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Regulation of acetylcholine receptors during differentiation of bone mesenchymal stem cells harvested from human reaming debris

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ABSTRACT

Acetylcholine (ACh) is an important signaling molecule in non-neuronal systems where it is involved in regulation of viability, proliferation, differentiation and migration of mesenchymal stem cells (MSC) that are capable to differentiate into osteoblasts, chondrocytes and adipocytes. Patients with the systemic disease osteoporosis show altered MSC properties, reduced bone formation and mineral density followed by increased bone fragility and high fracture incidence. Here we asked whether nicotinic and muscarinic acetylcholine receptors (AChR) are expressed in osteoblasts, adipocytes and chondrocytes differentiated from bone MSC extracted from human reaming debris (RDMSC) that was harvested during surgery of long bone diaphyseal fractures.
Using RT-PCR, AChR were detected in RDMSC, osteoblasts, chondrocytes and adipocytes of male and female bone-healthy and of female osteoporotic donors. An up-regulation in multiplicity and occurrence of AChR subtypes was found in female compared to male donors and in osteoblast of male donors compared to adipocytes. Real-time RT-PCR analysis resulted in a significant increase in relative expression of nAChR α9 in chondrocytes compared to adipocytes of healthy female donors. The nAChR subunit α10 was significantly up-regulated in osteoblasts of healthy compared to osteoporotic donors as well as the mAChR M5 that is additionally decreased in osteoporotic osteoblasts compared to MSC and chondrocytes of osteoporotic donors.
In summary, the gene expression of AChR during differentiation of RDMSC and its regulation in cells of osteoporotic donors lead to the assumption that AChR signaling is involved in bone formation and might be utilized to stimulate bone remodeling processes.

1. Introduction

Acetylcholine (ACh) is besides its important role as neurotransmitter in the nervous system a signaling molecule in non-neuronal cells. Together with its enzymes for synthesis and degradation, receptors and transporters, ACh comprises the cholinergic system. The non-neuronal cholinergic system is involved in viability, proliferation, differentiation, and migration of several cell types [1]. The effects of ACh and its agonists on cell function are mediated by acetylcholine receptors (AChR) that have been divided into two major subfamilies: the muscarinic (mAChR) and nicotinic receptors (nAChR).

The subfamily of mAChR is characterized by its agonist muscarine and coupled to G-proteins. Five different subtypes of mAChR are known: M1–M5. Subtypes M1, M3, and M5 are coupled to the Gq-protein, signaling via activation of phospholipase C and, therefore, inositol trisphosphate and intracellular calcium as a signaling pathway. Stimulation of M2 and M4 leads via Gi-proteins, in general, to an inhibitory effect that is caused by a decrease in cAMP, inhibition of voltage gated Ca^{2+} channels, and an efflux of K^{+} ions [2].

The nAChR are ligand-gated cation channels named after its agonist nicotine. They are built by 5 subunits. Functional receptor consisting out of subunits α1, β1, γ, δ and ε are restricted to the neuromuscular junction (muscle type) whereas the neuronal type is found on neurons and non-neuronal cells. This neuronal type of AChR is built up as heteropentamer by α- and β-subunits (α2-7, β2-4), as homopentamer

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AChR, acetylcholine receptors; agg, aggrecan; B2M, β2-microglobulin; BMP, bone morphogenetic protein; cAMP, cyclic adenosine monophosphate; cDNA, complementary desoxyribonucleic acid; CO_2, carbon dioxide; DMEM, Dulbecco's Modified Eagle Medium; EDTA, ethylene diamine tetraacetic acid; MSC, mesenchymal stem cells; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HACAT, keratinocyte cell line; H_2O, RT-PCR runs without template; mAChR, muscarinic acetylcholine receptors; mRNA, messenger ribonucleic acid; nAChR, nicotinic acetylcholine receptors; PPARγ, peroxisome proliferator-activated receptor gamma; RDMSC, reaming debris derived mesenchymal stem cells; real-time RT-PCR, real-time reverse transcriptase polymerase chain reaction; Ø RT, samples processed without reverse transcription; RUNX2, runt-related transcription factor 2; SH-SY5Y, neuroblastoma cell line; TGF-β, transforming growth factor beta; Wnt, Wingless-related integration site.

