The extracellular concentration of osteocalcin decreased in dental follicle cell cultures during biomineralization

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Abstract The secretion of osteocalcin (OCN) is an excellent differentiation marker for the osteogenic differentiation. This study investigated the secretion of OCN during the osteogenic differentiation of DFCs. During the differentiation of DFCs the extracellular concentrations of OCN were higher in standard cell culture medium than in osteogenic differentiation medium. However, after 4 weeks in the osteogenic differentiation medium the extracellular OCN concentration decreased strongly, whereas the concentration remains high in the control medium. At this point in time DFCs formed connective tissue like structures with mineralized clusters and OCN. Real-time RT-PCR analyses and western-blot analyses proved that OCN was expressed in both cell culture media. However, the expression of the mRNA was inhibited in the osteogenic differentiation medium. These results suggest that DFCs secrete constitutively OCN into the cell culture medium and that the osteogenic differentiation medium suppresses the gene expression of OCN. Moreover, OCN imbeds into the extracellular matrix after the formation of connective tissue like structures, and the soluble OCN in the cell culture medium disappears. Hence, extracellular OCN

in the cell culture medium is not a marker for the osteogenic differentiation of DFCs.

Keywords Osteocalcin · Dental follicle cells · Osteogenic differentiation · Mineralization

Introduction

Somatic stem cells from the dental mesoderm including the dental follicle are different to other types of mesenchymal stem cells, because they are derived from neural crest cells (Huang et al. 2009; Morsczeck et al. 2013). Recent clinical trials have shown that dental stem treatments are safe and that therapies with dental stem cells could improve regenerative medicine in the future (Feng et al. 2010; Chen et al. 2016). However, more basic science is required before cell based therapies are feasible for the treatment of periodontitis (Chen et al. 2016). Molecular mechanisms about the differentiation of stem cells into alveolar osteoblasts and cementoblasts are one focus of basic research.

Dental follicles precursor cells (DFCs), which are derived from impacted wisdom teeth (Morsczeck et al. 2005a), are already used for studies about molecular mechanisms for the differentiation into alveolar osteoblasts and cementoblasts (Morsczeck 2015). Interestingly, typical osteogenic differentiation markers such as the transcription factor RUNX2 are not induced in DFCs during the osteogenic differentiation

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(Morsczeck 2006; Saugspier et al. 2010). However, a differentiation mechanism was considered for the osteogenic differentiation of DFCs that is independent of RUNX2. This mechanism depends on the expression of ZBTB16 (Felthaus et al. 2014a, b). Interestingly, the gene expression of osteocalcin (OCN), which is the most abundant non-collagenous protein in bone, depends on the expression of ZBTB16 (Felthaus et al. 2014b). The secretion of OCN into the cell culture medium is a reliable marker for the osteogenic differentiation of mesenchymal stem cells under in vitro conditions (Nakamura et al. 2009). However, the protein secretion of OCN into the extracellular space remains elusive in DFCs. This study investigated therefore the extracellular concentration of OCN during the osteogenic differentiation of DFCs for the first time.

Materials and methods

Cell culture

Human dental follicle cells (DFCs) were purchased from ALLCells (Alameda, CA, USA). DFCs were cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Munich, Germany), supplemented with 10 % fetal bovine serum, FBS (Sigma-Aldrich) and 100 µg/mL Penicillin/Streptomycin as previously described (Morsczeck et al. 2016). DFCs at passage 6 were used for experiments.

OCN enzyme linked immunosorbent assay (ELISA)

The ELISA kit was purchased from Thermo-Fisher (Darmstadt, Germany) (OCN Human ELISA Kit). The kit was used according to the supplier's protocol. For the analyses of extracellular OCN 100 µl cell culture medium was used at different points in time of the osteogenic differentiation.

Immunohistochemistry

After 4 weeks of cell culture DFCs formed connective tissue like structures, when cultivated in the osteogenic differentiation medium. In standard medium DFCs formed generally very little and fragile connective tissue like structure, which is not appropriate for immunohistochemistry. However, in one experiment

we were able to isolate a considerable amount of a connective tissue like structure for immunohistochemistry in standard medium. For immunohistochemistry all connective tissue-like structures were fixed with formalin and imbedded into paraffin. Tissue sections were subjected to immunohistochemistry. Briefly, after deparaffinization, endogenous peroxidase blocking and preincubation with TBS containing 4 % bovine serum albumin, sections were incubated with mouse anti-OCN antibody (Takara, Otsu, Shiga, Japan), diluted 1:1000 and incubated for 1 h at room temperature. Tissue sections were stained with the Dako REALTM Detection System, Peroxidase/DAB+, Rabbit/Mouse Kit (Dako, Glostrup, Denmark).

