EXPERIMENTAL STUDY OF TISSUE REGENERATION

REGENERATION OF ORAL MUCOSA AND BON TISSUES STUDY BY EXPERIMENTAL ANIMAL MODELLS

THESIS

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INTRODUCTION AND AIMS

One of the principles in life is the ability of defense of the organism. The compact covering surface of the mucosa is a main part of the defense mechanism of the body surface. On this layer even hundreds of microinjuries can happen on the course of normal oral function. Pathological microerosions also frequently appear. The prevention of the damaging (microbial, antigenic, toxic, etc.) invasive attacks is fundamentally determined by the closure of the wound gates. The rapid closure of the epithelial cell layer is especially important in microlesions without bleeding since the cellular elements of the blood cannot participate in the buildup of the barrier. One fundamental element of saliva in this system is the epidermal growth factor (EGF), which is able o stimulate epithelial proliferation, differentiation and thus fast regeneration.

In our laboratory we started to study the experimental models of EGF production and also EGF content of human saliva in the early nineties. We reported the presence of EGF in the oral cavity following partial and complete removal of the major salivary glands (ablation) in two papers. These early data still hold true, no contradictory report appeared and supported the observation that in minor human salivary glands te production of both EGF and its receptor was reported.

Salivary EGF secretion is achieved towards the lumen. This is supported by observations that salivary EGF concentration multiplies by hundreds in response to adrenergic simulation while plasma EGF level remains unchanged. In pathological conditions (alcohol, smoking) leading to gastrointestinal alterations the EGF production was reported to increase proportional to the damage suggesting a possible role of tissue factors in the regulation of EGF production.

When submandibular gland, the place of EGF synthesis and storage in rodents, is removed in rats, a hypertrophic-hyperplastic compensation is induced, H^+ reflux through intestinal mucosa increase and the risk of ulcer formation becomes elevated.

In mouse, after removal of the submandibular gland (ablation), EGF (1 μg/ml in drinking water) reconstitutes the normal rate of wound healing. Intraperitoneal EGF administration (10 μg/kg, i.p. twice daily for 3 days) increases salivary amylase activity, while tissue amylase content decreases in the parotid gland. RT- PCR showed an 18

times increase of the 576bp tissue amylase mRNA compared to control. This indicates the importance of EGF in tissue adaptation as well.

No exact data are available regarding the cell source of salivary EGF. Up till now, EGF production was localized in the convulated tubular ductal cells (CGT) of rat submandibular gland that is densely innervated by adrenergic nerve fibers. In rats and in mice noradrenalin increases both salivary and plasma EGF concentrations, mediated by alpha adrenergic receptors. Our previous experiments and literature data support that after removing the other salivary glands (sialoadenectomy, ablation) the remaining glands respond by increased functional activity (hypertrophy and hyperplasia), and able to achieve certain compensation of salivary secretion. It is not known whether such compensation also exist for EGF production but we cannot exclude the possibility of this based on the effect on epithelial cells. This may has significance in human relations as well. It is important to know whether EGF compensation appears following diminished salivation induced by diverse reasons (tumor, accident, infection), which can be important to counterbalance mucosal microinjuries.

In the first part of our studies (A1) we investigated whether the removal os the submandibular and sublingual glands from rats (partial ablation) the compensatory parotid enlargement can be related to the compensation of EGF synthesis and secretion.

While the source of EGF in rodents is the submandibular gland, in humans EGF ic primarily secreted to saliva during chewing, the time for microinjury progression. Here EGF is secreted by the parotid glands. This raises practical questions: a) what is the pasticity of EGF production and whether EGF is produced in extra-salivary tissues; b) does sliva containd EGF after complete ablation; c) can EGF participate in oral mucosal regeneration without major salivary glands?

In the next part of my thesis (A2) I introduce a newly developed experimental method, complete salivary ablation and “experimental studies of minor salivary glands”, that gives us the appropriate tools to find the answers to the above listed questions.

Adverse civilization effects become more and more pronounced, even in the oral cavity. Destruction of the soft and hard tissues affects the majority of the population. Although great achievements were carried out in prevention and cariology to prevent

and restore caries, the destructive processes frequently lead to tooth loss. The same outcome may be caused by other factors as well: mucosal inflammation, gingivitis turning to periodontitis may induce bone loss and subsequently tooth loss. For the rehabilitation of the normal functional state, the classical prosthodontic methods are not always applicable. The problem solving of the loss of pillar teeth induced in a rapid development both in the techniques if dental implantation and in the restoration of alveolar bone.

The success of implantation is based on osseointegration (oi), as a consequence of suitable statical and bone-biological/bone-mechanical arrangement. Normally the process is slow, 6-10 months that is not very suitable for patients. The two phase technique results in food intake problems for the first period and may cause negative effects and even general health problem as well. The disbalance in chewing and in oral metabolism (self cleaning, circulation, decrease of stimulation, lack of essential amino acids, vitamins, minerals, trace elements) my adversely affect bone formation and induce defense, regeneration and immunological problems.

The last decades promoted a tremendous development in bone reconstruction techniques. Both in implant osseointegration and in periodontal guided tissue regeneration the central issue is the regulation of bone regeneration. The quality of the newly formed bone (structure, mineralization level and strength) and the time that is necessary to reach this depends on the activity or inhibition of a large number of factors such as growth factors, cytokines, bone morphogenetic proteins, local and systemic concentration of electrolytes.

Modern bone research originates from the research activity of W.B. Coley about a hundred years ago. Bone formation is a multiphase process that involves many components in space and time. The Bone Morphogenetic Proteins (BMPs), members of the Transforming Growth Factor peptide family (ie. TGF-α, TGF-β), play a crucial role in these processes. Bone regeneration research accelerated in the past decades. Most of our information on the regulatory factors is based on in *in vitro* culture studies, while *in vivo* experiments that can be more directly related to human situation are limited.

