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Impact of acetylcholine and nicotine on human osteoclastogenesis in vitro

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ABSTRACT

Recent studies showed that the non-neuronal cholinergic system (NNCS) is taking part in bone metabolism. Most studies investigated its role in osteoblasts, but up to now, the involvement of the NNCS in human osteoclastogenesis remains relatively unclear. Thus, aim of the present study was to determine whether the application of acetylcholine (ACh, 10^{-4} M), nicotine (10^{-6} M), mineralized collagen membranes or brain derived neurotrophic factor (BDNF, 40 ng/mL) influences the mRNA regulation of molecular components of the NNCS and the neurotrophin family during osteoclastogenesis.

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of young healthy donors (n = 8) and incubated with bone fragments and osteoclast differentiation media for 21 days. All the results are based on the measurement of RNA.

Real-time RT-PCR analysis demonstrated a down-regulation of nicotinic acetylcholine receptor (nAChR) subunit α2 and muscarinic acetylcholine receptor (mAChR) M3 by osteoclastogenesis while BDNF mRNA expression was not regulated. Application of ACh, nicotine, BDNF or collagen membranes did not affect osteoclastic differentiation. No regulation was detected for nAChR subunit α7, tropomyosin-related kinase receptor B (TrkB), and choline acetyltransferase (ChAT).

Taken together, we assume that the transcriptional level of osteoclastogenesis of healthy young humans is not regulated by BDNF, ACh, and nicotine. Thus, these drugs do not seem to worsen bone degradation and might therefore be suitable as modulators of bone substitution materials if having a positive effect on bone formation.

1. Introduction

Bone tissue is being formed, remodeled and resorbed depending on the continuous mechanical load applied to the bone. This process is influenced by several hormones like parathyroid hormone, glucocorticoids or sex steroids and also by the sympathetic nervous system [1]. Basically, bone mass is increased by osteoblasts and decreased by osteoclasts, holding a steady state in healthy bone. In diseases like osteoporosis or multiple myeloma, bone metabolism is imbalanced with higher osteoclastic formation and activity which leads to a significant reduction of bone mineral density. This is causing a high incidence of slow-healing pathologic fractures with high rate of complications. By population development, we have to expect a rising number of patients suffering from such fractures which will also raise the consumption of resorbable bone substitution materials such as collagen scaffolds or membranes, which are used as carrier material. Modified with hydroxyapatite, tricalcium phosphate, osteoprogenitor cells or growth factors they can achieve osteoconductive or osteoinductive properties although they can only provide minimized structural support [2].

The non-neuronal cholinergic system (NNCS) with its receptors, transporters, enzymes and its signaling molecule acetylcholine (ACh) is also taking part in regulation of bone metabolism [3,4]. En-Nosse et al. showed that all components for ACh synthesis, release, signaling, degradation and reuptake are present in human and mouse osteoblast-like cells [5]. The expression of muscarinic acetylcholine receptors (mAChR) on osteoblasts depends on the origin of the cell M3 can be found in spongy bone and mAChR M4 rather in periosteum [6]. Therefore, with regard to muscarinic receptors, in the present study

Abbreviations: ACh, acetylcholine; B2M, beta-2-microglobuline; BDNF, brain derived neurotrophic factor; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CarAT, carnitine acetyltransferase; ChAT, choline acetyltransferase; CO_2, carbon dioxide; CT, cycle threshold; DMEM, Dulbecco's modified Eagle's medium high glucose; mAChR, muscarinic acetylcholine receptor; M-CSF, human macrophage colony stimulation factor; MM, multiple myeloma; nAChR, nicotinic acetylcholine receptor; NNCS, non-neuronal cholinergic system; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; RANKL, receptor activator nuclear factor kappa ligand; real-time RT-PCR, real-time reverse transcriptase polymerase chain reaction; SD, standard deviation; TGF-β, tumor growth factor-β; TrkB, tropomyosin-related kinase receptor B.

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we decided to focus on the mAChR M3. ACh or metacholine were shown to activate the G protein-coupled mAChR, inducing osteoblast cell proliferation, an effect inhibited by the mAChR antagonist atropine [6]. Although it is well known that smoking is negatively influencing bone fracture healing [7], uptake of nicotine by smoking must not be equated with low-dose application of this substance because nicotine promotes osteoblast cell proliferation [8–10]. Nicotine also reduced the activity of alkaline phosphatase [10]. Application of the neurotrophin brain derived neurotrophic factor (BDNF) resulted in an increased osteoclastogenesis and osteoclast activity as shown by pit-assay and immunohistochemistry [11]. Bajayo et al. showed that agonists of nicotinic acetylcholine receptors (nAChRs) decrease bone resorption by upregulation of osteoclast apoptosis and that mice with gene deficiency of nAChR subunit α2 had an increased osteoclastic activity causing a loss of bone mass [12]. Thus, in the present study we analyzed the mRNA expression of nAChR subunit α2. However, up to now it was not investigated whether application of nicotine and ACh affects osteoclastogenesis.

Thus, aim of our study was to analyze if mRNA expression of molecular cholinergic components is regulated by application of nicotine, ACh, BDNF and mineralized collagen membranes, and if it correlates to the morphologic development of osteoclasts.

2. Material and methods

2.1. Isolation and culturing of peripheral blood mononuclear cells (PBMCs)

Venous blood samples were taken from eight healthy donors with anticoagulating, lithium-heparine coated S-Monovette blood collection systems (Sarsted, Nümbrecht, Germany). The four male and four female donors were aged between 22 and 29. They were all non-smokers, not pregnant, without chronic disease, and without usage of regular medication.