Osteogenic differentiation

Before the induction of the osteogenic differentiation, DFCs were cultivated in standard cell culture medium until sub-confluency (>80 %). The osteogenic differentiation was induced with the StemPro[®] Osteogenesis Differentiation Kit (Thermo-Fisher) according to the manufacturer's instructions.

The expression of OCN was determined by ELISA and immunohistochemistry. Moreover, the mineralization was determined by Alizarin Red staining, which was previously described (Saugspier et al. 2010).

Quantitative reverse transcription polymerase chain reaction (RTqPCR)

Total RNA was isolated from cells by using RNeasy isolation kit (Qiagen, Hilden, Germany). The cDNA synthesis was performed with total RNAs and the iScriptTM Advanced cDNA Synthesis Kit for RT-qPCR (Biorad, Munich, Germany) according to the manufacturer's protocol. Biorad PrimePCR primers were used according to the recommendations of the manufacturer. PCRs were made with SsoAdvancedTM Universal SYBR[®] Green Supermix (Biorad) on the StepOne real-time PCR machine (Thermo-Fisher).

Western blotting

For western-blot analyses of the protein expression of OCN and RUNX2 DFCs were treated with lysis buffer (250 µl phosphatase, 100 mM Na₃VO₄, 137 mM

NaCl, 200 mM Tris, 480 mM NaF, 1 % NP-40, 10 % Glycerol) on ice for 2 min. The protein degradation was inhibited with a Protease-inhibitor 1 Protease Inhibitor Cocktail tablet (Roche, Mannheim, Germany). Protein samples were separated by SDS–polyacrylamide gel electrophoresis with pre-casted Bolt® 4–12 % Bis–Tris Plus gels (Thermo-Fisher) and blotted to nitrocellulose membranes. Membranes were blocked with skimmed milk for 1 h and incubated with primary antibodies anti-OCN (Abcam, Cambridge, U.K.) and anti- β -Actin (Novus Biologicals, Littleton, CO, USA). For the detection of the primary antibody, a horseradish peroxidase labeled secondary antibody was used. The horseradish peroxidase activity was detected by chemiluminescence (GE Healthcare, Amersham, Little Chalfont, Bucks, U.K.). Protein spot intensities were quantified with the ImageJ software.

Results

DFCs secrete OCN in long-term cultures (Fig. 1). The OCN concentration levels were 41.35 ng/ml after 7 days of the long-term culture in DMEM and it decreased slightly to 37.4 ng/ml after 33 days. However, the OCN concentration was lower in the osteogenic differentiation medium (32.65 ng/ml). Moreover, OCN concentrations in the osteogenic differentiation medium decreased down to 15.5 ng/ml after 33 days. At this point in time DFCs formed collagenous connective tissue-like structures with

Fig. 1 OCN ELISA with different cell culture media. DFCs were cultivated in standard medium (DMEM), osteogenic differentiation medium StemPro® Osteogenesis Differentiation Kit (SPO). The OCN concentration decreased in SPO. Data points represent the average $\pm$ SD of two independent experiments. ELISAs were done according to materials and methods section

mineralized nodules in the osteogenic differentiation medium (Fig. 2). These collagenous connective tissue-like structures were stable and could be stripped off from the cell culture surface. Here, in immunohistochemical investigations the OCN protein was moderate (Fig. 2b). Interestingly one sample of the control culture formed also a connective tissue like structure. This tissue did not show any sign for mineralization, but the OCN staining was strong (Fig. 2b).

For comparison, we evaluated the gene expression of OCN mRNA with real-time RT-PCR analyses and the expression of the intracellular OCN protein by western blot analyses (Fig. 3). The mRNA expression level of OCN in osteogenic differentiation medium (SPO) was lower compared to the expression level in standard medium (Fig. 3a). Moreover, the expression of OCN decreased in both cell culture media during long-term cultures. In contrast, the protein expression level was increased in the osteogenic differentiation medium (Fig. 3b). However, different OCN protein expression levels did not vary throughout long-term cultures.

