COMPREHENSIVE EVALUATION OF NOVEL TREATMENT POSSIBILITIES FOR PERIODONTAL HARD- AND SOFT TISSUE RECONSTRUCTION

Short PhD Thesis

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INTRODUCTION

The limitations of currently applied techniques for periodontal hard- and soft tissue reconstruction related to treatment efficacy and patient morbidity have raised a demand to introduce novel treatment approaches as well as biomaterials aiming at increased treatment efficacy as well as reducing duration of treatment and patient morbidity. During the last decade, emerging new research fields have investigated the possibilities of tissue engineering related to the isolation and differentiation of human adult tooth derived stem cells and application of different recombinant growth-factors for periodontal hard tissue reconstruction as well as application of novel xenogenic materials for reconstruction of soft tissue anomalies.

Stem cell research and possibly related tissue engineering applications have become a promising field for tissue regeneration and implementation of regenerative medicine. Since the discovery and characterization of multipotent mesenchymal stem cells from bone marrow, similar populations from other tissues have now been characterized. Postnatal stem cells have been isolated from a variety of tissues including bone marrow, brain, skin, skeletal muscle and the gastrointestinal tract.
Recent studies have revealed the presence of adult stem cells in tissues of dental origin as well. Namely, primary cell cultures containing progenitor cells originating from both adult and deciduous dental pulp as well as periodontal ligament were described. Cells can be induced in vitro to differentiate into cells of osteogenic/odontogenic phenotype, characterized by polarized cell bodies and accumulation of mineralized nodules. The dental pulp and the periodontal ligament have also been suggested to harbour cells that are able to differentiate into neuronal direction.

While stem cells research and tissue engineering techniques are not yet available for human application, utilising human recombinant growth factors presents a novel promising treatment option for periodontal hard tissue reconstruction. preclinical studies have pointed to a role of growth/differentiation factor -5, -6, and -7 in the formation of the periodontal ligament. rhGDF-5 exhibits osteoinductive properties in vitro and in vivo. A recent study has shown that rhGDF-5/β-TCP implanted in a rat calvarial defect model enhances local bone formation. Taken together, preclinical data suggest that rhGDF-5 may have a significant potential not only to induce/support periodontal wound healing/regeneration but also to support regeneration elsewhere in the axial and appendicular skeleton. Although rhGDF-5 appears to be promising for enhancing periodontal regeneration, until now, it has not been used in humans to treat periodontal defects and thus, the safety and the clinical potential of the material are unknown.

Mucogingival deformities are often associated to advanced periodontal hard tissue defects but may also occur without the presence of periodontitis. For the correction of periodontal soft tissue defects, application of xenogenic grafting materials has been suggested as a promising alternative for connective tissue grafting. Predictable coverage of multiple adjacent gingival recessions (MAGR) still represents a challenge for the clinician due to difficulties in managing the soft tissues and poorer wound healing related to factors such as the large avascular surface, blood supply, differences in recession depth and position of the teeth. Connective tissue graft harvesting is often associated with increase patient morbidity, prolonged surgical time and the possibility of postoperative complications such as bleeding and numbness in the donor area. In order to overcome these inconveniences, attempts are made to develop new materials aiming to replace connective tissue grafts thus, improving patient acceptance and minimizing morbidity. A newly developed porcine derived bioresorbable collagen matrix (CM) (Mucograft®, Geistlich Pharma, Wolhusen, Switzerland) has been recently introduced proposed as an alternative to the subepithelial connective tissue graft (SCTG) in periodontal plastic surgery procedures. Taken together, the available data appear to suggest that CM might represent an alternative to SCTG thus warranting further investigations. However, according to the best of our knowledge, until now no prospective, randomized, controlled, clinical studies have compared treatment of MAGR by means of the modified coronally advanced tunneling technique (MCAT) using either CM or SCTG.

OBJECTIVES

Available data related to in vitro and clinical research on periodontal regenerative therapy have raised a number of fundamental questions dealing with possible future clinical impact of the above mentioned novel regenerative procedures and biomaterials. These goals focus on establishing the methodological basis to develop future tissue engineering applications, as well as safety and efficacy of currently available prototype biomaterials for human periodontal application. The performed in vitro and clinical studies aimed at:

- Establishing cell cultures of periodontal origin, investigating the effect of enamel matrix derivatives (EMD) on cell proliferation, characterisation of adult stem cells in vitro
- Developing in vitro protocols for osteogenic differentiation of periodontal ligament stem cells (PDLSCs) for future tissue engineering applications
- Investigating the safety and efficacy of a human recombinant growth factor on a β-TCP carrier (rhGDF-5) designed for periodontal hard tissue reconstruction in a pilot clinical study
- Investigating the safety and efficacy of a novel collagen matrix (Mucograft®) for gingival recession coverage of MAGR in a pilot clinical case series
- Comparing the clinical outcome and patient satisfaction related to the application of Mucograft® compared to connective tissue grafting in the treatment of MAGR in a split mouth randomised controlled study

METHODS

The present thesis reports on a review article, two in vitro research articles as well as three publications reporting on clinical studies.

