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Ich bedanke mich bei den unten aufgeführten Kolleginnen und Kollegen für ihre wertvolle Mitarbeit, die sie in den vergangenen zwei Jahren geleistet haben.

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Infected periodontal granulation tissue contains cells expressing embryonic stem cell markers

A pilot study

Key words: granulation tissue, periodontitis, stem cell, embryonic

Summary The commonly practiced removal of granulation tissue during periodontal surgery, aiming to eliminate infection and optimize healing conditions, may also remove progenitor stem cells that could otherwise support periodontal regeneration. The present study aimed to investigate if cells with embryonic stem cell properties are present in periodontal granulation tissue. During the course of flap surgery inflammatory granulation tissue was obtained from four patients and five periodontal defects. Tissues were processed in a collagenase/dispase solution to release the cells. Part of the resulting suspension was processed for bacteriological analysis (IAI PadoTest 4.5), whereas the remaining cell suspension was cultured and passaged once. Upon reaching confluence, total RNA was extracted, followed by cDNA synthesis. PCR was then performed (SYBR Green-based protocols) to measure gene expression levels of Collagen type I, and embryonic stem cell markers Nanog, Oct4, Rex-1 and Sox2. Results are expressed as $2^{-\Delta Ct}$ values of the target gene, calibrated against a housekeeping gene (GAPDH). A high total bacterial load up to $20.6 \pm 11.0 \times 10^6$ counts/mg of tissue was found. Collagen type I was strongly expressed, confirming the predominance of mesenchymal/fibroblastic cells. Among the studied embryonic stem cells markers, Nanog was most highly expressed (2.3 ± 1.2), followed by Oct4 (1.1 ± 0.5), Rex-1 (0.6 ± 0.2) and Sox2 (0.3 ± 0.2). This is the first study that demonstrates the presence of cells expressing embryonic stem cell markers among infected granulation tissue. This knowledge needs to be considered when devising future strategies to improve periodontal wound healing and regeneration.

Introduction

Removal of pocket granulation tissue during periodontal flap surgery procedures was developed with the aim to improve the conditions for wound healing and new attachment formation. Nevertheless, later studies indicated that removal of this tissue in conjunction with flap surgery is not critical for establishing conductive conditions for the efficient healing of the periodontal tissues (Lindhe & Nyman 1985). Hence, the implications of the removal of this granulation tissue on the repair and/or regeneration process of the periodontium have been interpreted in different ways over time. The concept that progenitor stem cells may reside in the periodontal tissues that give rise to virtually all periodontal tissues was first proposed in the '80s (Melcher 1985). Studies of the last decade have indeed identified stem cells in the periodontal ligament tissue, with the multi-potent capacity to generate osteoblast-like and cementoblast-like cells *in vitro,* as well as cementum-like and periodontal ligament-like tissues when transplanted *in vivo* in mice (Seo et al. 2004, Gronthos et al. 2006).

The fact that granulation tissues formed after tooth extraction can differentiate into bone, filling up the empty socket,

indicates that some precursor cells are present (Steiner et al. 2008, Trombelli et al. 2008). However, the presence of multipotent progenitor stem cells has not been demonstrated in the case of infected granulation tissues from chronic periodontitis lesions. It is hypothesized that the commonly practiced removal of granulation tissue during periodontal surgery may also result in removal of vital cells with characteristics of multipotent stem cells, which could support tissue healing. Better characterization of the nature of the removed granulation tissue may improve our understanding of the conditions that favour regeneration, rather than repair, of the periodontal tissues. Therefore the aim of the present study was to establish primary cell cultures from periodontal granulation tissues removed during the course of routine periodontal surgery, and to investigate the presence and expression levels of characteristic embryonic stem cell markers within these cultures.

