

Full Length Research Paper

Influence of osteogenic supplements on the osteoclastogenesis of human monocytes

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A basic requirement for bone remodeling is the communication between bone forming osteoblasts and bone resorbing osteoclasts – the so-called cross talk. Corresponding *in vitro* co-culture models might be a valuable technique in order to investigate the influence of novel biomaterials on the cross talk. Assuming that both cell types are derived by precursor cells, the role of the common osteogenic supplements concerning the osteoclastogenesis is still little known. Therefore, the approach of the present study was to analyse osteoclast formation in the presence of both, osteoclast differentiation factors as well as osteogenic differentiation factors dexamethasone (Dex), β -glycerophosphate (β -GP) and 1.25-dihydroxy vitamin D3 (VitD3) in typical concentration ranges as used for osteoblastogenesis of bone marrow stromal cells (BMSC). Human monocytes were isolated from buffy coat and separated by using magnetic activated cell sorting (MACS). DNA amount, activity of tartrate-resistant acid phosphatase isoform 5b (TRAP 5b), morphological features of the cells as well as gene expression for TRAP, cathepsin K (CTSK), calcitonin receptor (CALCR) and vitronectin receptor (VTNR) were evaluated. Finally, we are able to suggest conditions which allow both, osteoblastogenesis and osteoclastogenesis of human precursor cells in a combined cultivation medium.

Key words: Human bone marrow stromal cell, human monocytes, osteoclasts, osteoclastogenesis, osteogenic supplement, co-culture.

INTRODUCTION

Understanding communication of osteoblasts and osteoclasts is a pivotal topic in the development of bone substitution materials (Matsuo and Irie, 2008). An approach to the *in vivo* condition is ensured by the use of precursor cells for *in vitro* models. On the one hand, BMSC (also known as mesenchymal stem cells, MSC) can be commonly differentiated into bone forming osteoblasts. Therefore, osteogenic supplements like Dex, β -GP and VitD3 are used to stimulate BMSC to differentiate along the osteoblastic lineage. On the other hand bone-resorbing multinucleated osteoclasts differentiate from monocytes (Mc) which is derived from hematopoietic stem cells. The additional supplementation of the cytokines

macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor κ B ligand (RANKL) is required to regulate formation, activity and survival of osteoclasts (Heinemann et al., 2010). M-CSF is mostly important for osteoclast precursor cell survival, migration and cytoskeletal reorganisation (Lagasse and Weissman, 1997; Yoshida et al., 1990). RANKL binds to its receptor RANK on the surface of osteoclast precursors and induces the differentiation to osteoclasts (Hadjidakis and Androulakis, 2006). Both cytokines combined induce expression of genes that typify the osteoclast lineage, including those encoding TRAP, CTSK, CALCR and VTNR (Boyle et al., 2003). In an *in vitro* co-culture situation of BMSC/osteoblasts and monocytes/osteoclasts the stimulating supplements may affect differentiation behaviour of the precursors. The scope of the present study was to evaluate the influence of the osteogenic differentiation factors on the osteoclastogenesis

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from monocytes. Therefore, human precursor cells were cultivated in media supplemented with varied concentrations of osteogenic supplements Dex, β -GP and VitD3. Evaluation was performed using biochemical measurement of DNA and TRAP 5b.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect genes characterizing osteoclasts. Light microscopy revealed the morphological features of the cells. The future aim is to identify a cell culture media regime, which allows both osteoblastogenesis and osteoclastogenesis simultaneously.

MATERIALS AND METHODS

Isolation of human monocytes

Human monocytes (hMc) were isolated from buffy coats (purchased from the German Red Cross blood donation service, Dresden, Germany) obtained from the blood of healthy anonymous donors. The isolation is based on the OptiPrep density-gradient media technique with some modifications. OptiPrep™ (ProGen Biotechnik GmbH, Heidelberg, Germany) was mixed with the α -modification of minimal essential medium (α -MEM, Biochrom, Berlin, Germany) to obtain the OptiPrep™ Working Solution (WS), a 1.078 g/ml gradient solution and a 1.068 g/ml gradient solution. Buffy coats were centrifuged at 450 g for 20 min and the leukocyte-rich fraction (LRF) at the interface was collected. OptiPrep™ WS was mixed with the LRF to obtain a density of 1.100 g/ml. In a 50 ml falcon vessel, the OptiPrep™ WS/LRF mixture was placed under a layer of 1.078 g/ml lymphocyte-specific gradient solution. A layer of HEPES buffered saline (HBS) was placed on top and centrifuged at 700 g for 20 min. The peripheral blood mononuclear cells (PBMC) fraction was collected, washed with phosphate buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% bovine serum albumin (BSA) and centrifuged at 400 g for 10 min.