The missing or damaged parts of the organisms due to injury or destruction have been substituted for about a century using immuno-tolerant materials. The two most important factors are the biological usefulness of the implant and the immuno-tolerancy

of the organism against it. Bone regeneration involves the close contact of the foreign material and existing bone, resulting a fixation of the two structures to each other. Osseointegration means this biological regenerative process. Results of research on the regulatory factors and the systemic regulatory networks will bring new dimension for the modification of the time consuming biological processes.

Dental implants serve as special connecting elements between the outer world and inner part of the organism. The fast changing microflora in the oral cavity during metabolism, food intake and communication create a potentially dangerous environment in the oral region. Perhaps one of the hardest defensive task for the organism is the closure of the dental hard tissues erupting from the soft mucosa. The adhesion of the periodontal ligament and the marginal gingiva are strong to the surface of the tooth. If this barrier is wounded, the covering epithelial cell layer starts to proliferate in response to EGF, and close the wound. Unfortunately, this will result in pocket formation in a large number of the cases. This mechanism, the rapid closure of the surface wounds, may prevent osseointegration by forming epithelial contact to the implant surface. There are a number of methods to prevent the formation of this epithelial, destabilizing layer in clinical practice. All methods is about to enhance the chance of bone regeneration over epithelial growth on the implant surface by accelerating bone formation.

Basic science invasive experiments cannot be carried out in human subjects. Therefore, it is important to develop well reproducible, low-cost experimental models to study bone regeneration. For these, basic criteria should include the followings. Bone regeneration should be studied in a unified system, to investigate the effect of in situ and systemic growth factor and drug administration, and also the composition of the implant and its surface characteristics.

The albino laboratory rat is a routine subject of experimentation. The anatomical characteristics of rats are too small in the oral region to be used for routine, well reproducible bone regeneration studies. But structural analyses in this species led us to the conclusion that the rat tail bones are very similar in structure to the maxillary and mandibular alveolar bone. Based on these observations, we decided to model dental implantation in the rat tail and developed a new model to study osseointegration.

The second part of my thesis (B) describes a new, own "Bone regeneration/osseointegration model". This model opens new perspectives for experimental, bone regeneration, developmental biological, stem cell and regenerative, implant integration research. The results will provide data to select optimal bone structure, implant surface, biological factor and electrolyte concentrations, which all improve osseointegration processes.

During the past few years several publications have raised the possibility that bisphosphonates might provoke severe side-effects in the oral cavity (132, 172). In bone diseases, mainly in the case of bone destructions or cancer metastasis, bisphosphonates have became the primary choice of treatment worldwide, in order to suppress excessive osteoclast activity. The fact that bone regeneration and osseointegration involve bone remodelling, has raised the question how bisphosphonates will modify the osseointegration, measured in our model system. To tell the truth, in the light of several negative observations with bisphosphonates, we were waiting for degradation of the consolidation of implants, i.e. that implants will be lost concomitantly with disintegration of the tail vertebrae.

METHODS

A1) Partial ablation of salivary gland

1. Experimental animals and protocols: The experiments were carried out on young adult Wistar rats (Crl.(Wi)Br) of 200-300 body weight. At the beginning of experiments, under sodium pentobarbital (30 mg/kg i.p., Serva) anaesthesia, saliva samples were collected for 15 min., following pilocarpine stimulation. After 15 min saliva collection, bilateral submandibular (SM) and sublingual (SL) sialoadenotomy (partial ablation, [Zelles T, 1984]) were made on half of the animals, performed through a sagital neck incision. Intact and partial ablated animals were divided into groups receiving either drinking tap water or 0,5 % citric acid containing water. Experimental groups were the following: control (Cont), ablation (AB), citric acid (Cit) and ablation plus citric acid (AB+Cit). These treatments were carried on for a week. Food and the drinking top water were provided *ad libitum.* Food was withdrawn on last day 12-16 h before the experiment. On the experimental day 15 min pilocarpine stimulated saliva was collected from every second animals. After bleeding, the salivary glands were excised from all animals. The weight of glands was measured and tissues were

homogenized by a blendor in 20 mM, pH 7.4 phosphate buffered saline. (Ethical license of animal experiment N°:1799/003/2004.)

2. Collection of saliva: Following pilocarpine stimulation (Pilocarpine-HCl, Serva, 2.5 mg/kg i.p.), the secreted saliva from the oral cavity was collected in Eppendorf tubes. Samples were centrifuged at 5500xg for 10 min to remove debris in pellet.

3. Biochemical analysis: **a.)** *Protein concentration* was measured by the Bio-Rad Protein Micro Assay based on the method of Bradford [Bradford MM, 1976], on the microplate reader (Bio-Rad model 3550), using bovine serum albumin as a standard..

b.) *Amylase activity* was determined according to Bernfeld (1955), using starch as substrate. One unit (U) of amylase was defined as the amount that hydrolysed 1mg starch per min. per mg protein per ml saliva at 37°C.

c.) *EGF determination:* EGF was measured by radioreceptor assay, using human placental syncytiotrophoblast microvilli membranes [Booth AG, 1980]. One-hundred µl sample was added to 100 µl of human placental microvillous membranes and [^{125} I] - labelled human EGF (100µl/50,000cpm). After overnight incubation at room temperature, the membrane solution was diluted in 1000µl 5% polyethylenglycol, centrifuged at 1500x*g* for 10 min and the supernatant was discarded. The radiolabel associated with the membrane was determined in a gamma counter. Using this method the binding is independent of the species of the EGF source [Simpson RJ, 1985].

A2) Total ablation of salivary gland: „Minor salivary gland experimental model"

1. Experimental animals: Female Wistar Crl.(Wi)Br Charles River rats (weighted 230-280 g) from the breeding colony were kept in light controlled, air-conditioned rooms and were given food and water *ad libitum*. Rats were anesthetized with sodium pentobarbital (40mg/kg, i.p.). (Ethical license of animal experiment N°:1799/003/2004.)

