Bone & Joint Research

Supplementary Material

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Cytotoxicity determined by measuring lactate dehydrogenase (LDH) and metabolic activity (MTS)

Cytotoxicity and metabolic activity of HBCs cultured for one and two days in osteogenic differentiation medium containing different concentrations of ceftriaxone was determined using lactate dehydrogenase (LDH) and MTS method, respectively. Cadmium sulfate ($CdSO_4$) at five different concentrations (1 µM, 10 µM, 100 µM, 1,000 µM and 10,000 µM) served as control. For MTS, culture medium was replaced by 200 µL phenol red free proliferation medium containing 20 µL MTS and incubated for 60 minutes at 37°C in a humidified 5% CO_2 atmosphere, followed by analysis using a Mithras^2 Plate reader (optical density (OD) at 490 nm), relating the measured fluorescence intensities to control samples containing no cells.

For the LDH assay, HBCs were cultured for ten days in osteoblastic differentiation medium containing different ceftriaxone concentrations, whereas $CdSO_4$ in four different concentrations (1 µM, 10 µM, 100 µM and 1,000 µM) was added at day 9. The supernatant was then transferred to a new multi-well plate and stored at 4°C. Subsequently, the cells were lysed by adding 200 µL 0.1% Triton X-100 while the sealed

plate was shaken at 20 rpm for two hours at room temperature (RT). Afterwards, 20 µL of the complete lysate and 20 µL of the supernatant were transferred to a new multi-well plate. The KPP buffer (40.24 mM potassium phosphate dibasic (K_2HPO_4) and 9.7 mM potassium phosphate monobasic (KH_2PO_4)) was adjusted to pH 7.4. After sterile filtration, 29.9 mL of the KPP buffer were transferred into a 50 mL tube and 5 mg NADH (N9410, Sigma-Aldrich, Switzerland) and 2 mg Na-pyruvate (P2256, Sigma-Aldrich) were freshly added followed by shaking. This solution was added to each sample and measurements were taken every minute for 15 minutes using a Mithras^2 Plate reader.

Proliferation assay

HBCs in osteoblastic differentiation medium containing different ceftriaxone concentrations as well as in proliferation medium were exposed to fresh medium containing 10 µM of EdU (Click iT Edu Alexa Fluor 488 Imaging Kit, C10337, Invitrogen) on day 8, and incubated for further 48 hours. After removal of the culture medium, 4% formaldehyde in PBS was added and washed twice with 3% BSA in pre-warmed PBS after 15 minutes at RT. Permeabilization buffer (0.5% Triton X-100 in PBS) was added for 20 minutes at RT. After removing the permeabilization buffer and washing cells twice with 3% BSA in PBS, 100 µL Click-iT reaction cocktail, prepared according to the manufacturer's instructions, were added to each well and incubated at RT for 30 minutes, protected from light. Then the wells were washed once with 3% BSA in PBS. DNA was stained with 200 µL Hoechst 33342 (5 µg/mL) solution in PBS, left at RT for 30 minutes, protected from light. Wells were washed twice with PBS. Samples were analysed in triplets and the images were taken with the confocal laser scanning microscope (CLSM) with a 10x objective. Subsequently, the total number of cells and the number of proliferating cells were counted separately using Cell Profiler Software Version

3.1.9, followed by determining the percentage of cells which were in proliferation phase.

Metabolic activity at day 10 and 28, RT-PCR at day 10

Culture medium in a multi-well plate was replaced after ten or 28 days, by PrestoBlue solution (1:10) and incubated for 30 minutes, followed by analysis by a Mithras^2 Plate reader (LB943, Berthold Technologies, Germany). The measured fluorescence intensities were set in relation to control samples containing no cells.

Following the PrestoBlue analysis, the samples were washed with pre-warmed PBS once and lysed using 350 µL dissolved 1,4-Dithiothreitol (6908, Carl Roth, Switzerland) for approximately 15 minutes. The lysates were transferred to 1.5 mL tubes for RNA extraction, performed in accordance with the manufacturer's instruction (RNeasy Micro Kit (50), 74004, Qiagen, Germany). Extracted RNA was measured with NanoDrop (NanoDrop ND-1000 Spectrophotometer) and 100 ng RNA were used for the synthesis of the complementary DNA. RT-PCR primers (Table i) for both directions and iQ SYBR Green Supermix (BIO RAD, 170-8882) were used for detection of the target gene expression analysis using the CFX96 Real-Time System, C1000 Thermal Cycler (BIO RAD, USA). Each sample was analysed in triplicate. The gained relative expression of each target gene was calculated by applying the ΔΔCt method, normalized against the RPL13a gene.

ALP and Col-I staining

For staining and following CLSM analysis, culture medium was removed at day 10 and cells were fixed (4% PFA, 65mM Piperazine-N,N'-bis(2-ethanesulfonic acid (PIPES), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 3 mM $MgCl_2$). After 20 min, the fixation solution was discarded and 0.1% Triton X-100 (T8787, Sigma-Aldrich) was added to permeabilize cells, and washed twice after ten minutes at RT with pre-warmed PBS. Before staining, the samples were blocked with 5% goat serum (G6767, Sigma-Aldrich) and 1% FCS in PBS for 45 minutes. ALP was stained with a monoclonal antibody (1:1,000, B4-78, Developmental Studies Hybridoma Bank, USA). After one hour, the corresponding goat anti-mouse Alexa Fluor 488 antibody (1:400, A-11029, Thermo Fisher) was added. Antibodies were removed and samples washed thrice after one hour. Subsequently, actin and nuclei were stained with Alexa Fluor 546 nm – conjugated phalloidin 1:200 (A22283, Thermo Fisher Invitrogen, USA) and 4',6-diamidino-2-phenylindole (DAPI) 1:100 (D9542, Sigma-Aldrich) for one hour, respectively. All antibodies and dyes were dissolved in 1% FCS in PBS for the staining procedures. The staining was performed at RT and between all staining steps the samples were washed thrice with pre-warmed PBS. The images were taken using the CLSM (LSM780, Carl Zeiss, Germany) with a 10x objective.

