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ORIGINAL ARTICLE

Human saliva exposure modulates bone cell performance in vitro

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Abstract Various situations encountered by a clinician during the daily routine including surgical periodontitis therapy, dental implant insertion, or tooth extraction involve the contact of saliva with the jaw bone. However, there are only sparse data concerning the influence of saliva on bone cells. Saliva specimens were incorporated within culture medium and administered to murine MC3T3 osteoblasts, of which the morphology (REM), proliferation (EZ4U), and differentiation (qRT-PCR, alkaline phosphatase activity, extracellular matrix calcification) were assessed. Simultaneously, the composition of saliva media was analyzed with respect to the content of lactoferrin, activities of classical salivary enzymes, and the ability to provoke inflammatory cytokine production (enzyme-linked immunosorbent assay) in MC3T3 osteoblasts. The morphology, proliferation, and expression of differentiation-associated genes were seriously handicapped by saliva contact. Saliva-touched cells exhibited less alkaline phosphatase but normal levels of extracellular matrix mineralization. Saliva-containing culture media featured physiological activities of salivary enzymes and considerable amounts of lactoferrin but almost completely lacked salivary alkaline phosphatase and unspecific proteases. Upon saliva incubation, MC3T3 osteoblasts did not release noteworthy levels of interleukin-1beta

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or tumor necrosis factor alpha. Although saliva is generally considered to vitalize oral tissues, this study reveals that it harms osteoblast-like cells more due to the presence of salivary enzymes than by triggering of inflammation. This issue is clinically relevant because it broadens the understanding of the bone cell fate within the rather complex cosmos of the oral cavity thereby providing a basis for clinical decision making and treatment guidelines. It seems to be reasonable to restrict the contact period between saliva and bone.

Keywords Saliva · Osteoblasts · Proliferation · Differentiation · Inflammation

Introduction

Saliva is increasingly becoming a key target of fundamental research interest because it is crucial in coordinating and sheltering the complex infrastructure of the oral cavity. The salivary fluid incorporates a number of inorganic constituents that support the homeostasis of oral tissues [1]. Its organic compartment spans from antimicrobial enzymes to proteins [1], all of which serve manifold functions including lubrication and the attachment control of microorganisms to oral surfaces [2]. The whole of saliva contains a broad panel of cytokines, growth factors, and hormones [3, 4] which govern immune and inflammatory reactions as well as proliferation of oral tissue cells [3]. The diversity and orchestration of the many protective agents in saliva is scientifically traced with special emphasis on the maintenance of mucosal and tooth integrity [5, 6]: Saliva is supposed to account for the oral tissue wound healing [2, 7] that is tremendously accelerated compared to other tissues. Although research efforts already focus on understanding the underlying mechanisms [4], little is known about the response of hard tissue cells to saliva contact. Nevertheless, it is of outstanding interest to gain insight into the cellular reactions particularly of osteoblasts touched by saliva: The salivary fluid encounters and stays in contact with the alveolar bone for variable time periods during intraoral surgery, dental implant insertion, after tooth extraction, and in case of post-operative complications, wound dehiscence, or alveolar osteitis. Any clinician involved in implantology and periodontology needs to deal routinely with the complete spectrum of these clinical situations. In consequence, the present study was designed to assess time-dependent saliva influences on osteoblast-like cell morphology and proliferation capacity. Since saliva is postulated to favor repair mechanisms by inducing cell differentiation [4], the expression of osteogenic key markers was quantified. In a causative approach, we determined the composition of the saliva-mixed cell culture medium and its ability to provoke inflammatory cellular responses. It has to be emphasized that all experiments were run with samples of human whole saliva in order to optimally reflect the in vivo conditions.

Materials and methods

For all experiments, absorbance, chemoluminescence, or fluorescence emission was read in an Infinite M200 microplate reader (Tecan, Crailsheim, Germany), and data were collected and analyzed using the Magellan software version 6.5 (Tecan, Crailsheim, Germany). For a detailed description of each experiment, see the Supplemental Materials and Methods Section.

Saliva collection

Samples of stimulated human saliva were collected from six healthy volunteers (two females, four males), who had not undergone medication with antibiotics during the past 3 months. By chewing paraffin bits, the salivary flow rate was enhanced in order to collect 150 ml of stimulated saliva from each subject. Saliva was spun down (8,000 rpm, 10 min, RT), lyophilized in a Lyovac GT 2 (Amsco Finn-Aqua, Hürth, Germany) at 1 mbar, −40°C, and stored at −80°C until usage.

Cell culture

Murine MC3T3 osteoblasts were seeded at 3×10^4 cells/ml and incubated at 37°C for 3 h, 7 days, or 14 days in cell culture medium with (saliva group) or without (control group) lyophilized saliva solved within. All cell cultures were accurately checked for contamination daily. For all experiments, cell culture preparations were replicated three times with respect to each saliva (n=6 corresponding to the donors) or control medium (n=6).