Materials and Methods

Patient demographics and diagnosis

Ethics approval for this study was obtained from the Ethics Commission of the Canton of Zürich (KEK-ZH-NR: 2010-0016/0). All patients were treated in the Periodontology postgraduate clinic of the Center of Dental Medicine and have given their informed consent to participate in the study according to the recommendations of the declaration of Helsinki. Periodontal disease was classified as described by the International Workshop for a Classification of Periodontal Diseases and Conditions in 1999 (Armitage 1999). Patients with either severe chronic (CP) or severe aggressive periodontitis (AgP) were included. All patients were non-smokers. They had already received a non-surgical periodontal therapy, consisting of subgingival scaling and root planing under local anesthesia. Reevaluation after non-surgical therapy took place after at least eight weeks of healing time. Only the first patient had also received antibiotics during this treatment phase (amoxicillin 500 mg and metronidazole 500 mg 3 times per day for 10 days). Since patients were in need of further treatment, i. e. periodontal surgery in residual pockets of at least 6 mm probing depth that were positive for bleeding on probing, these residual pockets underwent routine periodontal surgery, in the course of which granulation tissue was obtained.

Tissue collection and processing

The removed tissues were first weighted on a precision scale. Thereafter they were processed for 2 h in 1 ml of digestion solution consisting of 3 mg/ml collagenase and 4 mg/ml dispase (Sigma-Aldrich, St. Louis, U. S. A.) in continuous rocking movement on a heating block set at 37 °C. One hundret µl of the resulting suspension was used for bacteriological analysis (IAI PadoTest 4.5, Zuchwil, Switzerland), whereas the remaining cell suspension was seeded on 75 mm^2 culture flasks, and cultured further in 5% CO_2 at 37 °C in DMEM/F-12 media, which was supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin, 0.05 µg/ml fungizone (all from Sigma-Aldrich) and 10% foetal bovine serum (PANSERA ES, PAN Biotech, Aidenbach, Germany). The established cell cultures were passaged once, using Stem Proacutase Cell Dissociation Reagent (Invitrogen, Paisley, U.K.) during the StemPro Acutase detachment procedure. Upon reaching confluence, the cell cultures were processed for gene expression analysis.

RNA extraction and cDNA synthesis

Total RNA was extracted from the cells with the RNeasy Mini Kit (QIAGEN, Hombrechtikon, Switzerland), according to the manufacturer's instructions. One µg of extracted total RNA was then reverse transcribed into single stranded cDNA synthesis using M-MLV Reverse Transcriptase, Oligo(dT)15 Primers, and PCR Nucleotide Mix according to the manufacturer's instructions (Promega, Madison, U.S.A.), at 40 °C for 60 min, and 70 °C for 15 min.

Quantitative real-time Polymerase Chain Reaction (qPCR)

For gene expression analysis in the established cell cultures, qPCR was performed using SYBR-Green-based protocols in an ABI Prism 7000 Sequence Detection System and software (Applied Biosystems, Carlsbad, U. S. A.). The studied genes were collagen type I, and the embryonic stem cell markers Nanog, Oct4, Rex-1 and Sox2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control (housekeeping gene). For the amplification reactions, the qPCR SYBR Master Mix was used (Applied Biosystems, Carlsbad, U. S. A), in combination with oligonucleotide primers, specifically designed for the indicated genes. The oligonucleotide primer sequences are provided in Table I. The standard PCR conditions were 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 seconds, 60 °C for 1 min, and 72 °C for 30 seconds. The expression levels of these five target genes in each sample were calculated by the comparative Ct method ($2^{-\Delta Ct}$ formula), after being normalized to the Ct value of the GAPDH housekeeping gene.

Microbiological analysis

Part of the digested tissue suspension was immediately sent at the Institut für angewandte Immunologie (IAI) AG, Zuchwil, Switzerland for microbiological analysis using a commercially

Tab. I Oligonucleotide primer sequences (forward and reverse primer)