Staining of collagen I was performed as described before. Instead of ALP, the monoclonal collagen type I (Col-I) antibody 1:1,000 (C2456, Sigma-Aldrich) and a corresponding goat anti-mouse Alexa Fluor 488 antibody 1:400 (A-11029, Thermo Fisher) was used.

Collagen quantification assay and DNA determination

Collagen quantification was done on day 14 by Soluble Collagen Assay Sircol Kit (S1000, Biocolor Life Science Assays, UK) following the manufacturer's instructions. Further, collagen standard solutions of 5, 10, and 15 µg in 100 µL acetic acid were also prepared according to the manufacturer's instructions. HBCs were cultured in osteoblastic differentiation medium containing different ceftriaxone concentrations as well as in proliferation medium as control in a multi-well plate. At day 14, 0.1 mg/mL pepsin and 0.5 M 4°C cold acetic acid were added to release the collagen into solution, followed by incubation at 4°C for 48 hours. Cell residues scraped off from the wells as well as the solution containing the lysate were transferred into 1.5 mL low protein binding tubes (22331, Eppendorf, Germany). A total of 200 µL of collagen isolation and concentration reagent, containing polyethylene glycol in TRIS-HCl buffer (pH 7.2), was added. Subsequently, samples were stored at 4°C overnight and then centrifuged (Centrifuge 5417R, Eppendorf) at 12,000 rpm for ten minutes. The supernatant was discarded and 1 mL Sircol dye reagent added to the pellet, followed by shaking at 10 rpm for 30 minutes on an orbital shaker. After another centrifugation at 12,000 rpm for ten minutes and draining the tubes, 750 µL of ice-cold acid-salt wash reagent, containing acetic acid, sodium, chloride and surfactants, were added to each sample and collagen standard solutions to remove unbound dye. A further centrifugation step at 12,000 rpm for ten minutes was done followed by removing supernatant. Then, 250 µL alkali reagent was added to the samples and collagen standard solutions, followed by vortexing until the dye completely dissolved. Then, 200 µL of each sample, standard solution, and acetic acid as reagent blank were transferred to a multi-well plate and the absorbance measured at 555 nm using a Mithras^2 Plate reader. This analysis was performed in triplicate.

DNA was quantified at culture day 14. After removing the culture medium, cells were washed twice with PBS; 500 µl filtered water (18.2 MΩ cm) were added in each well of a multi-well plate, followed by freezing at -20°C. The thawing process was performed using an orbital shaker at 220 rpm at RT. This procedure was repeated thrice. The standard curve was obtained by using DNA of a calf thymus (D-3664, Sigma-Aldrich). For this, a stock solution of 100 µg/mL was diluted in TNE-buffer (Tris (10 mM), EDTA (1 mM), NaCl (2,000 mM), filtered water, pH 7.4). One ml of Hoechst 33258 (94403, Sigma-Aldrich) stock solution (1 mg Hoechst powder/mL filtered water) was diluted in 50 ml TNE. After scraping cells off the bottom of the wells using a pipette tip, 100 µl of cell suspension and 100 µl of standard curve target dilution were transferred to a multi-well plate. First, 1 ml of the Hoechst stock solution was diluted in 50 ml TNE puffer (20 µg/mL final concentration). To obtain the Hoechst working solution, 140 µl of the 20 µg/mL solution were added to 6.84 ml TNE puffer. Then, 100 µl of the Hoechst working solution were added to the suspension and standard curve target dilution followed by incubating on an orbital shaker at 200 rpm at RT for 60 minutes, under protection from light. Fluorescence was measured at 460 nm using a Mithras^2 Plate reader. This analysis was performed in triplicate.

Calcium quantification assay

HBCs were cultured for 28 days in osteoblastic differentiation medium containing different ceftriaxone concentrations, as well as in proliferation medium as control, in a multiwell plate. After removing the medium and washing twice with pre-warmed PBS, calcium ions were dissolved out of the mineralized matrix by adding 200 µL of 1 M HCl, incubated for three hours at 37°C in a humidified 5% CO_2 atmosphere under constant agitation at 300 rpm. 10 µL of either calcium standard solution, blank control, or lysate of the samples were transferred to a multiwell plate before adding 200 µL of working

reagent Calcium Assay Kit (KA4081, Abnova). After three minutes incubation at RT, absorbance was measured at 600 nm and 650 nm by Mithras^2 Plate reader. Calcium concentrations were calculated using a standard curve. Samples were analyzed in triplicates.

Table i. Used primer sequences for reverse transcription-polymerase chain reaction analysis.

Genes	Sequences of primer pairs
Osteocalcin	Forward: 5'-GAAGCCCAGCGGTGCA-3' Reverse: 5'-CACTACCTCGCTGCCCTCC-3'
ALP	Forward: 5'-GGACATGCAGTACGAGCTGA-3' Reverse: 5'-CCAGCAAGAAGAAGCTTTG-3'
Col-I	Forward: 5'-CAGCCGCTTCACCTACAGC-3' Reverse: 5'-TTTTGTATTCAATCACTGTCTTGCC-3'
RPL13a	Forward: 5'-AAGTACCAGGCAGTGACAG-3' Reverse: 5'-CCTGTTTCCGTAGCCTCATG-3'

ALP, alkaline phosphatase; *Col-I*, collagen type I.