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Reduced free cholesterol in dental follicle cells inhibits osteogenic differentiation by inducing protein kinase C activity

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ABSTRACT

During the osteogenic differentiation of dental follicle cells (DFCs) the concentration of free cholesterol remains constant during differentiation. In less agreement with this result, several studies have shown that inhibition of cholesterol synthesis promotes differentiation of osteogenic progenitor cells. This study therefore investigated the effect of cholesterol synthesis inhibition on the osteogenic differentiation of DFCs. The induction of osteogenic differentiation showed a significant increase in free cholesterol concentration. Inhibition of cholesterol synthesis decreased both free cholesterol concentration and the alkaline phosphatase activity, a marker of osteogenic differentiation. However, a lower concentration of the inhibitor, which does not affect the concentration of free cholesterol, did not inhibit the activity of alkaline phosphatase (ALP). Previous studies have shown that the differentiation of DFCs is controlled by classical protein kinases C (PKC) and the induction of protein kinase B (AKT). In correlation with the reduced concentration of free cholesterol, AKT was also downregulated and the activity of classical PKCs was upregulated. Although inhibition of AKT was shown to downregulate the ALP activity, induction of AKT could not compensate for the reduced differentiation following inhibition of cholesterol. However, inhibition of PKC induced ALP-activity and suppressed the inhibitory effect of low free cholesterol concentration on the ALP activity. In conclusion, our study suggests that a reduced concentration of free cholesterol has an inhibitory effect on osteogenic differentiation through activation of PKC.

1. Introduction

The tooth germ consists of three different tissues: enamel organ, dental papilla and dental follicle. The dental follicle arises from neural crest cells and, as the developmental precursor of the periodontium, their tissue cells (dental follicle cells: DFCs) are the precursors for alveolar osteoblasts as adult stem cells [1,2]. As adult stem cells, DFCs are proposed not only for regenerative therapies, e.g. alveolar bone, but also for basic research on molecular processes during osteogenic differentiation [3]. Previous studies revealed a number of different pathways that control the differentiation of DFCs. A positive feedback loop, for example, between the bone morphogenetic growth factor (BMP) 2 and the transcription factor DLX3 is crucial for the initiation of osteogenic differentiation of DFCs and in particular for the expression of the induction of alkaline phosphatase (ALP) activity [4]. This BMP2/DLX3 feedback loop is controlled in various ways. Protein kinase A and APCCD1, an inhibitor of the WNT signaling pathway, showed a notable contribution to the induction of the transcription factor DLX3 [5,6]. The NOTCH signaling pathway also appears to be involved in the regulation of osteogenic differentiation of DFCs via inhibition of DLX3 expression [7]. The regulation of classical protein kinases C (PKC) has also been observed in connection with BMP2-mediated differentiation [8]. This regulation appears to be related, among other things, to the activation of protein kinase B (AKT) and the WNT signaling pathway [8]. However, basic metabolic processes such as energy metabolism and fatty acid metabolism appear to be involved in this context [9]. This study also suggested that the concentration of free cholesterol remains constant during differentiation.

Interestingly, however, the inhibition of cholesterol synthesis has been shown in various studies to promote osteogenic differentiation and bone formation [10–13]. In addition, the reduction of cholesterol by simvastatin is also considered to have a supportive role in oral therapies for mineralizing tissue, although clinical studies have shown that high concentrations of simvastatin increase the risk of osteoporosis [14]. However, based on our lipidomic study [9], it can be assumed that free cholesterol plays an essential role in the osteogenic differentiation of