Gene	Accession Number	Sequence
Collagen type Iα1	(NM_000088)	Forward: AAGATGGACTCAACGGTCTC Reverse: CAGGAAGCTGAAGTCGAAAC
NANOG	(NM_024865)	Forward: CCTTCCAATGTGGAGCAACC Reverse: CTCGCTGATTAGGCTCCAAC
OCT4	(NM_002701)	Forward: GTTGATCCTCGGACCTGGCT Reverse: ATCGGAGTTGCTCTCCACCC
SOX2	(NM_003106)	Forward: AGATGCACAACTCGGAGATC Reverse: GTCATGGAGTTGTACTGCAG
REX-1	(NM_020695)	Forward: ATGGCGTCCAAGACTACCAC Reverse: TGAGCGAGAAGCTGGTCTTG
GAPDH	(NM002046)	Forward: CAGCCTCCCGCTTCGCTCTC Reverse: CCAGGCGCCCAATACGACCA

available microbiological test (IAI Pado Test 4.5). This RNA-based molecular method enables the quantification of bacterial ribosomal 16s rRNA, providing a reliable numeric estimate for total or specific viable bacterial cells in the samples.

Results

Patient and periodontal site defect characteristics are provided in Table II. The five tissue samples were obtained from infrabony defects of four patients. In one patient, an additional sample from a furcation defect was also collected. Upon surgical removal, each of the five samples was weighted. On average, tissue weight was 53±39.3 mg. After dispersion of the tissue, part of the suspension was forwarded for microbiological analysis by the IAI Pado Test 4.5 (Tab. II). A high total bacterial load was evident, which was on average 26.4±11.3×10^6 counts/mg of tissue. The presence of specific putative periodontal pathogens was also investigated. *Aggregatibacter actinomycetemcomitans* was detected in 0/5 granulation tissues, *Porphyromonas gingivalis in 2/5, Tannerella forsythia* in 1/5 and *Treponema denticola* in 2/5. The cell cultures established from these granulation tissues were analyzed by qPCR for the expression of various genes. Among them, collagen type I was expressed at high levels by the cells, at a relative expression of 144.1±46.7. The expression of four consensus embryonic stem cell markers, namely Nanog, Oct4, Rex-1 and Sox2, was further analyzed (Fig. 1). It was found that these markers were indeed expressed by the cells in culture, but at levels 2-log lower than Collagen type I. Among these four studied genes, Nanog was the most highly expressed (2.3±1.2), followed by Oct4 (1.1±0.5), Rex-1 (0.6±0.2) and Sox2 (0.3±0.2).

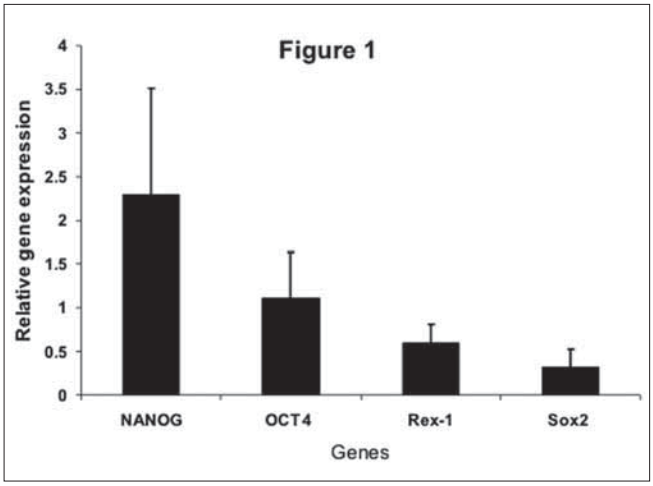

Fig. 1 Gene expression of embryogenic stem cell markers in cells cultures established from infected gingival granulation tissues. The relative expression of the genes is expressed as the $2^{-\Delta Ct}$ formula. The bars represent the mean relative expression ± standard deviation of the five cell cultures obtained by the respective granulation tissues.

Discussion

Earlier studies have identified the presence of cells with characteristics of putative mesenchymal stem cells in regenerating periodontal tissues. Such cells were localized particularly in the paravascular and extravascular regions of non-infected healing granulation tissue, based on the detection of the markers STRO-1, CD44 and CD146 (Lin et al. 2008). In a recent study, stem cells with osteogenic, cementogenic and adipogenic capacities were also isolated from inflamed human periodontal ligament tissue, demonstrating that the periodontal ligament tissue can retain its regenerative potential even under inflammatory conditions (Park et al. 2011).