Metabolic activity assay (EZ4U)

The proliferation of saliva-incubated MC3T3 osteoblasts was assessed by measuring their metabolic activity with a non-radioactive, colorimetric assay (EZ4U, Biomedica, Vienna, Austria) according to manufacturer's instructions. In brief, cells were washed with phosphate-buffered saline (PBS; PAA, Pasching, Austria) and incubated with a metabolizable dye substrate (1:8) for 3 h. The supernatant absorption was measured in triplicate at 450 nm subtracting 620 nm reference and blank optical density (OD) values.

Scanning electron microscopy

In order to assess the cell morphology, cells were fixed with 8% formaldehyde and stored at +4°C until usage. After rinsing with PBS, specimens were dehydrated in an alcohol series increasing in concentration (ranging from 30% to 100% ethanol, three times each for 20 min at RT), critical point dried (CPD 030 Critical Point Dryer, Bal-Tec AG, Balzers, Liechtenstein), and immediately sputter coated with gold palladium for 60 s at 60 mA (SCD 050, Balzers, Liechtenstein). The cells were examined using a LEO 435 VP scanning electron microscope (Zeiss, Oberkochen, Germany).

Quantitative RT-PCR

Total cellular RNA was harvested from cultured MC3T3 osteoblasts after accurately washing the cells with PBS, purified using a guanidium thiocyanate method (RNeasy Mini kit; Qiagen, Hilden, Germany), and stored at −80°C. Genomic DNA contamination was eliminated by DNase digestion according to manufacturer's instructions (RNase free DNase kit; Qiagen, Hilden, Germany). The RNA integrity and quantity were verified using the Experion RNA StdSens chip microfluidic technology according to manufacturer's instructions. cDNA was synthesized from up to 1 μg of total RNA in a 20-μl reaction mixture containing the iScript reaction mix and iScript reverse transcriptase in a C1000 Thermal Cycler (all Bio-Rad, München, Germany). The cDNA quantity was identified by a fluorescent nucleic acid stain using the Quant-iT PicoGreen dsDNA kit (Invitrogen, Karlsruhe, Germany), and all samples were diluted to a final concentration of 5 ng/μl, aliquoted and stored at −40°C. In order to evaluate the expression of the ubiquitin (Ubc), RUNX2, osteocalcin (OCN), osteopontin, alpha-1 collagen (Col1a1), and alkaline phosphatase (AP) genes, cDNA samples were amplified in duplicate in a 25-μl reaction mixture using pre-validated gene-specific RT^2 qPCR primer assays (SABiosciences, Frederick, MD, USA) with a iQ-SYBR Green Super Mix in a CFX96 cycler (both Bio-Rad, München, Germany). The products' specificity of each amplicon was checked by examining the melting temperatures (heating at 0.05°C/s to 95°C). Data were collected and analyzed using the CFX96 Manager software version 1.0 (Bio-Rad, München, Germany).

BCA protein assay and cellular alkaline phosphatase assay

Cells were lysed and stored at −80°C. The total protein quantity was analyzed using the BCA protein assay kit (Fisher Scientific, Schwerte, Germany) according to manufacturer's instructions. In brief, protein-chelated Cu^{2+} was detected by bicinchoninic acid at 562 nm. Aliquots of the same samples were diluted 1:10 with an alkaline phosphatase substrate (CSPD ready-to-use, Roche, Mannheim, Germany), of which the chemoluminescent signal was measured at 477 nm.

Calcium assay

Cells were scraped off their cell culture device using 0.5 N acetic acid and centrifuged. The supernatant was used for assessing the total protein content using the BCA protein assay kit as described above and the calcium concentration using the Quantichrom Calcium Assay kit (BioAssay Systems, Biotrend Chemikalien, Köln, Germany) according to manufacturer's instructions. The absorption of blue-colored calcium complexes was measured at 612 nm.

Enzyme-linked immunosorbent assays

Lactoferrin

Lactoferrin was identified in saliva-containing cell culture media using a human lactoferrin sandwich enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Montgomery, TX, USA) according to manufacturer's instructions as follows: sample/standard incubation 1 h at RT, biotinylated anti-human lactoferrin detection antibody 1 h at RT, horseradish peroxidase (HRP) solution 30 min at RT, and detection solution 30 min at RT in the dark. All incubation periods were alternated with extensive plate washing.

IL-1beta and TNFalpha

The release of inflammatory cytokines in the cell culture supernatants (see Supplementary Materials and Methods Section) was tested using a Mouse interleukin-1beta (IL-1beta) and a Mouse tumor necrosis factor alpha (TNFalpha) sandwich ELISA kit (both Ray Biotech, Norcross, GA, USA) as follows: sample/standard incubation 2.5 h at RT, biotinylated anti-mouse IL-1beta or TNFalpha detection antibody 1 h at RT, HRP solution 45 min at RT, and detection solution 30 min at RT in the dark. All incubation periods were alternated with intense plate washing.