OptiPrep™ WS was mixed with the PBMC fraction to obtain a density of 1.100 g/ml and was covered by layers of 1.078 g/ml gradient solution, 1.068 g/ml gradient solution and HBS. By centrifugation at 600 g for 25 min the monocyte-enriched PBMC fraction floated into the 1.068 g/ml layer. That fraction was collected, washed with PBS/EDTA/BSA and finally monocytes were purified by negative selection using MACS (Miltenyi, Bergisch Gladbach, Germany).

Osteoclastogenesis

Isolated monocytes were seeded at a density of 4×10^5 cells/cm² in 48-well tissue culture dishes and cultivated in α -MEM, supplemented with heat-inactivated 7.5% fetal calf serum (FCS), 7.5% human A/B serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. Medium and all supplements were obtained from Biochrom (Berlin, Germany). To induce differentiation, 50 ng/ml M-CSF and 50 ng/ml RANKL were supplemented. For testing the effect of osteogenic supplements on osteoclastogenesis concentrations of VitD3, β -GP and Dex have been varied in several ranges that are typically used to differentiate hBMSC into osteoblasts (Vater et al., 2010).

Colorimetric measurements

All measurements were performed with cell lysates obtained after 3 and 10 days of cultivation. Cell lysis was achieved with 1% Triton X-100 (Sigma) in PBS. For all colorimetric measurements, a Spectra

Fluor Plus microplate reader (Tecan, Crailsheim, Germany) was used.

DNA assay

Examination of DNA amount was carried out using Quant-iT™ PicoGreen® dsDNA Reagent.

TRAP 5b activity assay

Osteoclast differentiation was evaluated by the measurement of TRAP 5b activity using naphthol-ASBI phosphate (N-ASBI-P, Sigma) as a substrate according to a slightly modified protocol of Jankila et al. (2001). Cell lysates were added to the TRAP 5b reaction buffer consisting of 2.5 mM N-ASBI-P in 100 mM Na-acetate (sigma) buffer containing 50 mM Na-tartrate (sigma), 2% NP-40 (sigma) and 1% ethylene glycol monomethyl ether (EGME, sigma) adjusted to pH 6.1 and the mixtures were incubated at 37°C for 1 h. The enzymatic reaction was stopped by adding 0.1 M NaOH and fluorescence was measured at an excitation wavelength of 405 nm and an emission wavelength of 535 nm. The relative fluorescence units were correlated to a TRAP 5b standard.

Statistics

All measurements were collected in triplicate and expressed as mean \pm standard deviation. ANOVA test was employed to assess the statistical significance of results. *P* values less than 0.001 were considered highly significant.

Microscopy

Light microscopy of cell-seeded polystyrene (PS) was performed using a Zeiss Axiovert 40 CFL equipped with a digital camera (Canon).

RT-PCR

For RT-PCR, cells were washed twice with PBS and total RNA isolation was performed using the peqGOLD MicroSpin Total RNA Kit (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was transcribed from 250 ng of total RNA (measured using a Peqlab Nanodrop ND 1000) in a 20 μ L reaction mixture containing 200 U of Superscript II Reverse Transcriptase (Invitrogen), 0.5 mM dNTP (Invitrogen), 12.5 ng/ μ L random hexamers (MWG Biotech, Ebersberg, Germany) and 40 U of RNase inhibitor RNase OUT (Invitrogen). For cDNA synthesis, the reaction mixtures were incubated for 50 min at 42°C followed by 15 min at 70°C in a Primus 25 Advanced Thermocycler (Peqlab). For PCR experiments, 1 μ L of cDNA was used in a 20 μ L reaction mixture containing specific primer pairs (MWG Biotech) to detect transcripts of TRAP, CALCR, CTSK, VTNR and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences used and annealing temperatures are summarized in Table 1. After the initial activation step at 95°C for 4 min, 25 to 35 PCR cycles were run with each denaturation step at 95°C for 45 s, an annealing step for 45 s and a synthesis step at 72°C for 1 min followed by a final synthesis step at 72°C for 10 min. The same single stranded cDNA was used to investigate the expression of the genes described. The resulting PCR-products were analysed using the FlashGel™ Dock and documentation system (Cambrex Bio Science, East Rutherford, NJ, USA).