The present study was designed to investigate the expression of embryonic stem cell markers in cell cultures established from gingival granulation tissues removed during the course of periodontal flap surgery. The high total bacterial load detected confirms the presence of infection in these tissues. This observation is not surprising as it could be expected that granulation tissue next to a periodontal defect would not be sterile, and that non-surgical therapy may not completely eliminate all bacteria in the adjacent inflamed tissues. It is possible that these bacteria have either colonized the pocket epithelium or invaded deeper into the periodontal (potentially granulation) tissue. However, it is important to note, that the selected periodontal pathogens were only detected occasionally. On the other hand, the high gene expression of Collagen type I in the established cell cultures confirms that the cell populations contained within the granulation tissue are of mesenchymal or fibroblastic origin. Earlier studies have indeed confirmed the presence of diverse fibroblastic populations within the gingival granulation tissues, which exhibit lower proliferation rates compared to gingival fibroblasts from healthy tissues (Larjava et al. 1989, Hakkinen & Larjava 1992). Recent evidence suggests that granulation tissue obtained from intrabony defects during surgery contains mesenchymal stem cell populations that express the STRO-1 marker (Hung et al. 2011). The present study is the first to confirm the existence of cells with embryonic stem cell properties in periodontal

Tab. II Patient, site and tissue characteristics

	Patient and site characteristics					Tissue characteristics	
	Age (yrs)	Gender	Diagnosis	Pocket depth (mm)	Defect morphology	Tissue weight (mg)	TBL/weight*
Sample 1	41	f	CP	8	Infrabony	25	30.6
Sample 2	18	f	AgP	7	Infrabony	28	43.5
Sample 3	42	f	CP	6	Infrabony	42	18.7
Sample 4	36	m	AgP	8	Furcation	47	24.3
Sample 5	36	m	AgP	14	Infrabony	121	14.9
Average	34.6	N/A	N/A	8.6	N/A	52.6	26.4
SD	9.7	N/A	N/A	3.1	N/A	39.3	11.3

Abbreviations: yrs = years; f = female; m = male; CP = chronic periodontitis; AgP = aggressive periodontitis; TBL = total bacterial load; * TBL×10^6 counts/mg of tissue.

granulation tissue. To this extent, the cell cultures established from these granulation tissues expressed a panel of embryonic stem cell markers, including Oct4 (Boiani et al. 2004), Rex-1 (Ben-Shushan et al. 1998), Nanog (Zhang et al. 2009, Bais et al. 2012), and Sox2 (Avilion et al. 2003), which are crucial for the pluripotent capacities of stem cells. All four markers were indeed expressed in the cell cultures obtained from all five granulation tissues. This provides evidence that periodontal granulation tissue contains cells with embryonic stem cell properties. Among these markers, Nanog demonstrated the highest expression, followed by 2-fold lower levels of Oct4, Rex-1 and Sox2, sequentially. It is not clear at this stage how these markers can contribute to the cell dynamics of the gingival tissue, or what is the importance of the relative expression levels of each one of them. Nevertheless, the present findings imply that surgical removal of granulation tissue inevitably results in removal of pluripotent stem cells that might potentially contribute to the healing of the tissue, once the infection is controlled. This knowledge needs to be considered further in treatment approaches that aim in optimal periodontal wound healing.

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Résumé

La parodontite est une maladie inflammatoire des gencives, qui a pour conséquences la fonte des tissus mous et osseux. Les défauts osseux résultant sont remplis de tissu de granulation, qui est éliminé au cours de la chirurgie parodontale. Cette pratique a pour but de créer des conditions sans signes d'inflammation des défauts osseux et d'encourager la guérison des tissus atteints. Mais la chirurgie parodontale présente aussi un risque en éliminant des cellules souches et progénitrices qui pourraient éventuellement encourager la régénération parodontale.