2. Surgery: Through a sagittal neck incision bilateral submandibular, sublingual sialoadenectomy (partial ablation) was performed together with excision of both parotid glands (gls. Submandibular-sublingual-parotid ablation = total ablation [Blazsek *et al*. 1999]). Special attention was paid to preservation of both nerves and blood vessels in the neck area during this surgical procedure.

3. Investigational protocol of animals: One group of rats served as sham-operated controls. Subsequent to these surgical procedures, animals were allocated to one of five groups. Two of these groups and the sham-operated controls were studied immediately following surgery (acute group), while the secretory function in the other groups was investigated 1 week, 2 weeks or 3 months later (Chronic groups). In chronic experiments body weight gain, food intake and water intake was measured during the first three weeks after surgery.

4. Collection of saliva following total ablation: A novel technique for collection of secretory material from minor salivary glands [Blazsek J, 1999] - In ablated rats, tracheotomy was performed before starting the secretory experiments under anaesthesia. To evaluate secretory function of the minor glands, the mouth was rinsed with 250-250 µl water in every 5 min for 150 min to obtain 30 fractions. Control animals received no stimulation during this period and experimental groups received pilocarpine-HCl (1 mg/kg, i.p.; Sigma) after 50 min. β-receptor functions were blocked by Propranolol 2mg/kg (Inderal Inj. Zeneca, UK), starting from the 5th fraction (i.e. at 25. min). The samples were transferred in Eppendorf tubes on ice and centrifuged at 5500xg for 10 min to remove debris.

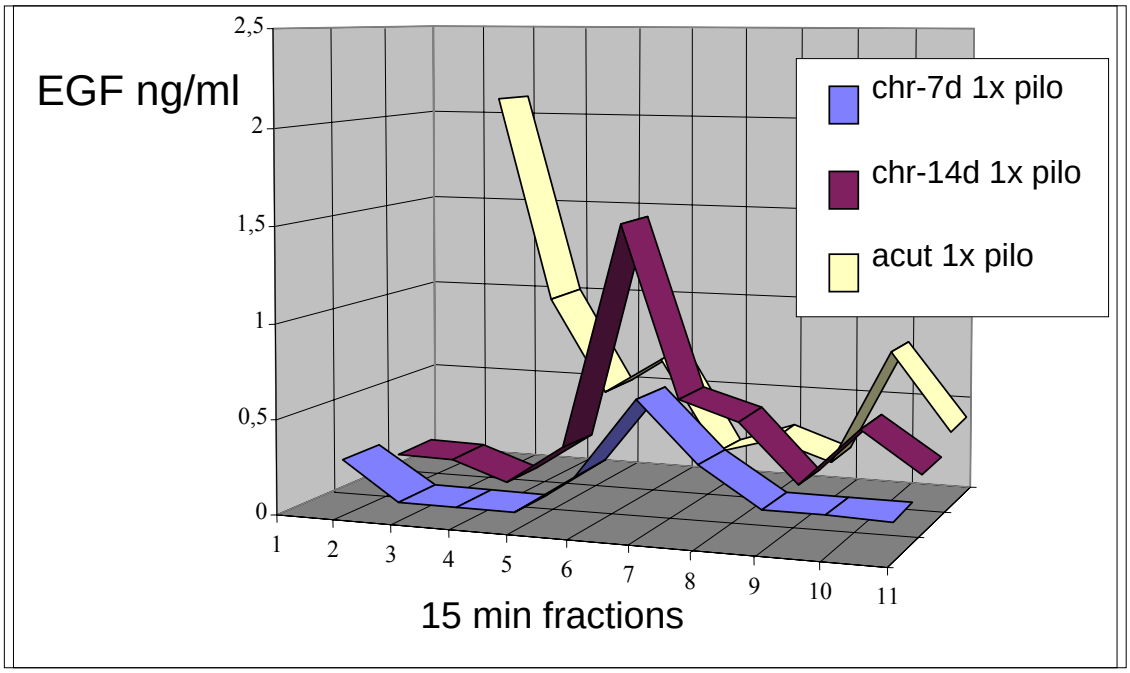

Fig. 1: Kinetics of EGF secretion in the oral cavity of rats following total ablation of major salivary glands 7 days, 14 days or immediately prior to pilocarpine stimulation.

5. Statistical analysis: Values represent the mean ± standard deviation (S.D.) using 3-5 parallel samples. Cumulative protein and amylase secretions over 100 min were calculated, roughly, assuming that all secreted protein and amylase was recovered by the rinsing fluid. Comparison among the groups was performed by ANOVA, using InStat software (GraphPad, San Diego, CA).

B1) Quantitative and qualitative analysis of bone tissue

1) Vitrification and staining of bone and cartilage: The skeleton was cleaned, fixed and dehydrated in 50, 70 and 100% ethanol and three changes in aceton. Alizarin red/alcian blue staining was carried out in standard staining solution (5 vol. 0.3% alcian blue in 70% ethanol, 5 vol. 0.1% alizarin red in 96% ethanol, 5 vol. acetic acid and 85 vol. 70% ethanol) overnight at 37°C. Background coloration was removed by several changes in 1% KOH, 20% glycerine in water. Native or stained skeleton preparations were photographed using dark-field illumination under a Zeiss STEMI SV8 stereomicroscope attached to a SONY CCD camera.

2) Microdissection and fractionation of femoral BM: Mice under anesthesia (Avertine 10µg/10g body weight) were perfused with 80-100 mL Ca,Mg-free PBS through the ascending aorta, then bone compartments (femur, vertebrae, mandible) were dissected aseptically and cleaned under PBS/0.5% BSA at 4 °C. The compact bone was bisected longitudinally using a fine scalpel then the BM shaft was carefully aspirated along the central diaphysis through a 23G needle. BM attached to the metaphyseal spongiosa was removed by repeated flushing. Dispersed cells and nodules were pooled in a tube then the endosteal bone and spongiosa were removed with a scalpel. Node-like niches/haematon units were purified after gentle disruption of pooled BM parenchyma in 1 mL culture medium. Aggregates in pellet and the bulk buffy coat suspensions were separated using three repeated 10 minutes 1xg sedimentation

3) FACS analysis and cell purification: Bone marrow (BM) fractions (buffy coat, haematon and endosteum) were dissociated into single cell suspensions in IMDM/10% fetal calf serum (FCS) containing 0.1% collagenase types-I, -II and IV, 0.1 U hyaluronidase and 5U DNaseII solution (Sigma) at 37°C for 40 minutes. Tiny residual capillaries and cells firmly attached to the endosteum were further dispersed in 0.1% collagenase type-I/dispase solution (Boehringen) for 10 minutes at 37°C. Nucleated cells were counted in a hemocytometer in 0.1% acetic acid.