Enzyme activity assays

The activity of typical salivary enzymes within the saliva medium preparations after sterile filtration and UV radiation was determined as described elsewhere [8–11] and as detailed in the Supplementary Materials and Methods Section. Negative controls of cell culture medium without saliva were run in each experiment showing no enzymatic activity. In brief, *alpha-amylase* activity was detected using a synthetic trisaccharide as a substrate. The *unspecific*

protease activity was determined using an EnzCheck protease assay kit (Molecular Probes, Leiden, The Netherlands). The salivary *alkaline phosphatase* activity was assessed by measuring the absorption of diethanolamin mixed with sodium nitrophenylphosphate. The *lysozyme* activity was evaluated using an EnzCheck fluorimetric assay kit (Molecular Probes, Leiden, The Netherlands). The *peroxidase* activity was recorded using dichlorofluorescin and hydrogen peroxide. The *collagenase* activity was measured using an EnzCheck fluorimetric assay kit (Molecular Probes, Leiden, The Netherlands).

Statistics

The changes and differences between the groups concerning cell proliferation, gene expression, cellular alkaline phosphatase activity, extracellular matrix (ECM) mineralization, and cytokine release were calculated in a mixed model [12] which was fitted with ID=(1–6, 7–12) as a random effect. The continuous response variables ($OD_{450–620\ nm}$, ΔCt, RLU/µg, µM/µg, pg/ml) are modeled as a linear function of group (saliva, control) and time (3 h, 7 days, 14 days) and the corresponding interaction as explanatory variables. Variance components were used as covariance structure. Least-square means were calculated. With respect to time, differences of least-square means to the control time (3 h) were calculated, and p values were adjusted by the Dunnett method, separately for each group. The level of significance was set at $p<0.05$. All calculations have been done using the PROC MIXED procedure from the statistical software SAS 9.1.2 (Cary, NC 27513, USA).