Le but de cette étude était d'analyser le tissu de granulation parodontale sur la présence de cellules à potentiel régénérateur, c'est-à-dire d'examiner les propriétés des cellules souches embryonnaires. Dans un même temps, la colonisation bactérienne du tissu de granulation parodontale a été analysée. L'étude a été approuvée par la Commission d'éthique du canton de Zurich (ZH-CEC n°: 2010-0016/0). Tous les patients ont été informés du but et de la procédure de l'étude à la fois verbalement et par écrit. Ils ont donné leur consentement à la participation de l'étude par écrit.

Dans cette étude pilote, les tissus inflammatoires de cinq sites de quatre patients atteints de parodontite ont été extraits et examinés. Il s'agissait de tissus de poches résiduelles d'au moins 6 mm de profondeur, qui répondaient positivement au «saignement au sondage». Ces dernières ont été soumises à la phase corrective de la chirurgie parodontale après achèvement de la phase hygiénique. Le tissu de granulation, qui normalement est éliminé, a été pesé et ensuite traité dans une solution de collagénase/dispase pour séparer les cellules. Une partie de cette solution a ensuite été examinée en utilisant un test de bactéries disponible dans le commerce (IAI PadoTest 4.5, Zuchwil, Suisse), sur la charge bactérienne totale (TBL). La suspension de cellules restante a été incubée et cultivée à 37 °C et 5% de CO_2 dans un milieu contenant 2 mM DMEM/F-12 L-glutamine, 1% de pénicilline/streptomycine, 0,05 microgrammes/ml Fungizone (tous les produits de Sigma-Aldrich, Saint- Louis, Etats-Unis) et 10% de sérum de veau fœtal (ES Pansera, PAN Biotech, Aidenbach, l'Allemagne a été après). Après avoir atteint une confluence, les cellules ont été «panachées» une fois. Une nouvelle croissance confluente a été de nouveau attendue, suite à laquelle l'ARN total a été isolée en utilisant le kit RNeasy Mini (Qiagen, Hombrechtikon, Suisse), puis lavée et soumise à la synthèse d'ADNc. Une réaction en temps réel en chaîne par polymérase (PCR) a été effectuée en utilisant le protocole basé «SYBR Green», afin d'obtenir l'expression du gène de collagène de type I et les cellules souches embryonnaires avec les marqueurs Nanog, Oct4, Rex-1 et Sox2. Les résultats sont exprimés en valeurs $2^{-\Delta Ct}$ du gène cible, après l'étalonnage par rapport au «gène de ménage» GAPDH.

Une charge bactérienne (TBL) allant jusqu'à $20,6 \pm 11,0 \times 106$ bactéries par mg de tissu a été trouvée. Les résultats de la PCR ont montré que le collagène de type I était fortement exprimé. Ainsi la prédominance de cellules mésenchymateuses/fibroblastique dans le tissu de granulation a été confirmée. Parmi les tests de marqueurs de cellules souches embryonnaires utilisés, Nanog a été exprimé le plus fortement ($2,3 \pm 1,2$) parmi tous les patients, suivi par Oct4 ($1,1 \pm 0,5$), Rex-1 ($0,6 \pm 0,2$) et Sox2 ($0,3 \pm 0,2$).

Cette étude est la première du genre, prouvant l'existence de cellules dans les tissus de granulation parodontaux ayant des propriétés de cellules souches embryonnaires. Cette constatation doit être gardée à l'esprit en vue de futures méthodes et développements pour améliorer la cicatrisation ou même pour favoriser une régénération parodontale.

Zusammenfassung

Parodontitis ist eine entzündliche Erkrankung des Zahnhalteapparates, die mit Weichgewebeverlust und Knochenabbau einhergeht. Die dabei entstehenden Knochendefekte sind mit Granulationsgewebe gefüllt, welches im Zuge von parodontalchirurgischen Eingriffen entfernt wird. Diese allgemein anerkannte Praktik bezweckt das Schaffen entzündungsfreier Verhältnisse und heilungsfördernder Defektmorphologien. Sie birgt allerdings auch das Risiko der Entfernung von Progenitor-Stammzellen, welche bei Belassen des Gewebes im Defekt eventuell der Regeneration förderlich sein könnten.