In vitro research was carried out at the Department of Oral Biology, Semmelweis University. All patients included in the clinical studies were referred to the Department of Periodontology, Semmelweis University for treatment of periodontal soft- or hard tissue defects.

Study I. A literature review was performed to collect relevant informations prior to initiating further in vitro and clinical periodontal research. To collect valuable informations, the application of EMD in periodontal regeneration compared to alternative treatment options (e.g. guided tissue regeneration - GTR) was analysed based on currently available literature data.

Study II. In the first in vitro study cell cultures from human periodontal ligament were established and multipotential adult stem cells (PDLSCs) were identified in these cultures. The effect of EMD was also analysed with regards to viability of cells cultures. We established the methodological basis for further in vitro research.

Study III. The second in vitro study described the introduction of differentiation protocols applicable for maintainable cell cultures containing PDLSCs and dental pulp stem cells (DPSCs). Using optimized pharmacological protocols the potential of periodontal and pulp derived adult stem cell cultures to form mineralized tissues and to undergo neuronal differentiation was analysed.

Study IV. The first clinical exploratory study was specifically designed to evaluate the clinical and histological outcomes following treatment of intrabony defects with open flap debridement alone or in combination with rhGDF-5 adsorbed onto a particulate β-tricalcium phosphate carrier. The publication reported on the study protocol, safety profile, the early healing phase and the clinical outcomes at 24 weeks while the histological outcomes were presented and discussed in great detail in a subsequent paper (Stavropoulos et al. 2011).

Study V. The second clinical study presented data from a prospective pilot case series, which was performed to evaluate the safety and efficacy of Mucograft® in the treatment of Miller class I and II MAGR using the MCAT technique.

Study VI. The third clinical study reported on a prospective, randomized, controlled, split-mouth clinical study. This was conducted to clinically evaluate the treatment of Miller class I and II MAGR using the MCAT technique either in combination with Mucograft® or SCTG.

RESULTS

Study I. Literature review:

Based on the presented evidences the following statements could be made:

a) Surgical periodontal treatment of deep intrabony defects with EMD promotes periodontal regeneration. The application of EMD in the context of non-surgical periodontal therapy has failed to result in periodontal regeneration.

b) Surgical periodontal therapy of deep intrabony defects with EMD may lead to significantly higher improvements of the clinical parameters than open flap debridement alone. The results obtained following treatment with EMD are comparable to those following treatment with GTR and can be maintained over a longer period.

c) Treatment of intrabony defects with a combination of EMD + GTR does not seem to additionally improve the results compared to treatment with EMD alone or GTR alone.

d) The combination of EMD and some types of bone grafts/bone substitutes may result in certain improvements in the soft and hard tissue parameters compared to treatment with EMD alone. However, further studies are needed in order to definitively clarify the possible advantage of a combination therapy of EMD and bone grafts/bone substitutes in relation to the single therapies.

e) Treatment of recession-type defects with coronally repositioned flaps and EMD may promote formation of cementum, periodontal ligament and bone and may significantly increase the width of the keratinized tissue. Application of EMD seems to provide better long-term results than coronally repositioned flaps alone.

f) Application of EMD may enhance periodontal regeneration in mandibular class II furcations. The clinical results are comparable to those obtained following GTR.

Study II. Isolation and characterisation of tooth derived stem cells:

We were able to show the ability of both periodontal ligament-derived and pulp-derived cells to form adherent clonogenic cell clusters of fibroblast-like cells, similar to those recorded for other mesenchymal stem-cell populations. These colony-forming cell populations, which we termed PDLSCs and DPSCs had high proliferation rate, as demonstrated by the doubling of the cell number during culture in about two days. A mean of 25-30 colony forming units were detectable within 10^5 cells, this number is well in line with data in literature referring to mesenchymal stem cells. For this reason, cells were passaged once a week until they reached confluence. Monolayers were usually formed 2 weeks following tissue preparation and isolation at the time point of reaching confluence, proliferation rate of cells in monolayers decreased due to contact inhibition. We managed to maintain isolated PDL cells within monolayer cultures, in several cases well over 20 passages. To test the importance of serum in the test medium, cells were serum-starved for 24 h and then either received 20% FBS or were left in serum-free a-MEM medium. To validate our assay system, as an internal

control, we plated only half of the cells into some wells. Our data revealed that FBS stimulated cell proliferation compared to serum-free controls.