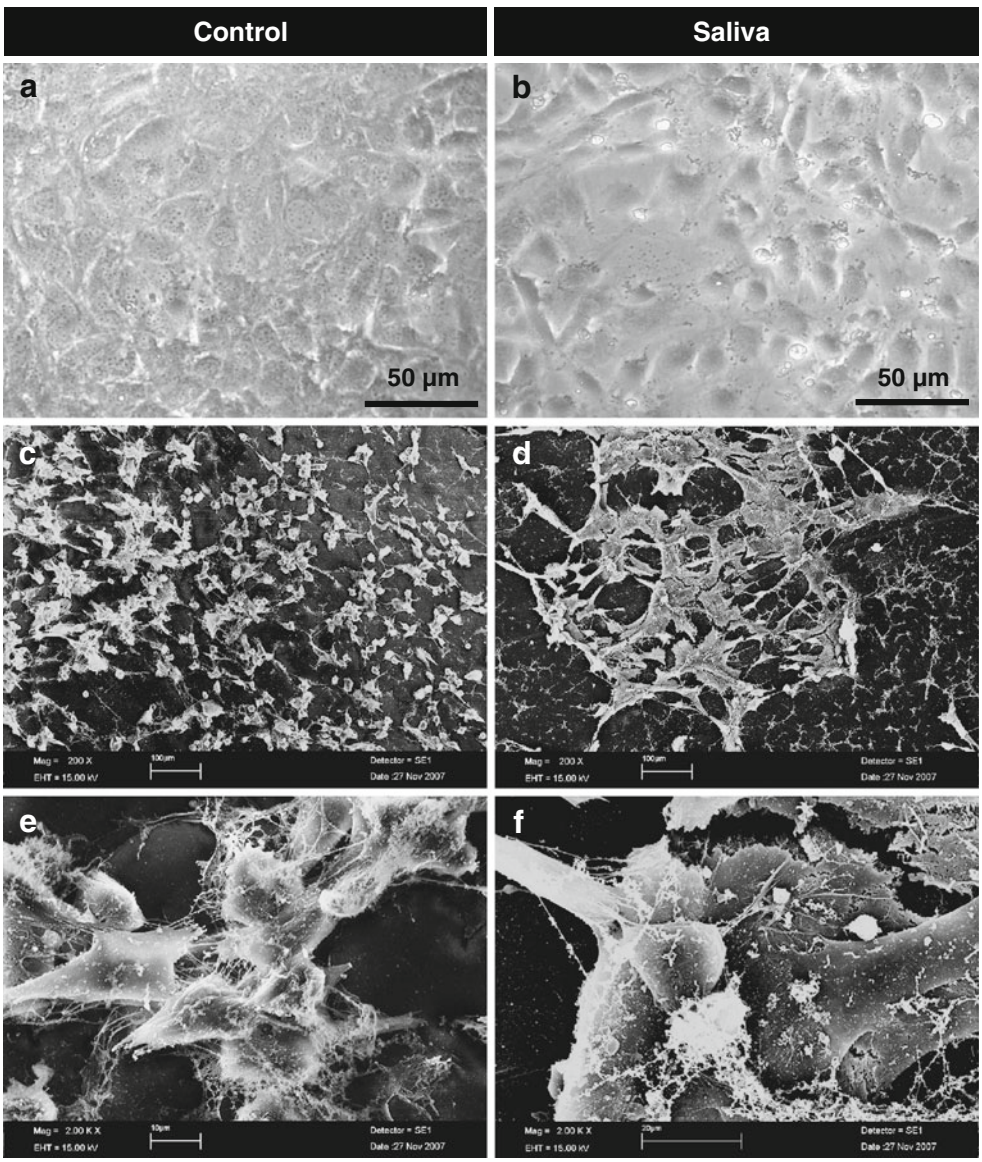

Fig. 1 Representative light microscopic and SEM images of control (*left column*: **a**, **c**, **e**) and saliva-incubated MC3T3 osteoblasts (*right column*: **b**, **d**, **f**) after 7 days in culture. **a** Whereas control cells readily reached confluency after several days in culture, **b** saliva incubation hampered the proliferation of MC3T3 osteoblasts. **c** Under control conditions, MC3T3 osteoblasts showed a regular spindle-shaped or flattened morphology and were evenly distributed within their cell culture device. **d** Saliva incubation induced a more rectangular shape of osteoblast-like cells which collocated in radiating aggregates. **e** Control cells were coated by an extracellular matrix molecule network which consisted mainly of thin filaments. **f** Saliva-incubated osteoblast-like cells revealed fewer filaments and were overall decorated with amorphous electron-dense deposits

Results

Cell morphology

After 3 h in culture, both control and saliva-incubated cells had readily adhered to the cell culture device without tremendous differences in cell morphology. Control MC3T3 osteoblasts proliferated well and reached confluency after 2–4 days in culture showing a plane ultrastructure (Fig. 1a). In contrast, saliva-incubated cells displayed a spheric shape and were arranged in radiating conglomerates without reaching confluency (Fig. 1b). Scanning electron microscopic images revealed that control cells showed a regular, spindle-shaped morphology (Fig. 1c), whereas saliva incubation led the cells to adopt a more rectangular profile (Fig. 1d). A network of multiple thin filaments formed the extracellular matrix of control cells (Fig. 1e). Saliva incubation yielded a mellow extracellular matrix network which blurred the borders of the underlying cells (Fig. 1f).

Cell proliferation

Both during saliva incubation and under control conditions, the count of MC3T3 osteoblasts significantly increased over time (Fig. 2) although to a significantly lower extent in saliva-incubated cells ($p=0.0002$). After 3 h of saliva incubation, the overall metabolic activity of saliva-incubated MC3T3 osteoblasts did not differ from control cells ($p=0.4204$). Nevertheless, after 7 days in culture, there were significantly less metabolically active cells in the saliva group compared to the control group ($p=0.0077$).

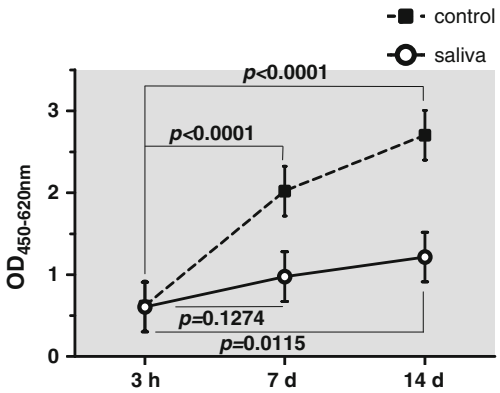

Fig. 2 Proliferation plot of control and saliva-incubated cells. Initially, the metabolic activity of saliva-incubated MC3T3 osteoblasts [*white circles* represent the least-square means (LSM) with 95% confidence interval (95% CI)] did not differ from control cells [*black rectangles* display the LSM (95% CI)]. The number of saliva-incubated MC3T3 osteoblasts increased to a significantly lower extent after 7 and after 14 days in culture compared to control cells

This trend continued and the number of saliva-incubated cells was even more reduced compared to control cells after 14 days ($p<0.0001$) in culture. Taken together, prolonged contact with saliva significantly hampered the proliferation capacity of MC3T3 osteoblasts.

Cell differentiation

In order to assess the salivary impact on osteoblast-like cell differentiation capacity, the differentiation status of MC3T3 osteoblasts incubated with and without saliva was surveyed at the distinct stages of gene and protein expression as well as extracellular matrix mineralization.