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by α-subunits (α7, α9) and as α-heteropentamer by different α-subunits (e.g. α9α10) [3]. At the interface of the α-subunits, the ACh binding site is localized. The homopentameric and α-heteropentameric receptors are able to conduct bivalent cations like Ca^{2+} in addition to monovalent cations like Na^{+}.

Mesenchymal stem cells (MSC) are multipotent cells that are able to differentiate at least into osteoblasts, chondrocytes and adipocytes. Recently, it was demonstrated that migration of MSC extracted from bone marrow is regulated by nAChR α7 [4]. Proliferation, migration, and differentiation of MSC are important processes during early fracture healing. MSC are similar to RDMSC that were derived from human reaming debris during routine surgery of long bone diaphyseal fractures (intramedullary nailing). MSC as well as RDMSC are also crucial for tissue engineering and incorporation of implants. The properties of MSC are regulated by a variety of signaling systems such as the Wnts [5,6] and bone morphogenetic proteins (BMP) [7,8]. In addition, the cholinergic system might be involved in regulation. ACh has been detected in embryonic stem cells [9] where Wessler et al. found a rise in concentration during early differentiation [10]. On the contrary, Hoogduijn et al. detected higher ACh concentrations in undifferentiated status of MSC [11]. Furthermore, it has been shown that blocking of acetylcholinesterase, the degradation enzyme of ACh, limits the differentiation capacity by inhibiting osteogenic differentiation [11].

A limited osteogenic differentiation and a shift into the adipogenic direction have been reported for MSC of osteoporotic donors [12]. In fracture healing it is desired that stem cells differentiate into osteoblast but not adipocytes. Osteoporotic patients have an increased fracture incidence. Osteoporosis is a systemic disease of predominantly postmenopausal women, which also occurs in men, however, in a lower number of cases. Osteoporosis is characterized by a reduced bone mineral density, changes in bone microarchitecture, and increased osteoclastogenesis that leads subsequently to increased bone fragility and fracture incidence. First results of an osteoporotic animal model showed that gene expression of mAChR M3 was down-regulated in osteoporotic bone [13]. Thus, AChR signaling might be involved in osteoporosis. One possibility to support bone formation is to stimulate or inhibit specific signaling pathways. AChR and the non-neuronal cholinergic system of bone might be a feasible signaling system that can be boosted to stimulate bone formation and osteoporotic fracture healing.

Thus, the aim of our study was to examine the mRNA expression of nAChR and mAChR in osteoblasts, adipocytes and chondrocytes differentiated from RDMSC harvested from human reaming debris of male and female bone-healthy and of female osteoporotic donors by means of qualitative reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR.

2. Materials and methods

2.1. Patients and samples

The study was designed according to the declaration of Helsinki and approved by the local ethical committee (74/09). Reaming debris was collected from the reamer head after opening the medullary cavity prior to insertion of a nail during routine surgical osteosynthetic treatment of long bone diaphyseal fractures. Samples were collected from 4 male patients (25–50 years of age), 4 female patients (21–80 years of age) who did not suffer from systemic diseases, and 4 female patients (60–80 years of age) with osteoporosis. All patients were non-smokers.

2.2. Cell culture of reaming debris

Reaming debris was removed from the reamer-head and cultured as described earlier [14–16]. In brief, specimens were placed in F12K medium (Invitrogen, Karlsruhe, Germany) containing 20% fetal calf serum (FCS, PAA, Cölbe, Germany), 0.05 U/ml of penicillin, and 0.05 μg/ml streptomycin (Invitrogen) [15]. The cultures were maintained at 37 °C in humidified 95% air, 5% CO_2 atmosphere. The medium was changed once per week. After reaching confluence of 70–80%, cells were passaged. Passages 5–7 were used in the present study.