Hematopoietic stem cells (HSCs) were analyzed quantitatively and purified by 7-color FACS. Single cell suspensions were washed in PBS/2% FCS (4°C) then to $2x10^6$ cells 5µg $Hoechst_{33342}$/mL staining solution was added at 37°C for 60 minutes. Cells were centrifuged (400xg, 7 minutes, 4°C), then lineage positive cells (CD3, CD4, B220, Gr1, Mac and CD119), Tie2 (1/50) or CXCR4 (1/50) were labelled by biotin tagged primary rat antibodies followed by 2 washes and incubation with streptavidin-APC-Cy7 for 30 minutes at 4°C. Cells were washed twice, then primary, fluorescence dye labelled antibodies were added simultaneously (rat anti-Sca-1 (FITC; 1/100 dilution; rat anti-c-kit (PE; 1/100 dilution) and rat anti-CD150 (SLAM; APC; 1/50 dilution) for 30 minutes at 4°C. Cells were diluted with ice-cold PBS/2% FCS, centrifuged and analysed immediately by FACS Calibur flow cytophotometer (Becton Dickinson) using the CellQuest and WinMDI2.8 soft wares.

4) Hematopoietic progenitor and stem cell assays: Long-term culture initiating cells, LTC-IC/CAFC, were determined by limiting dilution analysis (LDA) using irradiated MS-5 stroma cells (20 Gy, IBL 637 gamma irradiator, ^{137}Cs source, ORIS, France). Myelopoietic-lymphopoietic switch cultures were used as previously (Blazsek et al., 2000).

5) Histology: **a)** Vertebras and femur were dissected, cleaned and fixed overnight in acetic acid-zinc-formalin (AZF) fixative (1.25 g zinc chloride, 15 mL cc formaldehyde, 0.75 mL glacial acetic acid and distilled water (d.w.) to 100 mL) at room temperature . Following three washes in d.w. for 30 minutes Gooding and Stewar's decalcification fluid was added for 4 hours (10 % formic acid, 5 % formaldehyde in water). Samples were washed in water before processing for sucrose-gelatine impregnation and freezing in isopenthane at -80°C. Sections, 5 to 10 µm thick, were cut in a freezing microtome, taken on poly-lysine coated slides and were processed for differential staining. Osteoblasts were identified by strong alkaline phosphatase and osteoclasts by tartarate resistant acid phosphatase (TRAP) reactivity using standard detection kits (Sigma, USA). **b)** PAS and silver reaction on the bone formation: vertebras were fixed in formaldehyde for 2 days, and were decalcinated in 18% sodium-EDTA (pH 7.4) solution for one week at 56°C and were embedded into paraffin. Dewaxed sections were stained routinely with Hematoxylin and eosin, periodic acid-

Schiff (PAS) reagent and silver nitrate. After mounting slides were photographed in Leica DMLB1005 light microscope equipped with a JVC camera.

B2) A novel experimental method for the analysis of bone regeneration and osseointegration of titanium-stift implant in the rat caudal vertebrae

1. Experimental animals and protocols: Female Wistar rats (Crl(Wi)Br, Charles River; 250-370g) were kept and anesthetized as above. After implantation the rats were kept in individual cages to insure appropriate hygiene and wound healing during the first two weeks following surgery. C57BL/6 wild-type mice (IFFA CREDO, L'Arbresle, France) were bred under a 12-hour light-darkness schedule. For comparative anatomical and histological studies; mice were anesthetized by avertine, bleeded by intracardiac perfusion with 100 mL Ca, Mg-free PBS/20IU heparin/mL prior to fixation. (Ethical license of animal experiment N^{o}: 46/1999 and 1799/003/2004)

2. Surgical protocol of placement of titanium implants: The tail was disinfected and ligatured to control bleeding during surgery. A 5-6 mm incision was made at the level of caudal C4-C5 vertebras, the skin was retracted and the vertebras were exposed under sterile conditions. In the exposed surface of C4 a central, 1 mm diameter and 5 mm deep hole was formed, which corresponds to the size of titanium screw, using an electric drill. Then a larger and shorter hole was made (2.0 mm diameter and 3.5 mm depth), thus creating an "empty" cylinder which allowed the prospective bone regeneration to monitor. Screw-type titanium implants (1.2 mm in diameter; see Fig. 2B) were fabricated and their surface roughened using sandblasting (Full-Tech Company, Hungary). Sterilized screws were introduced into the 5 mm deep thin hole. Following insertion of the implant the skin was repositioned over the implant and tightly sutured. The surgical wound was protected aseptically by a plastic film-layer (Plastubol®: methylmethacrylat-butylacrylat-butylmethacrylat, diisooctyl phtalate – Pannonpharma Ltd.Hungary).

3. Aminbisphosphonate treatment (Zometa®) and osseointegration: The cyclic aminbisphosphonates Zoledronate (Zometa® Novartis) has been used according to the manufacturer's recommendation, applying 60% of the single lethal dose (1 mg/kg b.w.) established for rats. 0.6 mg/kg was injected i.p. in 1 mL physiological saline; controls received 1 mL of physiological saline. Ten rats were included in both control and

Zometa treated groups. Subsequent Zometa® treatments were done at 14 days intervals and repeted three times (3x14=42 days bone regeneration, osseointegration period).