Expression of differentiation-associated genes

The expression of the following key marker genes of osteoblast differentiation was quantified and normalized to the housekeeping gene Ubc: Cbfa1/ RUNX2, OCN, Col1a1, osteopontin, and AP. The main result was that saliva contact entailed a significant downshift in mRNA expression of genes associated with osteoblast differentiation. Saliva-incubated MC3T3 osteoblasts expressed significantly less RUNX2 mRNA than cells under control conditions after 3 h ($p=0.0047$), after 7 days ($p=0.0002$), and 14 days ($p=0.0160$) in culture. Nevertheless, the mRNA expression of RUNX2 was significantly increased both in the saliva and in the control group over time (Fig. 3a). In line with the expression of RUNX2, MC3T3 osteoblasts expressed significantly less OCN mRNA than control cells after 3 h ($p=0.0028$) and after 7 days ($p=0.0001$) and 14 days ($p<0.0001$) of saliva incubation. In both groups, the expression of OCN mRNA significantly increased over time (Fig. 3b). The same trend was observed regarding the expression of AP mRNA, though mRNA expression did not increase significantly during culture. Attention has to be drawn to the fact that there was no AP mRNA expression measurable after 3 h of culture. Nevertheless, MC3T3 osteoblasts in both groups expressed AP mRNA, although at a significant lower level upon saliva incubation after 7 days ($p=0.006$) and after 14 days ($p=0.0012$) in culture compared to control cells (Fig. 3c). The mRNA expression of the connective tissue protein Col1a1 significantly increased over time only in the control group. Saliva incubation yielded significantly less mRNA expression of Col1a1 after 3 h ($p=0.0143$) and after 7 days ($p=0.0007$) and 14 days ($p=0.0001$) compared to control osteoblasts (Fig. 3d). Surprisingly, the mRNA expression pattern of the integrin-binding ligand osteopontin revealed itself to be the opposite in that it significantly increased over time in both groups, being upregulated upon saliva incubation compared to control cells after 7 days ($p<0.0001$) and 14 days ($p=0.0042$; Fig. 3e). Noteworthy, the transcription profile of the