Expression of the markers was normalised to the expression of

Table 1. Primers for RT-PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	T _A (°C)
GAPDH	GGTGAAGGTCGGAGTCAACGG	GGTCATGAGTCCTTCCACGAT	55
TRAP	TTCTACCGCCTGCACTCCAA	AGCTGATCTCCACATAGGCA	57
CALCR	GCAATGCTTTCACTCCTGAGAAAC	CAGTAAACACAGCCACGACAATGAG	57
CTSK	GATACTGGACACCCACTGGGA	CATTCTCAGACACACAATCCAC	57
VTNR	AAGTTGGGAGATTAGACAGAGG	CTTCTTGTCTTTGAGGTGG	57

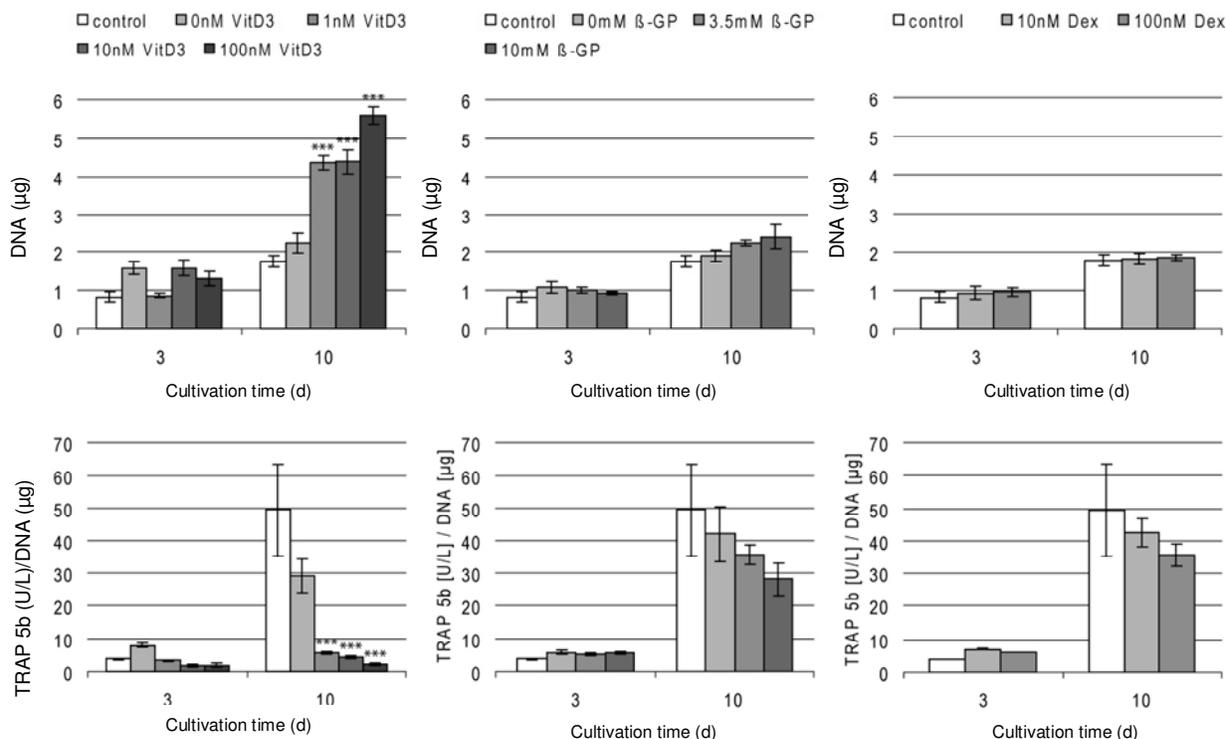


Figure 1. Influence of VitD3, β-GP and Dex concentrations on DNA amount and relative TRAP activity. The concentrations of other supplements were kept constant in each case as follows: 50 ng/ml M-CSF, 50 ng/ml RANKL, 10 nM Dex, and 10 mM β-GP for analysis of VitD3 influence; 50 ng/ml M-CSF, 50 ng/ml RANKL, and 10 nM Dex for analysis of β-GP concentration; 50 ng/ml M-CSF, 50 ng/ml RANKL, and 3.5 mM β-GP for analysis of Dex influence. Stars indicate significance compared to the 0 nM VitD3 sample.