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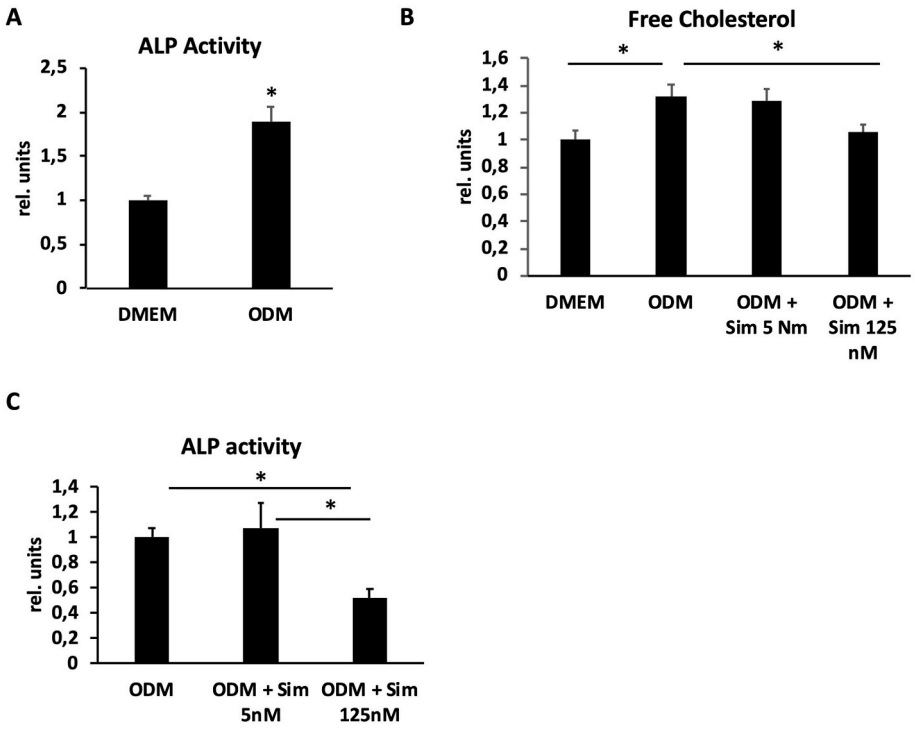

Fig. 1. (A) ALP activity in DFCs after 7 days in standard medium (DMEM) and osteogenic differentiation medium (ODM). Columns represent the mean ± standard deviation (n = 6). Asterisks (*) mark significant differences from cells in standard medium (DMEM). (B) Evaluation of free cholesterol in DFCs after 7 days in standard medium (DMEM) and osteogenic differentiation medium (ODM). To inhibit cholesterol synthesis, cells were treated with 5 nM or 125 nM simvastatin. Columns represent the mean ± standard deviation (n = 6). Asterisks (*) mark significant differences between the indicated groups. (C) ALP activity of DFCs after 7 days in osteogenic differentiation medium supplemented with and without simvastatin. Columns represent the mean ± standard deviation (n = 6). Asterisks (*) mark significant differences between the indicated groups.

DFCs. This study therefore investigated for the first time the effect of inhibited cholesterol synthesis on the osteogenic differentiation of DFCs. This study supported our hypothesis that free cholesterol is essential for differentiation and that inhibition of cholesterol synthesis inhibits osteogenic differentiation via PKC activation.

2. Materials and methods

2.1. Cell culture

Human dental follicle cells (DFCs) were purchased from ALL cells and cultivated in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with 10 % fetal bovine serum (FBS; Sigma-Aldrich) and 100 μg/ml penicillin/streptomycin. Cells after passage 6 were used for experiments. Simvastatin was purchased from Merck and were used in different concentration.

2.2. Osteogenic differentiation

DFCs were cultured to subconfluence (>80 %) in standard cell culture medium (see above) before stimulating osteogenic differentiation with osteogenic differentiation medium. The differentiation medium named ODM and consists of DMEM-based cell culture medium with 10 % fetal bovine serum (Sigma-Aldrich), 100 μM ascorbic acid 2-phosphate, 10 mM KH_2PO_4, HEPES (20 mM), and 10^{-8} M dexamethasone (Sigma.Aldrich). Differentiation of DFCs was determined by gene expression of osteogenic markes (see real-time RT-PCR), alkaline phosphatase activity (see below) mineralization of the extracellular matrix, which was determined by alizarin red staining (see below).