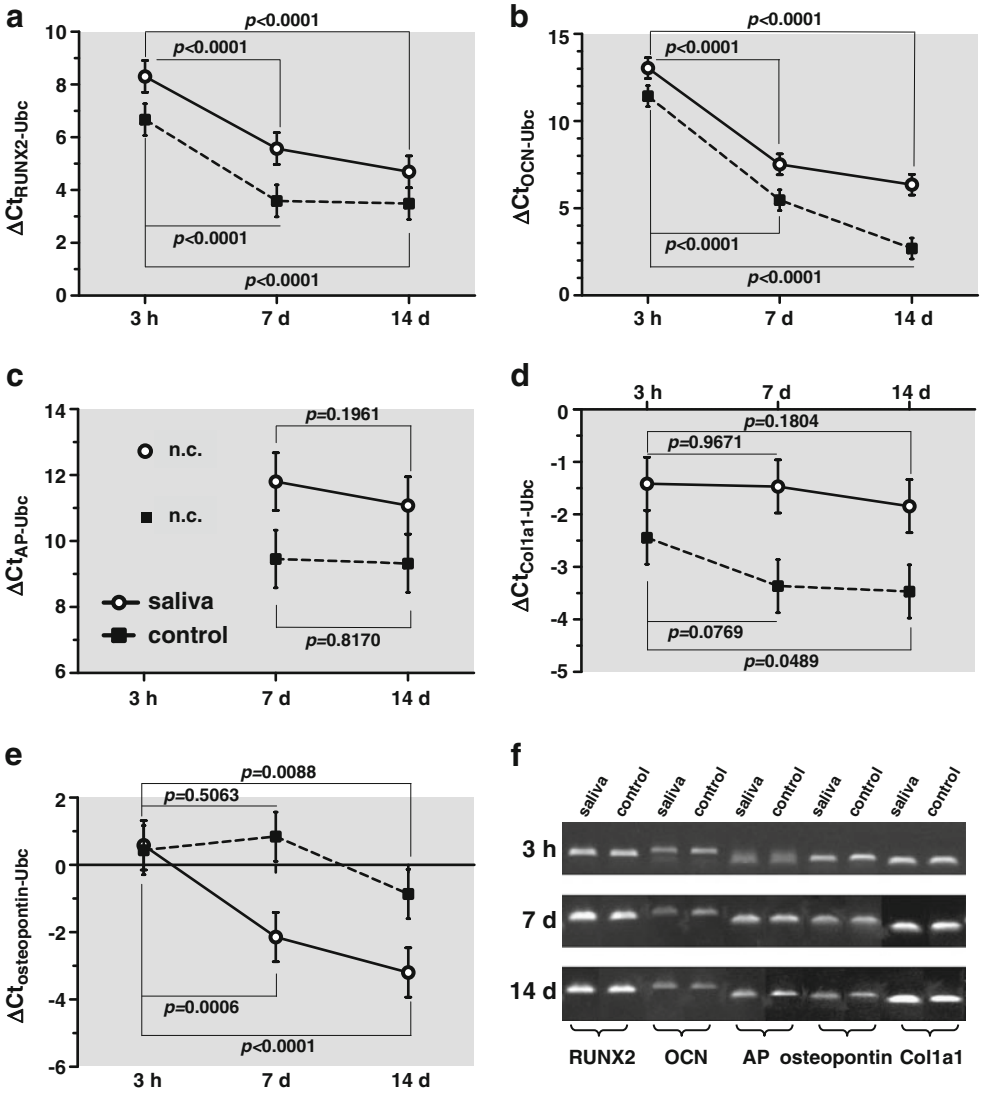

Fig. 3 Expression of osteoblastic differentiation-associated mRNAs in control [LSM (95% CI) indicated as *black rectangles*, connected by a *dashed line*] and saliva-incubated MC3T3 osteoblasts [LSM (95% CI) displayed as *white circles*, connected by a *solid line*]. The Ct values of the respective gene of interest [**a** RUNX2, **b** OCN, **c** AP, **d** Col1a1, and **e** osteopontin] were normalized to the corresponding housekeeping gene ubiquitin (*y*-axis: $\Delta Ct_{GOI\text{-}Ubc}$). In consequence, elevated ΔCt values symbolize a retarded differentiation. All together, the differentiation status increased both in control and in saliva-incubated cells for all genes of interest. However, saliva yielded a delayed transcription of RUNX2, OCN, and Col1a1 already after 3 h in culture. **c** AP mRNA was detectable after 3 h in culture neither in control nor in saliva-incubated cells (*n.c.* not calculable). The delay in mRNA expression upon saliva incubation continued after 7 and 14 days in culture with respect to RUNX2, OCN, AP, and Col1a1. **e** In contrast, the expression of osteopontin mRNA was upregulated after 7 and after 14 days of saliva incubation. **f** These results are represented by gels of the corresponding amplificates which were visualized using ethidium bromide

examined genes was delayed, but not modified upon saliva incubation with the exception of OCN and osteopontin. In addition to the lag time, saliva incubation ultimately yielded a slowdown in the expression of OCN ($p=0.0014$) and osteopontin ($p=0.0006$) mRNA.

Alkaline phosphatase activity

Cells in both groups were found to express active AP even after 3 h of saliva incubation without any difference between the groups ($p=0.3936$). However, MC3T3 osteoblasts expressed significantly less active AP after the prolonged saliva incubation of 7 days (saliva vs. control, $p<0.0001$) and 14 days (saliva vs. control, $p<0.0001$; Fig. 4a). In both groups, the quantity of active AP increased significantly over time (Fig 4a), although this effect was not detectable with respect to AP mRNA expression (Fig. 3c). Furthermore, the increase in active AP quantity was significantly decelerated in the case of saliva incubation ($p<0.0001$).

Extracellular matrix calcification

The calcium content of the extracellular matrix appeared to increase upon saliva incubation, however, only to an insignificant degree possibly attributed to huge differences between saliva samples of distinct donors. After 3 h of culture, the ECM of saliva-incubated cells tended to contain more calcium than the ECM of control cells ($p=0.1940$; Fig. 4b). Seven days later, the ECM calcium content of the control cells was transiently augmented reaching a similar level compared to saliva-incubated osteoblast-like cells ($p=0.2773$). After 14 days of culture, the calcium quantity in the ECM of saliva-touched MC3T3 osteoblasts was slightly, but not significantly, increased compared to control cells ($p=0.1252$; Fig. 4b).