GAPDH by image analysis using the BioImaging System Gene Genius with the acquisition software GeneSnap and the GeneTools software (SynGene, Cambridge, UK).

RESULTS

Influence of vitamin D3

An increase of DNA amount by factors of up to 3 after 10 days was detected for increasing concentrations of VitD3. The cultivation without VitD3 strongly stimulates relative TRAP 5b activity, whereas increasing VitD3 concentrations cause significant decrease in a dose-dependent manner Figure 1. Corresponding light microscopy images are shown in Figure 2h, d, c, b, a, and reveal clear

influence due to supplementation of VitD3. Even though all modifications result in the formation of multinucleated cells (exemplary indicated by white arrows), characteristic network-like accumulations of cells (exemplary indicated by *ac) were formed with increasing concentrations of VitD3. Only few of these cell accumulations are visible for the cultivation with 1 nM VitD3, but they dominate with 100 nM VitD3. Concurrently, the cell number seems to increase, which confirms the results of the DNA analysis. On the other hand the amount of large multinucleated cells is directly associated with the TRAP 5b activity measurements. RT-PCR analysis shows slightly increased expression of TRAP and VTNR at 10 nM VitD3 as well as 100 nM VitD3.

CTSK expression is not influenced. Interestingly the

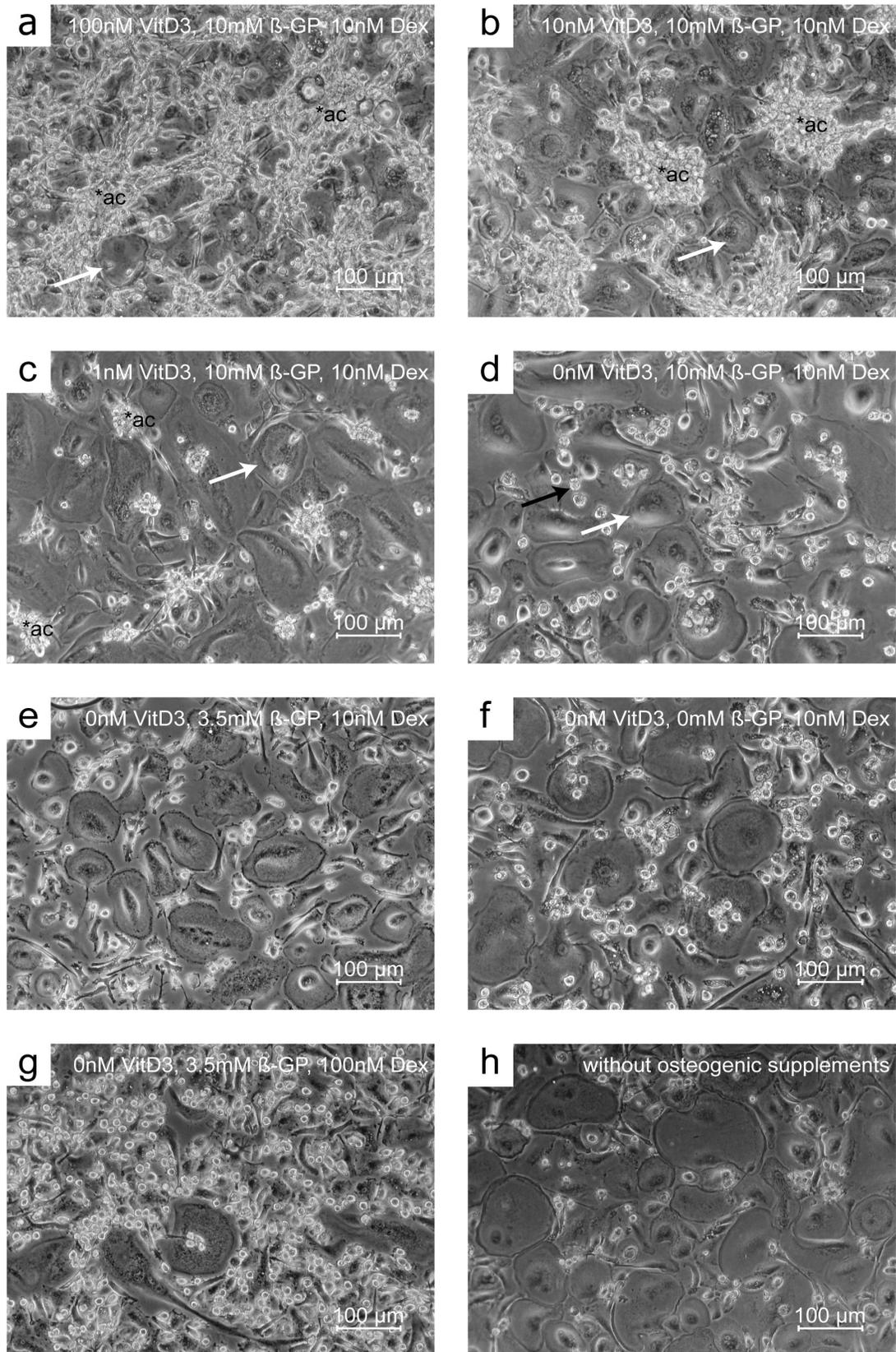


Figure 2. Light microscopy images of cells after 10 days of cultivation in different media compositions. Network-like cell accumulations (*ac), mononucleated cells (black arrows) and multinucleated cells (white arrows) are indicated.

Table 2. Expression of the markers normalised to the expression of the housekeeping gene GAPDH calculated by image analysis of Figure 3. For comparison, highest values were set to 10, respectively. HMc represents the expression profile of human monocytes as isolated. Letters correspond to the media compositions as indicated in Figure 2.