4. Quantitative mesure of biomechanical force of osseointegration: Six weeks following surgery rats were exsanguinated under Nembutal anaesthesia. Before removing the implants, the bone structure of all animals (n=10) from each group was scanned using the microCT. Following removal of the implants, vertebrae were fixed in 10% formalin and two vertebrae of each animal, were scanned using microCT. The end pont maximum force needed to extract the titanium implant was measured using a Tenzi TE 18.1 (TENZI Ltd. Hungary) apparatus and expressed in Newton (N).

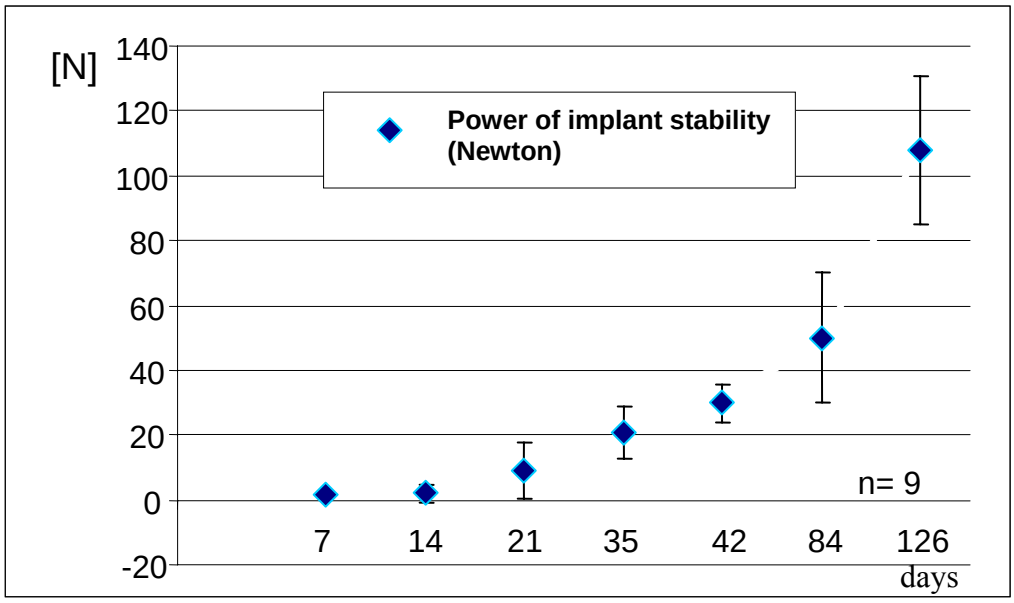

Fig. 2: Consolidation kinetics of titanium implant as measured by the force of extraction (sign ± SD).

5. Micro morphometry analysis with Micro Computer Tomography (microCT): **a**.) Six weeks following the placement of titanium implants bone regeneration was measured by X-ray microCT instrument (SKYSCAN 1172 X-ray Microtomograph, Belgium). The instrument had an X-ray source from a sealed microfocuse X-ray tube with a spot size of 8 µm, a resolution of 17.7 µm, using a cone-beam volumetric reconstruction algorithm. We used a 0.5 mm Aluminium filter to „soften" and even-out the X-ray beams.

b) Implanted samples were scanned at 360° rotation while the bony socket of the vertebra following removal of the implants were scanned with 180°, at 0.7 degree

intervals. The X-ray scatter generated on the surface of the implants was subtracted (Fig. 1B).

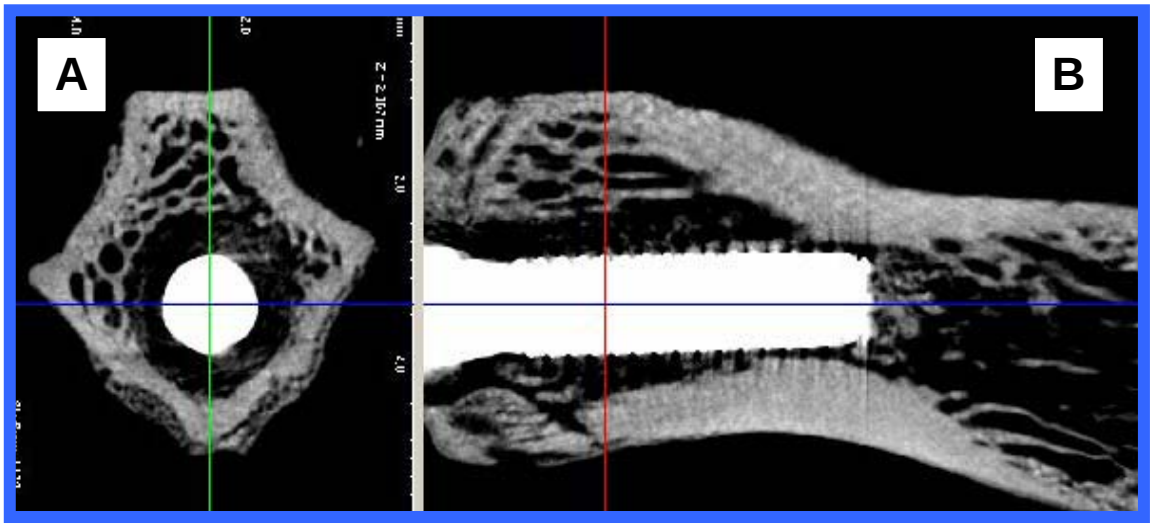

Fig. 3: Reconstituted 2D image of the tail vertebra with implant, after 3 weeks of implantation; **A)** Cross section and, **B**) longitudinal section. Note, the see is empty „bone-casette", only a few pieces of trabecula the surrounding original bone tissue and the implant. The pace betveen of jellow lines is the Region of Interest (ROI).

c) For reconstruction of 2D and 3D images we used the cone-beam volumetric algorithm (Feldkamp). d) Measurements were made on the Region of Interest (ROI) x 1.5mm Tissue Volume (TV) on the computer-reconstructed 3D samples. These measurements were made both before and after removal of the implants.(Fig.1-2.) Reconstruction and 2D analysis. 2D and 3D images were reconstructed and data were analyzed using the Skyscan software. First, we determined the original dimensions of the "empty" bony chamber that were originally created using the burs. We also determined the volume of the inserted implant.