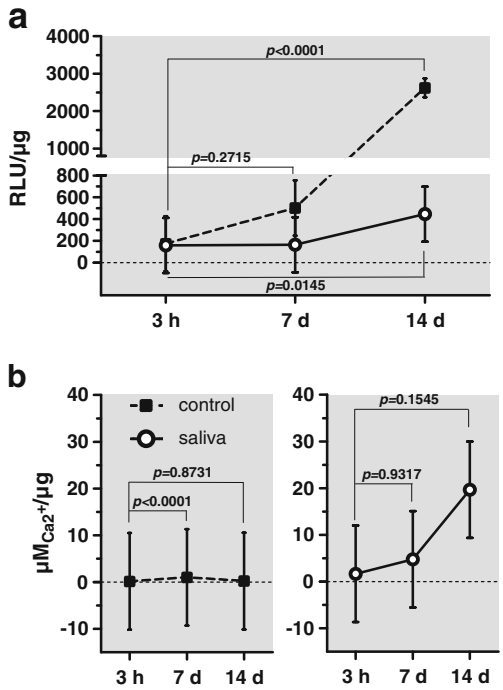

Fig. 4 Differentiation status of MC3T3 osteoblasts cultured with and without saliva by the means of **a** cellular alkaline phosphatase (AP) activity and **b** extracellular matrix (ECM) calcification. **a** After 3 h of saliva incubation, control [LSM (95% CI) indicated as *black rectangles*, connected by a *dashed line*] and saliva-incubated osteoblasts [LSM (95% CI) displayed as *white circles*, connected by a *solid line*] featured similar quantities of active AP, of which the level was increased to a significant lower extend in the control group after 7 and after 14 days (*y*-axis: relative luminescence units per microgram of protein). **b** However, the mineral content within the ECM of control cells was not significantly different from the ECM calcification of saliva-incubated cells. The calcium content within the ECM of saliva-incubated osteoblasts did not significantly rise over time or in comparison to the control osteoblasts (*y*-axes: micromolars of calcium per microgram of protein)

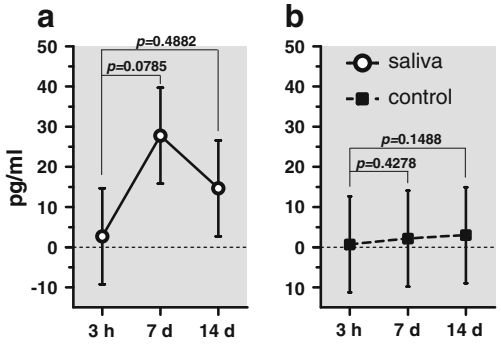

Fig. 5 Detection of murine IL-1beta in the cell culture supernatants of **a** saliva-incubated [LSM (95% CI) displayed as *white circles*, connected by a *solid line*] and **b** control MC3T3 osteoblasts [LSM (95% CI) indicated as *black rectangles*, connected by a *dashed line*]. The quantities of IL-1beta did not increase and did not differ in both groups over time from after 3 h, 7 days, and 14 days in culture (*y*-axes: picograms of cytokine per milliliter of culture medium)

Saliva composition

Salivary inflammatory stimulus

IL-1beta was traceable already in the culture supernatants of the saliva group after 3 h [least-square means (95% confidence interval) values 2.71 pg/ml (−9.24; 14.66)], peaked after 7 days [27.75 pg/ml (15.79; 39.69)], and were still detectable after 14 days of incubation [14.64 pg/ml (2.69; 26.59); Fig. 5a]. In the supernatants of control osteoblasts, we also detected relatively low levels of IL-1beta after a culture period of 3 h [0.69 pg/ml (−11.26; 12.64), $p=0.0998$ compared to saliva-incubated osteoblasts], 7 days [2.13 pg/ml (−9.81; 14.08), $p=0.0573$ compared to saliva-incubated cells], and 14 days [2.99 pg/ml (−8.95; 14.95), $p=0.1421$ compared to saliva-incubated cells; Fig. 5b]. This lack of significance is possibly a consequence of striking inter-individual differences of IL-1beta release upon incubation with saliva derived from different donors. We failed to detect TNFalpha in the culture medium of saliva-incubated and control cells after 3 h, 7 days, and 14 days of culture period.

Detection of lactoferrin

All saliva media samples contained a huge quantity of lactoferrin [median value (minimal–maximal) 152.7 ng/ml (55.6–242.3), Supplemental Table 1], whereas lactoferrin was not detectable in control cell culture medium.

Identification of salivary enzyme activities

The content of biologically active saliva ingredients was determined in the ready-to-use culture medium preparations: alpha-amylase [median (minimal–maximal) value 30.86 U/ml (5.25–110.5)], lysozyme [448.8 U/ml (61.35–928.1)], peroxidase [1.425 mU/ml (0.76–3.99)], and collagenase [12.11 mU/ml (4.68–36.16)] featured activities in the same range as recorded for native saliva. However, activity of unspecific proteases or alkaline phosphatase [0 U/ml (0–4.61)] was either not detectable or only at a very low amount, respectively (Supplemental Table 1).

Discussion

To saliva is commonly ascribed the attribute of encouraging rapid wound healing. This effect is theoretically justified by a diversity of salivary components which are globally thought to stimulate the proliferation and differentiation of mucosa cells [4, 7]. Although various clinical situations involve the contact of saliva and bone, it is to the best of

our knowledge the first time that the influence of saliva on capital osteoblastic cell reactions is evaluated.