Markers	hMc	a	b	c	d	e	f	g	h
TRAP	5	10	10	7	7	10	6	10	6
CALCR	1	2	1	1	7	6	6	10	7
CTSK	2	8	8	7	8	6	7	10	8
VTNR	5	9	8	6	6	10	6	8	5

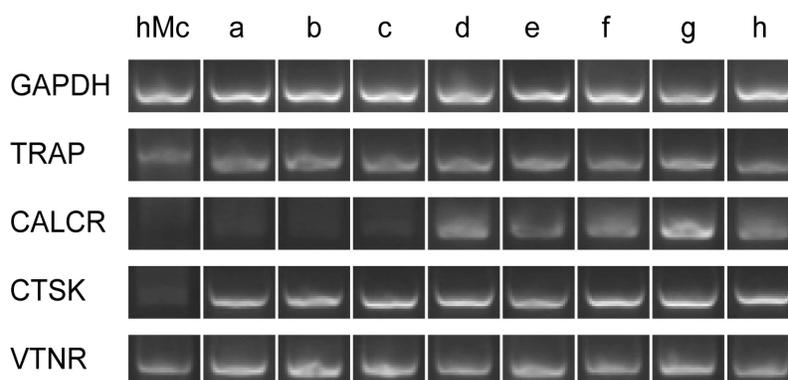


Figure 3. Gene expression of TRAP, CALCR, CTSK and VTNR after 10 days of cultivation in different media compositions. HMc represents the expression profile of human monocytes as isolated. Letters correspond to the media compositions as indicated in Figure 2.

expression of CALCR is strongly reduced even for low concentrations (1 nM) of VitD3 (Figures 3a, b, c and Table 2).

Influence of β -glycerophosphate

At day 10 the addition of up to 10 mM β -GP shows a slightly increasing but not a significant effect on the DNA amount. Relative TRAP 5b activity decreases by a factor of about 0.75 comparing the cultivation with 0 and 10 mM β -GP. Performing light microscopy (Figures 2h, f, e and d) no differences in the cell's morphology were detected for the three β -GP concentrations as well as compared to the control sample. The gene expression profile is marginally influenced by varying the β -GP concentration, with slight advantages for the 3.5 mM β -GP modification (Figures 1, 3h, f, e, d and Table 2).

Influence of dexamethasone

Dex has clearly no influence on the DNA amount during the cultivation and results in negligible decrease of relative TRAP 5b activity. Morphological influence on

osteoclastogenesis is detected by light microscopy (Figures 2h, e, and g). The addition of 10 nM Dex seems not to influence osteoclast formation. However with 100 nM Dex strong effects are visible, while less multinucleated cells are formed and a lot of cells remain mononucleated. In contrast to the microscopic observation gene expression levels of TRAP, VTNR, CALCR, and CTSK are slightly increased at 100 nM Dex compared to the control and 10 nM Dex (Figures 3h, e, g and Table 2).