The PBMCs were separated using ficoll density gradient centrifugation by Leucosep Centrifuge Tubes (Greiner Bio-One, Frickenhausen, Germany). In brief, 15 mL blood were transferred to the Leucosep tubes and centrifuged at 800 g for 20 min. The PBMCs containing cell-layer was collected, washed with phosphate buffered saline (PBS), and centrifuged again. Cells were resuspended in 5 mL Dulbecco's modified Eagle's medium high glucose (DMEM, GIBCO, Paisley, UK), seeded into 24-well plates (3.45×10^6 cells per well) and incubated with bone fragments and osteoclast differentiation media consisting of DMEM with 10% bovine serum albumin (BSA, Sigma-Aldrich, Taufkirchen, Germany), 1% penicillin/streptromycin (Invitrogen, Karlsruhe, Germany), 25 ng/mL human macrophage colony stimulation factor (M-CSF, Sigma-Aldrich), 25 ng/mL receptor activator nuclear factor kappa ligand (RANKL, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 37 °C in a humidified atmosphere with 6% CO_2. After the third day of incubation 1 ng/mL transforming growth factor-β (TGF-β, Sigma-Aldrich) was added to the culture. Cells were divided in five different testing groups which received either nicotine as exogenous ligand of the nAChR subunit α2 (10^{-6} M, Sigma-Aldrich, Taufkirchen, Germany), ACh as endogenous stimulator of nAChR and mAChR (10^{-4} M, Sigma-Aldrich), BDNF (40 ng/mL, PAN Biotech, Aidenbach, Germany), were seeded on mineralized collagen membranes or remained blank as control group. Media were changed every second day.

2.2. Mineralized collagen membranes

Mineralized collagen membranes with a diameter of 8 mm and a thickness of approx. 0.1 mm were manufactured from acid-soluble collagen type I from calf skin (Collaplex 1.0, GfN, Wald-Michelbach, Germany) according to a published procedure [13]. In the production process, collagen fibrils were reassembled and synchronously mineralized with nanoscopic hydroxyapatite. Membranes were generated by a vacuum filtration process and finally chemically cross-linked with a carbodiimide derivative. The material was sterilized by gamma irradiation (25 kGy) [13].

2.3. Live cell observation and scoring

Prior to media changes, cells were examined by inverted light microscopy (Type 090-135.002, Leica Microsystems GmbH, Wetzlar, Germany) equipped with a digital camera (DS-Fi1, Nikon, Duesseldorf, Germany). Pictures were taken, randomized, blinded and scored by four experienced scientists. The score included the following parameters: a) estimated amount of polynuclear cells, b) their estimated cell-size, c) estimated amount of nuclei, and d) estimated cellular vitality. The scale reached from −2 (strong reduction compared to control)

Table 1
Primer used for real-time RT-PCR.

Primer	Sequence	Ann.-temp.	Length	PCR kit used	Accession-no.
BDNF[a]	5′-CTA-CGA-GAC-CAA-GTG-CAA-TCC-3′ 5′-GTA-CGA-CTG-GGT-AGT-TCG-GC-3‘	60 °C	100 bp	Quantifast	NM_001143812.1
TrkB[b]	5′-TGG-TGC-ATT-CCA-TTC-ACT-GT-3′ 5′-CGT-GGT-ACT-CCG-TGT-GAT-TG-3′	58 °C	130 bp	Roche	NM_006180.3
nAChR α7[c]	5′-GTA-CGC-TGG-TTT-CCC-TTT-GA-3′ 5′-CCA-CTA-GGT-CCC-ATT-CTC-C-3′	59 °C	138 bp	Roche	NM_001190455.2
nAChR α2[d]	5′-ACT-ACA-TTG-CCG-ACC-ACC-TG-3′ 5′-GGA-AGA-TCC-TGT-CGA-TGA-CC-3′	60 °C	93 bp	Quantifast	NM_001282455.1
mAChR M3[e]	5′-TTG-GGT-CAT-CTC-CTT-TGT-CC-3′ 5′-CGG-AGG-CAC-AGT-TCT-CTT-TC-3′	60 °C	79 bp	Quantifast	NM_000740.2
Ctsk[f]	5′-GCG-ATA-ATC-TGA-ACC-ATG-CG-3′ 5′-TTG-TTT-CCC-CAG-TTT-TCT-CCC-3′	60 °C	103 bp	Roche	NM_000396.3
ChAT[g]	5′-GTT-TGT-CCT-CTC-CAC-TAG-CC-3′ 5′-TTG-TAG-CAG-GCA-CCA-TAC-CC-3′	60 °C	93 bp	Quantifast	NM_020984.3
B2M[h]	5′-TCT-CTC-TTT-CTG-GCC-TGG-AG-3′ 5′-CAA-CTT-CAA-TGT-CGG-ATG-GA-3′	59 °C	135 bp	Roche	NM_004048.2

[a] BDNF = brain derived neurotrophic factor.
[b] TrkB = tropomyosin-related kinase receptor B.
[c] nAChR α7 = nicotinic acetylcholine receptor subunit α7.
[d] nAChR α2 = nicotinic acetylcholine receptor subunit α2.
[e] mAChR M3 = muscarinic acetylcholine receptor M3.
[f] Ctsk = cathepsin-K.
[g] ChAT = choline acetyltransferase.
[h] B2M = beta-2-microglobulin.