Finally, we subtracted the two volumes to determine the volume of the newly generated bone tissue. This "Tissue Volume" (TV) was the area between the implant surface and the walls of the bony chamber. TV had a thickness of 1.5 mm and a total of 86 scanned consecutive layers (ROI x 1.5 mm TV= 2.694 mm3) were analyzed using the Skyscan software.

6. Statistical analyses: Values represent mean±standard deviation (S.D.). The data of stabilization force were performed using 10-10 parallel samples. A morphometric analysis was applied for both internal and external controls. Comparison

among the groups was performed by ANOVA (Student's t-test). Calculations were performed using the InStat program package (GraphPad Software, San Diego, CA).

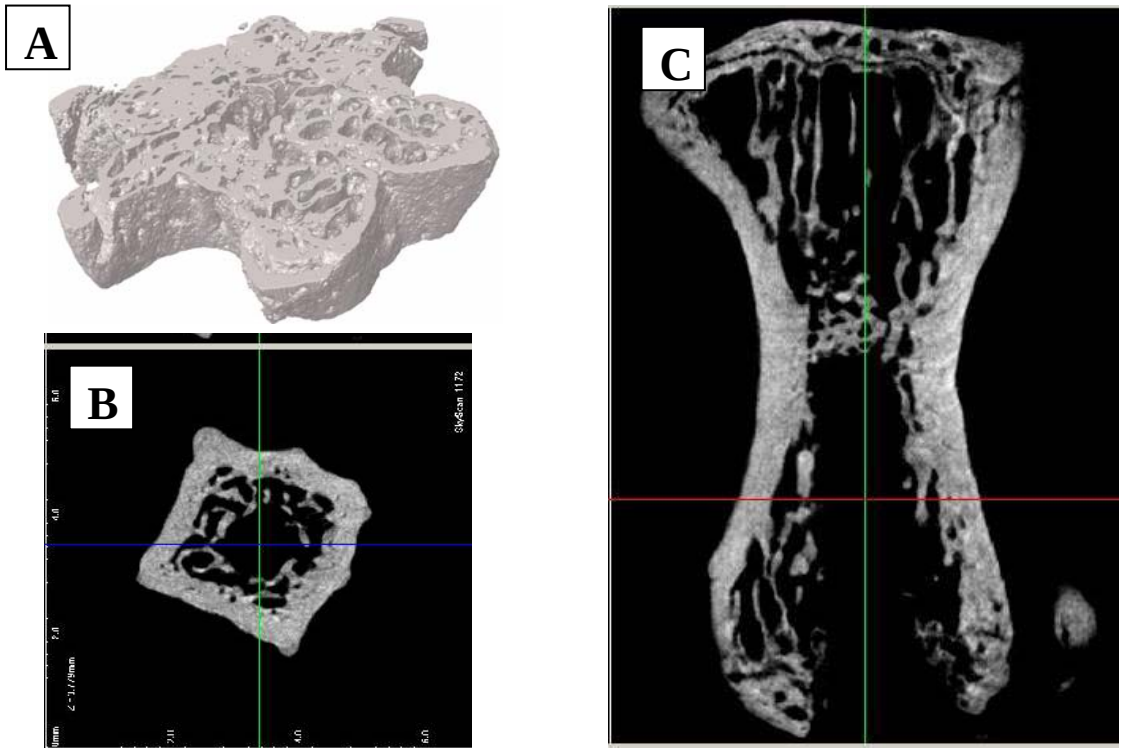

Fig. 4: A) Reconstituted 3D image of the tail vertebra with a free plane-cutting-slice, **B**) Cross section vertebra after 6 weeks of implantation, after extraction of implant from C4 vertebra; **C)** longitudinal section following extraction of the titanium implant. Note the trabecula of spongy bone, the surrounding compact bone and the place of extracted implant. 3D Computer Tomographic images six weeks after treatment. Note that Zometa induced a three-fold increase in new bone formation:

RESULTS

A) Compensation of oral epidermal growth factor (EGF) secretion

1) Our investigations confirmed that salivary glands secrete a wide range of EGF. Partial ablation of the salivary glands (sialoadenectomia) or chronic gustatoric stimulation via citric acid both induce salivary gland hypertrophy. Ablation of the salivary gland induces an increase in mass of the glandula parotis. On the other hand, following ablation, an increased gustatoric stimulus decreases the EGF concentracion by 90% in the gl. Parotis, while the salivary EGF level raises by 20%.

2) A basic recognition confers that the survival time of experimental rats was more then 90 days following removal of major salivary glands, strongly arguing that the major salivary glands are not vital organs.

3) Upon pilocarpin stimulus the contribution of minor salivary glands to total protein secretion was 15% while only 1 to 2 % for the amylase secretion. Minor salivary glands are under vegetative control in rat.

4) Following ablation of major salivary glands, the level of salivary proteins, amylase and EGF raised slowly and modestly. Our results document the persistence of residual EGF in saliva. The results support the idea that following ablation of the major salivary glands the dispersed minor salivary glands compensate the EGF secretion.

5) The results suggest, that our new method may provide a useful tool for the investigation of biofilm physiology in the oral cavity, with particular respect to minor salivary glands in the absence of major salivary glands. Our data, together, shed light on novel, hitherto not well established aspects of the adaptive faculty of minor salivary glands. On one hand, we have identified the amylase enzyme and EGF in the biofilm covering the mucosa and tooth. On the other hand, we show that pilocarpin stimulated the secretion of both, which could be inhibited by propranolol. It can be hypothetized that in human too, in case of accidents, tumour or other causes resulting loss of salivary gland function, the minor salivary glands may substitute for EGF secretion.

