantly increased as compared to the control (gingivitis: 51 ± 8 μm vs. 27 ± 2 μm, respectively; diabetes: 47 ± 1 μm vs. 28 ± 2 μm, respectively). The selective VEGFR2 inhibitor ZM323881 caused significant vasoconstriction in gingivitis (6th group) in the 5th, 15th, 30th and 60th minutes after its application as compared with the data of healthy animals.

In the group of diabetic animals (7th group), the ZM323881 also induced significant vasoconstriction in the 15, 30 and 60 minute as compared to the baseline.
According to the immunohistochemistry, VEGFR2 expression significantly increased in gingivitis by all layers of the vascular wall. Gingiva samples harvested from ligated animals showed strong immunoreactivity both in the vascular wall and at the neighbouring area compared to control gingival tissue. A massive immunopositivity against VEGFR2 was observed within all mural layers of the small gingival blood vessel, including the innermost layer of the endothelium. Similarly, in the middle layer, the SMCs were represented by punctate immunolabeling. Apart from labeled pericytes, located external to the muscle layer, several immunopositive fibroblasts have also been observed. In further control slides, where the primary or secondary antisera was omitted, no specific immunolabeling was observed either in arterioles or in venules, differing substantially from the level of background tissue reactivity.

Western blot analysis corroborated this latter result, as there was a significant increase in the amount of proteins typical for VEGR2 (240 and 210 kDa), in case of gingivitis specimens, when compared to the control.

The immunohistochemistry, performed by samples harvested from diabetic animals, revealed, that VEGFR2 expression was increased mostly in mast cells appearing in large numbers next to the venules, as compared to the control, where only weak immunreactivity could be detected and even mast cells could hardly be found. The western blot analysis supported this result as well, as

there was a significant labeling with the proteins typical for VEGFR2.

The absorbtion change of the investigated tissues (skeletal muscle, submandibular gland, small intestine harvested from Wistar male rats) was measured *in vitro* with microplate reader on 460 nm after the application of our newly developed solutions (TEA, TEA-DEA mix, MEA). TEA caused significant absorbtion decrease in skeletal muscle as compared to the control (saline) in every time of our measurement. The small intestine results that were recorded showed significant absorption decrease between the 13. and 88. minutes after the beginning of the experiment and between the 103. and 123. minutes as well. The submandibular gland also showed significant absroption decrease that was detected between the 38. and 93. minutes.
TEA-DEA solution also decreased the absorbtion of the muscle after 8. minutes throughout the experiment. Regarding the submandibular gland and small intestine, we were not able to measure any significant absorbtion decrease, but the descending trend could be observed.
With the application of MEA, we were able to detect significant absorbtion decrease by the submandibular gland right from the beginning of our measurement. In case of skeletal muscle, after the 40. minutes, significant absorption decrease was confirmed all along the time frame of the experiment. There was no significant change with the small intestine.
After the *in vivo* preliminary studies with our physiologic TEA solution, we detected spectacular transparency increase in the examined gingival tissue.
By means of our new, real 3D vital microscopic system we made a 3D modelling of our examined venule, which

was observed only in 2D previously. The software, that was developed by our working team for our system, allowed us to draw coordinates in our 3D images and to simply highlight the vessels as a 3D model, which could be animated as well.

5. CONCLUSIONS

1.) The applied method is suitable for our investigation, as the dropping application itself, using the control saline, does not have any effect on the venular diameter.

2.) There is no significant basal VEGF production and VEGFR2 expression under physiologic circumstances in gingiva.

3.) Locally applied exogenous VEGF is able to cause venodilation through VEGFR2s activation and consequent endothelium derived NO release pathway.

4.) Our results also suggest that in case of experimental gingivitis or diabetes, there is an increased production of VEGF in gingiva.

5.) In these diseased conditions, the large amount of VEGF through the activation of increased VEGFR2 expressions in the vascular wall and in neighbouring cells (endothelial cells, SMCs, pericytes in gingivitis and mostly mast cells in diabetes) plays an important role in vasoregulatory processes (vasodilation, increased permeability, angiogenesis and remodeling of the local microvascular network) of gingiva,

6.) selective inhibition of VEGFR2s may be a new therapeutic adjuvant approach of periodontal disease especially in diabetic patients.

7. Our newly developed real 3D translucent vitalmicroscopy may open up a new promising area of research.

6. LIST OF OWN PUBLICATIONS

6.1. Publications in connection with the dissertation

6.1.1. Peer reviewed Articles with impact factors

1. Gyurkovics M, Lohinai Z, Györfi A, Iványi I, Süveges I, Kónya M, Bodor C, Székely AD, Dinya E, Fazekas A, Rosivall L. (2009) Venodilatory effect of vascular endothelial growth factor on rat gingiva. J Periodontol, 80(9): 1518-1523. IF=2.192 (2009)

2. Gyurkovics M, Lohinai Z, Győrfi A, Bodor C, Székely AD, Dinya E, Rosivall L. (2012) Microvascular regulatory role and increased expression of vascular endothelial growth factor receptor type 2 in experimental gingivitis. J Periodontal Res, doi: 10.1111/j.1600-0765.2012.01520.x. [Epub ahead of print]. IF=1.686 (2011)

6.1.2. Citable abstracts

1. Gyurkovics M, Lohinai Z, Győrfi A, Bodor C, Fazekas A, Nyárasdy I, Rosivall L. (2008) The role of vascular endothelial growth factor (VEGF) in experimental gingivitis. J Vasc Res, 45(2): 129.

2. Gyurkovics M, Lohinai Z, Győrfi A, Iványi I, Süveges I, Kónya M, Bodor C, Baintner K, Fazekas Á, Rosivall L. (2007) Examination of the venodilatory effect of Vascular Endothelial Growth Factor (VEGF) in rat gingiva. Acta Phys Hung, 94(4): 345.

3. Gyurkovics M, Lohinai Z, Győrfi A, Bodor C, Fazekas Á, Nyárasdy I, Rosivall L. (2009) The VEGF production rises in experimental gingivitis. Acta Phys Hung, 96(1): 78.

4. Gyurkovics M, Lohinai Z, Győrfi A, Bodor C, Fazekas Á, Nyárasdy I, Rosivall L. (2010) Gingival venule diameter is regulated through the increased VEGF expression in experimental diabetes. Acta Phys Hung, 97(1): 104-105.

5. Gyurkovics M, Stuber I, Berkei G, Kis P, Rosivall L, Korom C, Lohinai Z. (2011) New approach in vital microscopy: Three-Dimensional Transparent Imaging. Acta Phys, 202(684): 25.

6. Gyurkovics M, Stuber I, Berkei G, Kis P, Korom C, Rosivall L, Lohinai Z. (2011) Development of Translucent Three-dimensional Vital Microscopy in Dental Research. J Dent Res, 90(S B): 269.

6.2 Publications not in direct connection with the dissertation

1. Iványi I, Gyurkovics M, Várnagy E, Rosivall L, Fazekas A. (2008) Comparison of guttapercha points of different brands. Fogorv Sz, 101(2): 65-69.

2. Herczegh A, Ghidan A, Gyurkovics M, Bedő Z, Lohinai Z. (2013) Effectiveness of a high purity chlorine dioxide solution in intracanal *Enterococcus feacalis* biofilm eliminating. Acta Microbiol Immunol Hung, 60(1): 63-75. IF=0,787 (2011)