Discussion

The secretion of OCN into the cell culture medium is a very early and a useful marker for the evaluation of the osteogenic differentiation of bone marrow derived mesenchymal stem cells (Santos et al. 2009; Nakamura et al. 2009; Hosogane et al. 2010). In this study we examined the extracellular OCN concentration in DFC long-term cultures with both standard cell culture medium and osteogenic differentiation medium. Our result were very strange, because in contrast to mesenchymal stem cells (Nakamura et al. 2009) DFCs secreted OCN into the cell culture medium without the induction of the osteogenic differentiation. However, Nakamura et al. (2009) showed that the OCN concentration, which was unverifiable in standard medium, increased above 150 ng/ml in the osteogenic differentiation medium. This extracellular OCN concentration was more than 3 times higher than in our study. Surprisingly, in DFCs both the concentration of the extracellular OCN and the mRNA expression of OCN decreased during long-term cultures. Previous studies with DFCs have also shown that the mRNA expression level of OCN decreased in the osteogenic differentiation (Morsczech et al. 2009; Saugspier et al. 2010). However, a recent study showed that

Fig. 2 DFCs after 33 days of the osteogenic differentiation. **a** Alizarin red staining. **b** Immunohistochemistry: OCN staining of DFC derived connective tissue like structures after long term

cultures in control medium (DMEM) and in the osteogenic differentiation medium (SPO)

dexamethasone, which is the main inducer of the osteogenic differentiation under in vitro conditions, stimulated the mRNA expression of OCN in DFCs (Felthaus et al. 2014b).

The intracellular protein concentration of OCN (western-blot analysis) was slightly induced during the osteogenic differentiation. Moreover, the protein concentration did not decrease during long-term cultures. Our results suggest that the osteogenic differentiation (medium) inhibits the transcription of OCN while it does not influence the translation in DFCs. We obtained similar results for the expression of Parathyroid hormone-related protein (PTHrP) during the osteogenic differentiation of DFCs (Klingelhöffer et al. 2016). However, additional investigations are required to reveal the mechanisms for regulation of the transcription and of the translation of OCN in DFCs.

OCN was imbedded into connective tissue cell structures of DFCs after the osteogenic differentiation. These results are comparable to our previous investigations. Here, OCN was also imbedded in the extracellular matrix of DFCs after the osteogenic differentiation (Morszeck et al. 2005b). We suppose that DFCs secrete constitutively OCN into the cell culture medium. Moreover, we believe that the concentration of OCN decreased in the cell culture medium in long-term cultures, because osteogenic differentiated DFCs created collagen-rich connective tissue like structures and OCN proteins attached to these structures. This assumption is supported by our observation that OCN attached to connective tissue like structures in standard cell culture medium, although DFCs in standard medium did not form mineralized cell clusters.

Fig. 3 The expression of OCN at days 3, 6 and 10 after the induction of the osteogenic differentiation (SPO). For control, DFCs were cultivated in standard medium (Ctrl.). **a** Real-time RT-PCR analyses for the mRNA expression of OCN. Columns represent the average $\pm$ SD ($n = 3$). **b** Western Blot analyses. Ratios illustrate the relative intensities of protein bands of OCN and β -Actin, which was used for normalization

Previous studies with osteoprogenitor cells have shown that the osteogenic transcription factor RUNX2 controls the expression of OCN (Hassan et al. 2004, 2006). However, RUNX2 is not required for the osteogenic differentiation of DFCs, but the transcription factor ZBTB16 is an important factor for the differentiation of DFCs (Saugspier et al. 2010; Morsczeck et al. 2010; Felthaus et al. 2014a). We speculate that the protein expression/secretion of OCN is connected to the gene expression of ZBTB16 in DFCs, because the gene expression of OCN depends on the expression of ZBTB16 (Felthaus et al. 2014a, b). However, additional studies are required to reveal the route of OCN in DFCs during the osteogenic differentiation.

In conclusion, our results suggest that the induction of the osteogenic differentiation did not induce the secretion of OCN, but DFCs secrete constitutively OCN into the cell culture medium. After 33 days of the osteogenic differentiation the OCN protein binds to the mineralizing collagenous tissues. This is probably the reason, why the OCN level decreased in the cell culture medium. The decrease of the extracellular OCN

concentration level could be used as a “marker” for the differentiation of DFCs under in vitro conditions.

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