2.3. Differentiation of RDMSC

For osteogenic differentiation RDMSC were cultured 3 weeks in DMEM low glucose (PAA) containing 0.1 μM dexamethasone (Sigma-Aldrich, Munich, Germany), 0.05 mM ascorbic acid-2 phosphate (Sigma-Aldrich), 0.05 U/ml penicillin, 0.05 μg/ml streptomycin, 10% FCS, 10 mM β-glycerophosphate (Sigma-Aldrich) [15].

Adipogenic differentiation was induced by culturing the cells alternately in induction medium and culture medium as described earlier [16]. In brief, induction medium was composed of 0.2 mM indomethacin (Synopharm, Barsbüttel, Germany), 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1methyl-xanthine (Sigma-Aldrich) while culture medium consisted of DMEM high glucose (PAA) with 0.01 mg/ml insulin (Aventis, Frankfurt, Germany), 0.05 U/ml penicillin, 0.05 μg/ml streptomycin, and 10% FCS (PAA). Media changes were performed every 3–4 days for a period of 4 weeks.

Chondrogenic differentiation was primarily induced by centrifugation [16]. Therefore, cells (2.5×10^5) were transferred into a 15 ml polypropylene tube within the standard medium and centrifuged at 150 g for 5 min. After 24 h incubation in standard medium pellets were treated with chondrogenic induction medium that consists of DMEM high glucose containing ITS + 1 Premix (Sigma-Aldrich), 35 μg/ml ascorbic acid-2 phosphate (Sigma-Aldrich), 0.1 μM dexamethasone, 10 ng/ml transforming growth factor-beta (TGF-β, R&D Systems, Wiesbaden, Germany), 1 mM sodiumpyruvat, and 50 ng/ml bone morphogenetic protein-2 (BMP-2, R&D Systems). Medium was changed every 2–3 days.

2.4. RT-PCR

Cells were washed with 0.05 M phosphate-buffered saline, lysed with RLT-buffer (Qiagen, Hilden, Germany) and homogenized by using Qiashredder spin columns (Qiagen).

Total RNA was extracted with the RNeasy Mini Kit (Qiagen) as described earlier [17]. RNA isolation of chondrogenic pellets was performed using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA synthesis and RT-PCR was performed as described earlier [13]. In brief, contaminations with genomic DNA were removed and RNA was reverse-transcribed with superscript II reverse transcriptase. The cDNA was amplified with gene-specific primer pairs (human primers [17], Table 1). RT-PCR was performed using AmpliTaq Gold polymerase (Applied Biosystems), cycling conditions according to the manufacturer's protocol, and annealing temperatures between 56–59 °C. The PCR products were separated by electrophoresis on a 1.25% TRIS-acetate-EDTA gel. The following controls were performed: (1) samples processed without reverse transcription (Ø RT) to control for contamination with genomic DNA, (2) RT-PCR runs without template (H_2O), (3) primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as positive controls for the efficiency of RNA isolation and cDNA synthesis, (4) the neuroblastoma cell line SH-SY5Y and keratinocyte cell line HACAT (American Type Culture Collection, ATCC, Rockville, MD, USA) as positive controls for the quality of the gene-specific primers, and (5) primers for runt-related transcription factor 2 (RUNX2), aggrecan (agg), peroxisome proliferator-activated receptor gamma (PPARγ) to control differentiation.

Negative controls remained blank and positive controls with a primer pair for GAPDH (Fig. 1A), RUNX2 (Fig. 1D), agg (Fig. 2A), and PPARγ (Fig. 2D) as well as cDNAs of several human cell lines (SH-SY5Y and HACAT) showed a single band at the appropriate height.

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