B) Elaboration of a new implantation method in rats and analysis of bone regeneration following bisphosphonate treatment

The clinical application of data, obtained on the field of experimental tissue-engineering is conditionned by the availibility of rapid and kvantitative evaluation systems. Modeling in dental implantology is still hampered by the lack of such practical methodology, therefore we have searched for and elaborated a novel system applying the rat tail spongy vertebrae, as a model.

1) Using the rat tail model the kinetics of bone regeneration and osseointegration of implants can be evaluated quantitatively. This technique makes it posible, for the first time, to simultaneously measure the qualitative morphometric and quantitative biomechanical changes of newly–formed bone on the effect of systemic or local therapeutical treatments.

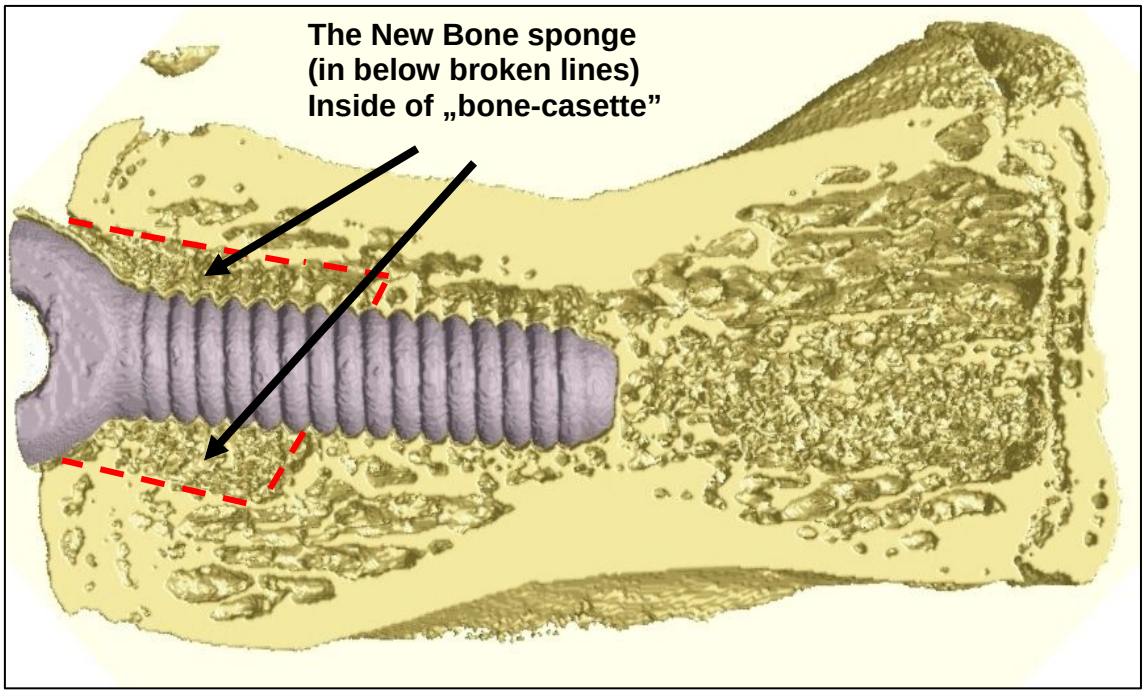

Fig. 5: Three-dimensional view of a titanium screw osseointegration 6 weeks after Zometa treatment (median sagittal layer). Notes, the Old Bone is well preserved and New Bone is finely structured and calcificated.

2) We have tested the usefulness of our implantation method by measuring the effects of a amino-bisphosphonate (Zometa®) on bone neogenesis and consolidation of titanium implant. The results provide evidence that Zometa significantly increased bone neogenesis and inforced the integration of titanium. The results provide new information on the beneficial effects of Zometa in the case of bone regeneration or using titanium implants. On the other hand the method has open up the way how to analyse novel regulatory factors and substitution materials.

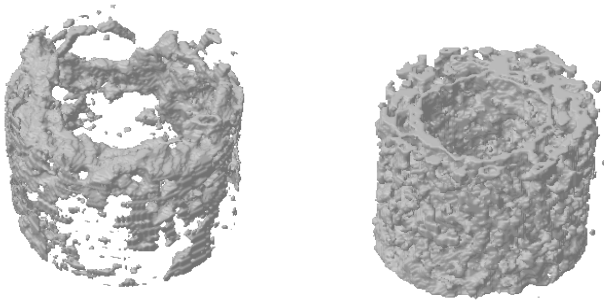

Fig. 7: Zometa treatment significantly increases the volume and mass of newly formed bone tissue. The analyzed tissue volume 2,694 mm^3 (TV=100%), the reference bone volume (RefBV) and the volume of newly formed bone tissue (NewBV) are shown in controls and Zometa treated rats at six weeks of

treatment. There was no significant difference in the standard bone tissues (*NS); the volume of regenerating bone was significantly less in the control group (**p p>0,002), while the bone volume of Zometa treated group reaches the value of standard (reference) (**** NS). The NewBV was more than three-fold higher in the Zometa group, compared to controls (***p>0,0002).

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3) We uncovered, serendipitously, that all 25 caudal vertebrae are aplasic, despite the presence of osteoblasts and osteoclasts there. This discovery provides a guiding cue in understanding the genetic, cellular and molecular control of HSC seeding in BM and the involvement of endosteal osteoblasts in forming the haematopoietic stem cell niche.