According to our results, effect of EMD compared to serum starving at the concentration of 25, 50, 100, 200, 400 μg/ml was not linear. EMD yielded a significantly proliferative effect at concentrations of 200 and 400 μg/ml on the viability of PDL derived primary cultures. Migration of PDL cells was observed following the treatment of plastic culturing surfaces with EMD towards EMD droplets. This was confirmed via phase contrast light microscopy.

A fraction of the cells in DPSC and PDLSC cultures expressed the cell surface molecule STRO-1, a mesenchymal stem cell marker, which is also present in bone-marrow derived and periodontal ligament derived stem cell cultures. STRO-1 immuno-reactivity gradually decreased with increasing passage numbers, but 8,47% of the cells were still STRO-1 positive even at higher passage numbers. CD34, a characteristic marker of haemopoetic stem cells showed a prevalence of 21%, while c-kit embryonic stem cell marker positivity was detected in 18% of the cells in PDL cultures.

Study III. Osteogenic and neuronal differentiation of tooth derived stem cells:

In these experiments DPSC and PDLSC cultures were grown in the presence of osteogenic differentiation cocktail consisting of dexamethazone, L-ascorbic acid 2-phosphate and b-glycerophosphate. Under these conditions cultures uniformly demonstrated the capacity to form Alizarin red S positive condensed nodules with high calcium content. The deposits were sparsely scattered throughout the adherent layer as single mineralized zones. Control cultures formed adherent layers without any sign of calcium deposition. Thus, this observation confirms the previous findings that DPSCs and PDLSCs are capable of differentiation to mineralized tissue in vitro in response to appropriate pharmacological stimulation.

While neurodifferentiation Protocol 1 and Protocol 2 resulted in short term and reversible neuronal differentiation or even death of the cells after 48 h, Protocol 3, our three step differentiation procedure over 9 days resulted in a robust differentiation of both pulp cultures and periodontal cells towards neural lineages in essentially all surviving cells that initially showed the characteristics of dental fibroblasts. After 9 days of differentiation, the vast majority of cells, derived from either the dental pulp or the periodontal ligament, displayed complex neuronal morphology, expressing both bipolar and stellate forms. However, a small portion of cells retained their flat shape, and were attached beneath the processes of the neuronal cells. These elements are presumably committed towards glial fates, or serve as stanchions for the developing neuronal cells. Therefore, they might be indispensable for neuronal survival. DPSCs and PDLSCs showed a very similar expression pattern during neuronal differentiation treatment. There was a sharp decrease in the expression of the mesenchymal marker vimentin in response to neurogenic induction. This very striking decrease became less pronounced during maturation. The expression of the neuronal marker NSE progressively increased under maturing conditions and remained increased during maturation. The protein expression pattern investigated by immunocytochemistry corresponded well to the gene expression data.

Study IV. Healing following rhGDF-5/β-TCP treatment – randomised controlled study

Healing following surgeries progressed without major complications. Primary intention healing was observed in nine of ten patients in the rhGDF-5/β-TCP group, and in all ten patients in the control group. One patient in the rhGDF-5/β-TCP group experienced a slightly delayed epithelialization of the interdental papilla resulting in complete wound closure within 3 weeks. At 6 months, both treatments resulted in significant improvements in terms of PD reduction and CAL gain compared to baseline. Treatment with rhGDF-5/β-TCP resulted in higher, but statistically not significant, PD reduction (3.7±1.2 vs. 3.1±1.8 mm; p=0.26) and CAL gain (3.2±1.7 vs. 1.7± 2.2 mm; p=0.14) compared to the control.

All patients showed negative anti-rhGDF-5 antibody levels at screening and Visits 3 and 9. No relevant rhGDF-5 plasma levels were detected in any patient. Two patients, both belonging to the rhGDF-5/β-TCP group showed rhGDF-5 plasma levels at screening (Visit 1) and postsurgery (Visits 3 and 9). One patient showed a plasma level of 46.7 pg/mL rhGDF-5 at baseline (before implantation of rhGDF-5/β-TCP) vs. 47.3 pg/mL at Visit 3, and 57.9 pg/mL at Visit 9.The other patient showed a plasma level of 514 pg/mL rhGDF-5 at baseline vs. 427 pg/mL at Visit 3, and 690 pg/mL at Visit 9. Due to the fact that the measured rhGDF-5 plasma levels were already positive at the baseline (before periodontal surgery) and did not increase throughout the study, a causal relationship to study medication can be excluded. For all other samples, rhGDF-5 plasma levels were below the limit of quantification (<40.0 pg/mL). It may, thus, be anticipated that these results were false positive.