2.3. Alkaline phosphatase activity

The activity of ALP was measured after 7 days of osteogenic differentiation. DFCs were cultured in differentiation medium with and without simvastatin. The cells were lysed (Triton-X solution) and the lysates were assayed for ALP activity with a solution containing *p*-nitrophenyl phosphate (60 min at 37 °C). The production of *p*-nitrophenol was measured spectrophotometrically at 415 nm. The measured values of the ALP test were related to the measured values (490 nm) for the protein concentration determined with the BCA test (ThermoFisher). ALP activity values were calibrated to a control sample (rel. unit = 1).

2.4. Real-time reverse transcription polymerase chain reaction (real-time RT-PCR)

Total RNA was isolated from cells using the RNeasy isolation kit (Qiagen). cDNA synthesis was performed using total RNA and the iScript™ Advanced cDNA Synthesis Kit for qRT-PCR (Biorad) according to the manufacturer's protocol. PCRs were performed using SsoAdvanced Universal Probes Supermix (Biorad) on the StepOne real-time PCR machine (Life Technology). Primers for AKT1 gene were purchased from Bio-Rad. For normalization gene expression of the RPS18 gene (housekeeper gene) was used. For relative gene expression (calibration) a control group of DFCs cultivated in standard medium was used (relative gene expression = 1). The calculations were carried out using the previously described $\Delta\Delta C_t$ method [15].

2.5. Western blotting

Protein samples were isolated from DFCs, separated by polyacrylamide gel electrophoresis and blotted onto a nitrocellulose