CONCLUSIONS

A) Experimental study of the regeneration factor acting in the oral cavity

The results confirm, that our newly elaborated method allows the secretory function of minor salivary glands to measure, in the absence of major salivary glands. Taken together the data obtained from partial and total ablation of the major salivary glands, our results uncovered a hitherto not well recognized and not clearly established faculty of minor salivary glands. First, the amylase enzyme and EGF are stable members of biofilm-forming protein-peptide components that covers the oral mucosa and teeth. Secondly, their production and secretion can be stimulated by pilocarpine, and this stimulation can be blocked by propranolol. When the gl. submandibularis, the main tissue-source of EGF production is removed, this results in a sudden and dramatic fall in the amount of salivary EGF. This functional lack induces a compensatory regeneration in the resident gl. parotis resulting intensive tissue gain and saliva production. Compensatory reaction of the gl. parotis occurs when the submandibulary-sublingual glands, or alternatively, one-side parotis gland are removed, however, the tissue-volume of the draining system remains quasi uneffected. Because the endogenous EGF is pruduced by the draining ductal cells, the amount of reactive EGF is relatively low, compared to the other secretory products. In addition, the amount of EGF in parotis falls logarithmically, following excision of the submandibular glands, indicating for the contribution of these latters, via a hitherto unknown mechanism, in regulation of EGF by the parotis. Alternatively, the low EGF level may be the effect of an intensive „wash-off" mechanism at the level of draining ductuli. Several research teams succided EGF production to demonstrate in minonr salivary glands (Brunner gland). Clearly, what is new here is the reactive EGF production by the minor salivary glands following total ablation. Indeed, our comparative results obtained in ablated and pilocarpin-stimulated

rats support this hypothesis. Compared to the reaction level measured immediately following ablation one dose Pilocarpin stimulation resulted a higher EGF secretion in rats ablated 14 days before. The cumulative EGF production (100minute fractionated collections) was 2.23 ng at day-x compared to 2.63 ng obtained at day 14 (18%, $p<0.01$). The data document that the biofilm covering the oral cavity involves EGF even in absence of the major salivary glands. This observation supports that the integrity of oral mucosa is maintened by reduntant mechanisms, similarly to other biological systems which play a defense role in the organism. There are reason to interpolate the data to human, where under pathological conditions, resulting loss-of-function of major salivary glands (accident, cancer) EGF production is maintained by the minor salivary glands.

B) Experimental anaysis of bone regeneration and osseointegration

The clinical application of data, obtained on the field of experimental tissue-engineering is conditionned by the availibility of rapid and quantitative evaluation systems. Modeling in dental implantology is still hampered by the lack of such practical methodology. Searching for an optimal bone compartment throughout the whole rat skeleton, amenable for the experimental evaluation of osseointegration, we uncovered, serendipitously, that all 25 caudal vertebrae are aplasic, despite the massive presence of osteoblasts and osteoclasts there. This finding is important in both fundamental and applied research fields and the method has open up the way how to analyse novel regulatory factors and substitutive materials. Remarkably, the caudal vertebrae are freely accessibles, permitting microchirurgical operations to realize and their poor BM compositions make them more realistic to interprete experimental data without interference of the bulk bone marrow using the femur as bone support.

Using the rat tail model the kinetics of bone regeneration and osseointegration of implants can be evaluated quantitatively. This technique makes it possible, for the first time, to simultaneously measure the qualitative morphometric and quantitative biomechanical changes of newly–formed bone on the effect of systemic or local therapeutical treatments. We have tested the usefulness of our implantation method by measuring the effects of a amino-bisphosphonate (Zometa®) on bone neogenesis and consolidation of titanium implant. The results provide evidence that Zometa significantly increased bone neogenesis and inforced the integration of titanium. The

results provide new information on the beneficial effects of Zometa in the case of bone regeneration or using titanium implants.

The paucity of caudal vertebrae in active, haematopoietic bone marrow provides a tool and guiding cue in understanding the genetic, cellular and molecular control of HSC physiology (seeding, self-maintenance, developmental potentials) in BM and the involvement of endosteal osteoblasts in forming the haematopoietic stem cell niche. The high osteoblast content of caudal vertebrae raise the question concerning the presence of mesenchymal stem cells in these skeletal domains.

Our ongoing preliminary experiments support the concept that the tail vertebra model can be applied succesfully in the field of human cancer research. Implantation of human tumor tissue (breast, prostate) into the tail vertebrae of NOD/SCID mice may better support tumor cell growth and tumor cell speading (metastasis) as compred to the usually applied subcutaneos transplantation.

NEW FINDINGS - ESENCIAL

Partial ablation of the major salivary gland

1) The EGF secretion is under noradrenalin regulation, in grand salivary glands.
2) Ablation of the submandibulary and sublingual salivary glands does not result in the total disparition of EGF from the secreted saliva.
3) The amount of EGF measured in saliva exceeds the production by one gland.
4) The quantity of EGF in saliva increases by the stimulation of the salivary gland.
5) The gl. Parotid secrets a mass of proteins and EGF even in case of low tissue pool.
6) Salivary glands constitut an unique tissue system, provided by the fact that the function of affected glands are compensated by the complementary, remaining glands.

Total ablation of the major salivary gland

7) Following total ablation of salivary glands the avarage survival time exceeds 90 days, indicating that these glands are not of vital importance.
8) Minor salivary glands in rat are under vegetative regulation, and following pilocarpin stimulus they produce 15% of the whole proteins while only 1-2% of whole amylase.
9) Minor salivary glands have compensatory regenerative potentials at the level of protein and EGF production, following removal of the major salivary glands.

Modelling of bone regeneration and pathophysiological studies

10) Our results revealed, that caudal vertebrae provide an ideal environment to carry out preclinical investigations on bone modifying substances. Remarkably, interference with fully active bone marrow is less compromistic here, due to the paucity in bone marrow parenchyme at this anatomical location. Therefore, we propose that the tail vertebra model represents a more realistic system for large scale comparative studies.

11) Despite the aplastic caracters of bone marrow parenchyma in caudal vertebrae the osteoblast and osteoclast compartments are highly actives. The cellular and molecular mechanisms responsible for this unusual tissue composition are not known. This situation provides a physiological tool to discover some basic regulatory mechanisms operating between normal haematopoietic stem cells and osteoblasts.

12) Previous publications and our ongoing works indicate that new informations on the aplastic tail vertebrae might provide some basic knowledge to better understand the function of bone marrow microenvironment and the disfunctions in the case of myelodysplasia, preleukaemia and acut myeloid leukaemia.

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