Ziel dieser Studie war es, parodontales Granulationsgewebe auf die Anwesenheit von Zellen mit Regenerationspotenzial, d. h. mit Eigenschaften von embryonalen Stammzellen, zu untersuchen. Des Weiteren sollte die bakterielle Besiedlung von parodontalem Granulationsgewebe analysiert werden. Die Studie wurde von der Ethikkommission des Kantons Zürich (KEK-ZH-NR: 2010-0016/0) genehmigt. Alle Patienten wurden über das Ziel und die Durchführung der Studie sowohl mündlich wie auch schriftlich aufgeklärt und haben ihr schriftliches Einverständnis zur Studienteilnahme gegeben.

Das Entzündungsgewebe von fünf Defekten von vier Parodontitispatienten, welche in der Assistentenklinik des Zentrums für Zahnmedizin der Universität Zürich behandelt wurden, wurde entfernt und untersucht. Es handelte sich hierbei um Gewebe aus Resttaschen von mindestens 6 mm Tiefe, welche positiv auf «Blutung auf Sondierung» reagierten. Diese wurden nach erfolgreich abgeschlossener Hygienephase im Rahmen der korrektiven Parodontitistherapie einer routinemässigen Lappenoperation unterzogen. Das Granulationsgewebe, welches im Normalfall verworfen wird, wurde gewogen und danach in einer Kollagenase/Dispase-Lösung verarbeitet, um die Zellen aus ihrem Verbund zu lösen. Ein Teil dieser Lösung

wurde daraufhin mit einem kommerziell erhältlichen Bakterientest (IAI PadoTest 4.5, Zuchwil, Schweiz) auf den Total Bacterial Load (TBL) untersucht. Die restliche Zellsuspension wurde bei 37 °C und 5% CO_2 in einem DMEM/F-12 Medium, welches mit 2 mM L-Glutamin, 1% Penicillin/Streptomycin, 0.05 µg/ml Fungizone (alle Produkte von Sigma-Aldrich, St. Louis, U.S.A.) und 10% fetalem bovinem Serum (PANSERA ES, PAN Biotech, Aidenbach, Deutschland) versetzt wurde, kultiviert. Nach Erreichen der Konfluenz wurden die Zellen einmal «gesplitted». Es wurde wieder ein konfluierendes Wachstum abgewartet, im Anschluss daran wurde die Total RNA mit dem RNeasy Mini Kit (Quiagen, Hombrechtikon, Schweiz) extrahiert und einer cDNA-Synthese unterzogen. Eine real-time polymerase chain reaction (PCR) wurde mithilfe von SYBR-Green-basierten Protokollen durchgeführt, um die Gen Expression von Kollagen Typ I und den embryogenen Stammzell-Markern Nanog, Oct4, Rex-1 and Sox2 zu erhalten. Die Ergebnisse wurden als $2^{-\Delta Ct}$ Werte des Zielgens, nach Kalibration gegen das «housekeeping Gen» GAPDH, dargestellt.

Bei der bakteriellen Analyse wurde ein hoher Total Bacterial Load (TBL) von durchschnittlich $26.4 \pm 11.3 \times 10^6$ Bakterien pro mg Gewebe festgestellt. Die Ergebnisse der PCR zeigten, dass Kollagen Typ I stark exprimiert wurde, wodurch die Prädominanz von mesenchymalen/fibroblastischen Zellen im Granulationsgewebe bestätigt wurde. Unter den untersuchten embryonalen Stammzellmarkern wurde Nanog bei allen Patienten am höchsten exprimiert (2.3 ± 1.2), gefolgt von Oct4 (1.1 ± 0.5), Rex-1 (0.6 ± 0.2) und Sox2 (0.3 ± 0.2).

Diese Studie ist die erste ihrer Art, die Zellen mit Eigenschaften embryonaler Stammzellen im parodontalen Granulationsgewebe nachweist. Diese Erkenntnis sollte bei der Entwicklung zukünftiger Strategien zur Verbesserung der parodontalen Heilung und Regeneration im Auge behalten werden.

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