DISCUSSION

Although monocytes are known to be postmitotic cells (Nijweide et al., 1986), slightly increase of DNA values from day 3 to 10 was recorded for all modifications. On the one hand, this observation can be addressed to further adherence of the cells at day 10. On the other hand, this can be explained by the presence of other proliferative cell types. The isolation and purification of monocytes from buffy coat is done by negative selection, whereby non-monocytes are labelled using antibodies against other cell types (Miltyeni, 2010). Although the isolation and purification of monocytes is carefully performed, low

fractions of other cell types in the final suspension cannot be excluded. In the present case flow cytometry using an anti-CD14-FITC antibody detected the purity of monocytes to be about 90%. The significant increase of DNA values detected for high VitD3 concentrations possibly can be addressed to dendritic cells. These cells also derive from monocytes; however, it is still unknown how their lineage commitment is regulated (Miyamoto et al., 2001). The role of VitD3 on osteoclast differentiation and activity is controversially discussed in the literature. On the one hand VitD3 has been shown to support osteoclastogenesis by increasing the expression of RANKL, and decreasing the expression of its antagonist OPG in a co-culture with stromal cells/osteoblasts (Horwood et al., 1998). Although the precise mechanism of interaction is still unclear, evidence suggests that VitD3 has direct effects on osteoclast precursors by increasing the expression of VTNR (Medhora et al., 1993). This corresponds with our findings, where VTNR is slightly increased by VitD3. In contrast, other reports describe the suppression of osteoclast differentiation due to VitD3 and a decreased resorptive activity (Itonaga et al., 1999; Kogawa et al., 2010; Takasu et al., 2006).

In the present study the TRAP 5b activity is strongly decreased by the addition of VitD3, whereas TRAP gene expression was slightly higher for 10 nM VitD3 and 100 nM VitD3. Measurement of the total TRAP 5 (5a and b) using the method developed by Lau et al. (1987) resulted in equal values for all concentrations. This indicates that cells cultivated with VitD3 start to proliferate and obviously exhibit more TRAP 5a (calculated as a difference of total TRAP and TRAP 5b) at an expense of TRAP 5b activity. Other TRAP 5a positive cells are macrophages and dendritic cells as it was discussed on the basis of DNA analyses (Halleen et al., 2002). In the present study the low TRAP 5b activity detected for high VitD3 concentrations conforms to the low numbers of multinucleated osteoclast-like cells observed using light microscopy. Moreover, expression of CALCR is strongly reduced in case of VitD3 addition. This receptor is considered as a late osteoclast differentiation marker, and its presence is often related to mature bone resorbing osteoclasts (Kurihara et al., 1990). Inhibiting effects of phosphate on osteoclastogenesis using co-culture models have been reported by Takeyama et al. (2001) and Kanatani et al. (2003). For increasing concentration of β -GP a similar trend is also observed in the present study, however, without being significant. No influence was recognized by microscopy. Similar to our experiments, Sivagurunathan et al. (2005) have ascertained that Dex has no significant influence on the number of TRAP-positive cells. Hozumi et al. (2009) described decreasing sizes of the osteoclasts as well as decreasing numbers of their nuclei in case of cultivation with 100 nM Dex in a co-culture with bone marrow adipocytes. Furthermore, Kartsogiannis reported increasing yield of osteoclasts due to the addition of Dex, but it may be

detrimental to osteoclast morphology (Kartsogiannis and Ng, 2004). The reports cited correspond with our findings that high expression levels of TRAP, CALCR and CTSK were recorded for high Dex concentrations being accompanied by a disturbance of the typical osteoclast morphology.

Taking into account, that all media modifications result in the partial formation of cells that show features characterizing osteoclasts, the total fractions should be described as osteoclast-like cells (OLC) – a term which is generally used in the literature. However the varying degree of severity of these typical features as detected in the present study, allows the identification of disadvantageous and advantageous cultivation media supplement concentrations, with the future aim of precursor cell co-cultivation and differentiation.

Conclusion

The osteogenic supplements Dex and β -GP slightly inhibit osteoclast formation but not thus far that osteoclastogenesis does not work at all. Nevertheless a co-cultivation of human BMSC/osteoblasts and hMc/osteoclasts should not use more than 10 nM Dex and can use up to 10 mM β -GP if necessary for osteoblast differentiation. Already in minute quantities the addition of VitD3 results in the reduction of both TRAP 5b activity and expression of the late osteoclastic marker CALCR. Therefore it should not be used for osteogenic differentiation of human BMSC in a co-culture with monocytes.

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