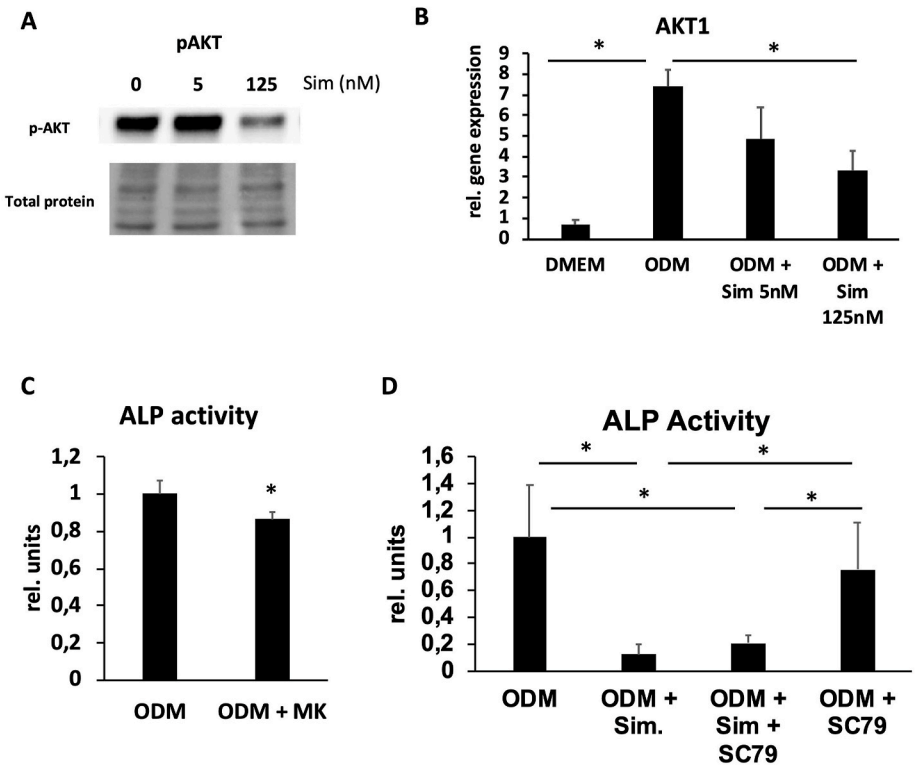

Fig. 2. (A) Western blot analysis using pAKT antibody and protein samples from DFCs cultured for 3 days in ODM with the indicated simvastatin concentrations. (B) Real-time RT-PCRs with DFCs after 3 days in DMEM or in ODM without and with the indicated simvastatin concentrations. Columns represent the mean ± standard deviation (n = 3). Asterisks (*) mark significant differences between the indicated groups. (C) ALP activity of DFCs after 7 days in osteogenic differentiation medium supplemented with and without MK2206. Columns represent the mean ± standard deviation (n = 6). Asterisks (*) indicate significant differences compared to cells in standard medium (DMEM). (D) ALP activity of DFCs after 7 days in osteogenic differentiation medium supplemented with and without simvastatin or SC-79, or in combination. Columns represent the mean ± standard deviation (n = 6). Asterisks (*) indicate significant differences between the indicated groups.

membrane as previously described [8]. Primary antibodies Phospho-Akt (Ser473; pAKT) and Phospho-(Ser) PKC Substrate (pPKC) were purchased from Cell Signaling and antibody detection with secondary antibodies was performed by chemiluminescence using the ChemiDoc Imaging System (BioRad). Total protein content was estimated using the stain-free method (Biorad) to ensure the success of Western-blotting.

2.6. Cholesterol assay

DFCs were differentiated in osteogenic differentiation medium (ODM) for 7 days. For control cells were cultivated in standard cell culture medium (DMEM). For the determination of free cholesterol, the bioluminescence assay "Cholesterol/Cholesterol Ester-GloTMAssay" (Promega) was used according to the manufacturer's instructions. The measured values were related to the measured values for the protein concentration determined with the BCA test. The relative units were determined by calibrating the measurements with the measurement obtained from cells cultured in standard medium (rel. unit = 1).

2.7. Statistics

For statistics the one-way ANOVA with Tukey's post-hoc tests or alternatively the unpaired Student's tTest were applied. A p-value below 0.05 was considered as significant (*).

3. Results

The ALP activity is an early marker of osteogenic differentiation and was increased 7 days after induction (Fig. 1A). Free cholesterol concentrations were also increased after differentiation induction. To assess the concentration of simvastatin that can reduce free cholesterol concentrations, DFCs were treated with 5 and 125 nM simvastatin during differentiation. Free cholesterol concentrations were reduced only with the higher concentration of simvastatin (Fig. 1B). In another experiment, it was shown that the higher simvastatin concentration suppressed not only free cholesterol but also ALP activity (Fig. 1C).

Further experiments should clarify how the inhibition of ALP activity might be related to the reduced free cholesterol concentration. Previous studies have shown that AKT and PKC influence the osteogenic differentiation of DFCs; for example, AKT is induced after induction of osteogenic differentiation [16]. Therefore, the extent to which AKT is inhibited following reduced cholesterol concentrations should be investigated in detail. It was shown that the expression of the active AKT protein (Fig. 2A) and the gene expression of AKT1 (Fig. 2B) were inhibited by high concentration of simvastatin. To evaluate the role of AKT in inhibiting ALP activity after reducing cholesterol concentrations, the inhibitor MK2263 and the activator SC-79 were used to specifically regulate AKT activity in DFCs. Although AKT inhibition demonstrated that its activity is essential for ALP activity (Fig. 2C), AKT activation could not reverse the inhibitory effect of low cholesterol concentrations on ALP activity (Fig. 2D). The results show that although reduced free cholesterol concentrations have an effect on AKT activity, this has no influence on ALP activity.