Study V. Healing following treatment with Mucograft® - case series

The postoperative healing was uneventful in all 8 cases. No complications such as allergic reactions, matrix exfoliations, abscesses or infections were observed throughout the entire study period. All patients completed the study and no patient was lost during follow-up. All patients expressed improvement in root sensitivity. At 12 months CRC was obtained in 2 out of the 8 patients and in 30 out of the 42 recessions (71%). MRC was 84%. Mean GRD, GRW, GT and KTW improved statistically highly significantly ($p<0.0001$) compared to baseline while PD did not show statistically significant differences.

Study VI. Healing following treatment with Mucograft® compared to SCTG – randomised controlled study

All patients completed the study and attended all recall visits. Exposure of the CM was not observed in any of the cases. No adverse events related to both treatment modalities were recorded. At 12 months, KGW increased on average from 2.1 ± 0.9mm to 2.4 ± 0.7 mm on test sites and from 2.0 ± 0.7 mm to 2.7 ± 0.8 mm on control sites. The difference between the two treatments was not statistically significant. At 12 months, there was no difference in the mean value of PD on test sides compared to control sides. Both treatment groups showed significant post-surgical improvement in GRD and clinical attachment gain, when compared to baseline. In the test group, mean GRD decreased significantly from 1.9 ± 0.6 mm at baseline to 0.6 ± 0.5 mm at 12 months while in the control group the corresponding values were 1.8 ± 0.5 mm and 0.2 ± 0.3 mm, respectively. Both treatments resulted in statistically significant CAL gain (1.9 ± 0.6mm mm and 1.4 ± 0.4 mm for test and control groups, respectively).

When results were expressed as percentage of root coverage at 1 year, both treatment modalities resulted in a statistically significant percentage of root coverage amounting to 71% ± 21 % in the test group and 90% ±18 % in the control group, respectively. The difference between the two groups was statistically significantly greater for the control treatment ($p= 0.0004$). At 12 months, CRC was recorded in 5 patients in the test and in 13 patients in the control group, respectively and was statistically significantly greater for the control treatment ($p= 0.0305$). In both groups GRW decreased statistically significantly between baseline and 6 months and 12 months. The differences between the two groups were not statistically significant ($p>0.05$). Mean surgery time was significantly lower ($p <0.0001$) in the test (i.e. 42.5 ± 4.8 min) compared with control (i.e. 58.6 ± 6.6 min). Postoperative complaints on the VAS scale were lower for CM. All patients reported a decrease in root sensitivity.

CONCLUSIONS

Study I.

Surgical periodontal treatment of deep intrabony defects with EMD promotes periodontal regeneration. The application of EMD in the context of non-surgical periodontal therapy has failed to result in periodontal regeneration. Surgical periodontal therapy of deep intrabony defects with EMD may lead to significantly higher improvements of the clinical parameters than open flap debridement alone. The results obtained following treatment with EMD are comparable to those following treatment with GTR and can be maintained over a longer period. Nevertheless, in cases with advanced attachment loss a different treatment approach may be needed to overcome limitations of current regenerative techniques.

Study II.

Isolation, culturing and characterization of stem cell cultures periodontal ligament origin was successful. Our in vitro model allows for further in vitro and in vivo investigations of periodontal regenerative procedures. Our present findings establish the methodological basis for future research on proliferation and differentiation of PDLSCs. The identified adult stem cells of periodontal origin might be used in future tissue engineering applications aiming at periodontal regeneration.

Study III.

Our data demonstrated that both DPSC and PDLSC cultures may differentiate into either osteogenic or to a neuronal fate in response to appropriate pharmacological treatments. Further investigations are still necessary to optimise these procedures, enabling the utilisation of the differentiation potential of dental human stem cell cultures. Nevertheless, it is already clear that both the human dental pulp and the periodontal ligament is a potential source for tissue engineering not only in aspects related to dental bone regeneration, but also for neuroregenerative applications.

Study IV.

The findings of this study indicate that in the tested application the use of rhGDF-5/β-TCP appeared to be safe and the material possesses a sound biological rationale. Thus, further, adequately powered, randomised controlled clinical trials are warranted to confirm the clinical relevance of this new approach in regenerative periodontal therapy.

Study V.

Within their limits, our results indicate that treatment of Miller Class I and II multiple adjacent gingival recessions by means of MCAT and Mucograft® may result in statistically and clinically significant root coverage. Further studies are thus warranted to evaluate the performance of Mucograft® compared to connective tissue grafting and other treatment alternatives.

Study VI.

The presented findings indicate that the use of Mucograft® may represent an alternative to connective tissue grafting by reducing surgical time and patient morbidity but yielded lower root coverage than connective tissue grafting in the treatment of Miller Class I and II multiple adjacent gingival recessions when used in conjunction with the MCAT technique.

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