Our initial assumption was that saliva contact results in similar stimulative effects on bone cells as published for mucosa cells [2, 4, 7, 13]. This hypothesis was strikingly refuted by the findings that whole saliva incubation yielded a reduced number of MC3T3 osteoblasts which in addition were slow to differentiate compared with control cells as shown by the lower expression of the osteogenic differentiation master gene RUNX2 together with lower amounts of the differentiation key markers osteocalcin and alkaline phosphatase mRNA. The morphological finding of a reduced number of thin filaments surrounding the saliva-incubated cells may be correlated to the reduced expression of alpha-1 collagen mRNA. Indeed, one could argue that our results concerning the differentiation status of the MC3T3 cells were not rigorously coherent because the osteopontin mRNA expression was enhanced and the ECM mineral content tended to be delicately augmented by saliva incubation. It is tempting to assume that an elevated osteopontin expression involves this slightly, but not significantly, higher ECM mineral content. However, the protein translate of osteopontin does not act as a nucleator of calcium salt precipitation even though other members of the family of phosphorylated sialoproteins do so [14]. In this context, the amorphous or more globular aggregated structures girdling the saliva-touched cells may result from a reduced ECM compactness. Beyond its role in ECM mineralization, osteopontin operates as a pro-inflammatory molecule and speeds the humoral immune response [15]. With respect to the reputation of osteoblasts to be immunocompetent [16], we speculate that the enhanced expression of osteopontin mRNA is rather a consequence of an inflammatory stimulus induced by saliva incubation than an indication for osteoblastic differentiation. The detection of IL-1beta in the cell culture supernatants renders a possible explanation of this phenomenon, although the difference in inflammatory cytokine production was statistically not significant. Nevertheless, IL-1beta is thought to provoke an enhanced expression of osteopontin [17, 18] despite the overall downregulation of the osteogenic differentiation [19]. In this light, it is generally equivocal to associate the ECM calcium level in saliva-incubated osteoblasts with their differentiation status: A pronounced ECM mineralization could simply be a symptom of the presence of various inorganic saliva constituents [2] which are deemed to precipitate within the ECM.

Saliva physiologically contains a number of growth factors such as lactoferrin [20] and hormones that stimulate the proliferation of oral or bone cells [3, 4, 7, 21]. Although we detected considerable amounts of lactoferrin, the proliferation of MC3T3 osteoblasts was not enhanced but in contrast was hampered upon saliva incubation. This observation led us to ask why the behavior of saliva-touched osteoblasts does not reflect the published saliva effects onto oral soft tissue cells. Although the action of saliva is the most remarkable aspect among the particular features of oral wound healing, it requires the interplay with cell types other than osteoblasts: It involves specialized oral mucosa fibroblasts and keratinocytes which were found to be sensitive to salivary ingredients like defensins [22] and histatins [7]. In addition, the low or in some cases absent release of inflammatory cytokines [16] indicates that saliva itself damages bone cells independently of enclosed bacterial endotoxins. Accordingly, we asked how saliva harms osteoblasts: Only a few salivary proteins are deemed to injure host cells. Among these, the most prominent potential candidates are mucins and salivary enzymes. Already shown more than two decades before, it was very recently confirmed that adhered salivary mucins impair the attachment of oral tissue cells in culture thereby hindering cellular proliferation and differentiation [13, 23]. It is reasonable to assume that this phenomenon is even boosted by the presence of collagenases and other enzymes which even physiologically serve to remodel the ECM [24, 25]: Saliva medium preparations still contained the latter in their biologically active conformation in spite of the preceding lyophilization and sterilization procedures. We want to emphasize that these circumstances do not portray an in vitro artifact: From a clinical point of view, these mechanisms are suspected to cause a delayed wound healing of saliva-touched bone, root, or implant surfaces as a consequence of deferred cell adhesion [23].

We conclude that saliva contact hampers the osteoblastic cell performance. However, we concede that the experimental in vitro framework of the present study does not reflect two fundamental factors of the in vivo reality: In vivo, saliva (a) encounters native cells and (b) comes along with micro-organisms. Although the assessment of human primary cells would provide an even more authentic insight in the performance of saliva-touched osteoblasts, MC3T3 osteoblasts were an equitable alternative for this first-time evaluation [26–28]. In this context, it is possible that the mere presence of human immunoglobulins harmed the MC3T3 osteoblasts, although the saliva medium preparations were assumed not to abound with human immunoglobulins compared to the huge quantities of serum or viscous salivary proteins. In vivo, osteoblasts may be surrounded by other cell types, and it is reasonable to assume that these affect the outcome of saliva contact. For their part, microorganisms modulate host tissues on their part while saliva balances the ecological homeostasis between the both [4]. Hence, we cannot rule out that in vivo the overall protective effects of saliva prevail [29]. With a view to the data of the present study, however, we recommend that the clinical contact period of saliva and bone should be shortened as much as possible.

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Conflicts of interest The authors declare that they have no conflicts of interest.

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