While AKT is activated during osteogenic differentiation, PKC is inhibited [8]. In a next step, we investigated the extent to which a reduced cholesterol concentration led to PKC activation, which might have inhibited the differentiation of DFCs. It was shown that a high concentration of simvastatin not only lowers free cholesterol but also activates PKC activity (Fig. 3A). Using the inhibitor of classical PKCs, we were able to induce ALP activity (Fig. 3B and C). Furthermore, we were able to suppress the inhibition of ALP activity by simvastatin with PKC inhibitor GÖ6976 (Fig. 3D). These results suggest that free cholesterol

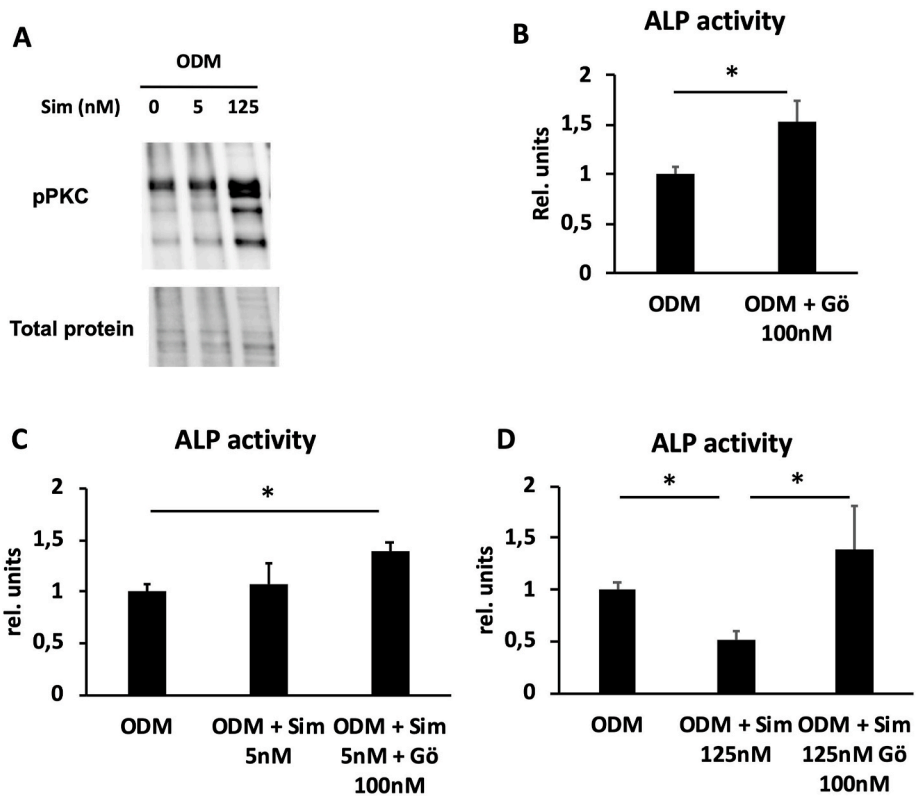

Fig. 3. (A) Western blot analysis with PKC-substrate antibody (pPKC) and protein samples from DFCs cultured for 4 days in ODM with the indicated simvastatin concentrations. (B) ALP activity of DFCs after 7 days in osteogenic differentiation medium supplemented with and without GÖ6976. The bars represent the mean ± standard deviation (n = 6). Asterisks (*) indicate significant differences compared to cells in standard medium (DMEM). (C) ALP activity of DFCs after 7 days in osteogenic differentiation medium supplemented with and without 5 nM simvastatin or GÖ6976, or in combination. The columns represent the mean ± standard deviation (n = 6). (D) ALP activity of DFCs after 7 days in osteogenic differentiation medium supplemented with and without 125 nM simvastatin or GÖ6976, or in combination. The columns represent the mean ± standard deviation (n = 6). Asterisks (*) indicate significant differences between the indicated groups.

impairs PKC activity and supports the osteogenic differentiation.

4. Discussion

The results of this study showed that cholesterol synthesis is enhanced after induction of osteogenic differentiation and that inhibition of cholesterol synthesis with simvastatin administered to DFCs at a concentration of 125 nM impairs osteogenic differentiation. These results are consistent with the results of our previous studies, which suggested a constant cholesterol concentration is essential for the osteogenic differentiation of DFCs [9]. However, previous studies from other groups showed different results when simvastatin was examined for osteogenic differentiation or bone regeneration. Mundy et al. [10] showed that the inhibition of cholesterol synthesis stimulated bone regeneration in an animal model. In addition, simvastatin, at an even higher concentration than in our study, supported the mineralization of the extracellular matrix of rat bone marrow cells in an in vitro study [17]. Interestingly, studies with different cell types including dental cells observed that simvastatin supports the mineralization [18–22]. However, not all studies with simvastatin showed that inhibition of cholesterol synthesis resulted in an improvement in cell viability, so in some studies apoptosis was also induced [23,24]. Our study showed that low concentrations of simvastatin had no effects on osteogenic differentiation, but at a higher concentration of simvastatin osteogenic differentiation was inhibited. The reasons for the influence of free cholesterol concentration on differentiation may be complex. Cholesterol plays an important role in maintaining cell membrane fluidity, which could be important for cell stability during differentiation. It is likely that cell structure also plays a role through changes in the cytoskeleton. A previous study showed that simvastatin induced differentiation by controlling Rho-GTPase-based cytoskeletal organization [12]. Moreover, Okamoto et al. showed in DPSCs that the actin fiber formation was suppressed by simvastatin [21]. Although there is no evidence that the same occurs during osteogenic differentiation of DFCs, the data from our recent proteomic study suggest an important influence of cytoskeletal organization [25]. Nonetheless, in contrast to dental pulp stem cells, these changes must have a negative impact on the differentiation of DFCs, since in our study simvastatin inhibited differentiation.

Interestingly, we were able to show that inhibition of free cholesterol concentration leads to an induction of PKC activity and that inhibition of PKC can reverse the inhibitory effect of simvastatin on osteogenic differentiation. We have previously shown that PKC activity suppresses osteogenic differentiation of DFCs and that the inhibitor for classical PKCs (GÖ6973) supports osteogenic differentiation [8]. On the other hand, it was also shown that treatment with simvastatin inhibits AKT, but in this case it was not possible to influence differentiation through induction of AKT. Therefore, the activating effect of inhibition of PKC on differentiation does not appear to occur directly via the activation of AKT, because both activating and inhibitory effects on differentiation were observed after inhibition of AKT [8]. However, inhibition of AKT causes inhibition of ALP activity [16], which is exactly what we observed in this study. Interestingly, there are previous studies that showed that cholesterol formation and synthesis play a supporting role in osteogenic differentiation [26,27]. In contrast to our results, this group was able to show that PKC and PKA are involved in the supporting effect of cholesterol [27]. This group repeatedly used oxysterol to conduct their studies on osteogenic differentiation. The question remains, of course, to what extent this modified form of cholesterol might have a different effect. They also identified a connection to the hedgehog signaling and other signaling pathways [28]. However, another study

showed that elevated cholesterol exposure influences osteogenic differentiation [29]. Cholesterol-treated stem cells showed a stimulated osteogenic differentiation process with induced ALP activity.

In conclusion the osteogenic differentiation of DFCs did not benefit from the inhibition of cholesterol synthesis. A low dose of simvastatin has no detectable effect on free cholesterol concentration and osteogenic differentiation in DFCs, whereas a high dose decreases free cholesterol concentration and impairs this biological process in DFCs. These data suggest that the presence of free cholesterol is important for osteogenic differentiation and that cholesterol appears to be related to PKC inhibition. This study again demonstrates how important PKC regulation is for the osteogenic differentiation of DFCs. However, further sophisticated studies will be required to evaluate cholesterol for the osteogenic differentiation of DFCs.

CRediT authorship contribution statement

Christian Morsczeck: Conceptualization, Data curation, Project administration, Writing – original draft, Writing – review & editing. **Anja Reck:** Investigation, Writing – review & editing. **Torsten E. Reichert:** Project administration, Writing – review & editing.

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Ethics approval and consent to participate

Not applicable, only cell culture experiments with anonymized purchased cells.

Consent for publication

Not applicable.

Funding

Not applicable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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