Calcitriol modulates the effects of bone marrow-derived mesenchymal stem cells on macrophage functions

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ARTICLE INFO	ABSTRACT
	Objective*(s):** Some evidence showed that calcitriol has an important role in regulating growth and differentiation of mesenchymal stem cells (MSCs). However, the interaction between mesenchymal stem cells and macrophage is not clear yet. The current study was done to investigate the *in vitro* effects of calcitriol on the interactions between bone marrow-derived MSCs and rat macrophages. ***Materials and Methods: MSCs were isolated from rat bone marrow and pulsed with different concentrations of calcitriol (50, 100 and 200 nanomolar) for 24, 48 and 72 hr. Then, mesenchymal stem cells were co-cultured with macrophages for 4 hr. Finally, macrophages were evaluated for ability to uptake neutral red, phagocytosis activity against opsonized yeast, respiratory burst and viability. ***Results:*** Our data showed that bone marrow-derived MSCs pulsed with calcitriol may cause a significant increase in uptake of neutral red and phagocytic activity of opsonized heat killed baker's yeast. Moreover, treatment of MSCs with calcitriol enhanced macrophage viability. Nevertheless, the respiratory burst of macrophages was significantly reduced in macrophages co-cultured with calcitriol-treated MSCs compared to control group. ***Conclusion:*** Calcitriol may accelerate and potentiate anti-inflammatory M2 macrophage polarization by MSCs.

Introduction

Calcitriol (1α,25(OH)2-Vitamin D3) possesses diverse biological actions that are mediated through alterations in gene expression in target cells (1, 2). After binding to its intracellular receptor, calcitriol orchestrates many essential biological processes from actions essential for maintenance of mineral homeostasis to focal actions that control the growth, differentiation and activity of numerous cell types such as cells of the immune system, skin, the pancreas and bone (1, 3). Interestingly, recent evidence reported that calcitriol has an important role in regulating growth and differentiation of mesenchymal stem cells (MSCs) (4, 5).

MSCs are plastic, adherent, fibroblast-like, multipotent non-hematopoietic progenitor cells that can differentiate into mesenchymal tissues like bone, cartilage, and fat (6). MSCs have been shown to possess an immunoregulatory role as they directly interact with a variety of myeloid and lymphocytic leukocytes; therefore, MSCs may be considered as precious tools for cell-based immunotherapy (7-9).

MSCs in tissue and bone marrow establish a niche which has indispensable interactions with hematopoietic cells including macrophages (10-12). Although the crosstalk between MSCs and many cells of immune system has been disclosed (12-14), there is very little known about the interaction between MSCs and macrophages. On the other hand, there is no information about the role of calcitriol as an environmental factor on the crosstalk between MSCs and macrophages. The present study was designed to investigate the effects of calcitriol on the interactions between bone marrow-derived MSCs and rat macrophages.

Materials and Methods
Chemicals

Natural red, Nitro blue tetrazolium, dioxin, dimethyl sulfoxide (DMSO), 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) and phosphate-buffered saline (PBS) were obtained from Sigma-Aldrich (St Louis, MO). May–Grunwald–Giemsa stain was procured from Merck (Darmstadt, Germany). Fetal

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calf serum, Dulbecco's Modified Eagle Medium (DEMEM) and RPMI 1640 were bought from GIBCO/Life Technologies Inc. (Gaithersburg, MD).

Isolation and proliferation of MSCs

Bone marrow-derived MSCs were isolated as previously described (15). In brief, bone marrow was flushed out from Wistar rats tibias and femurs, under deep anesthesia. After twice washings by centrifugation at 1200 rpm for 5 min in PBS, cells were plated in 75-cm^2 tissue-culture flasks at concentrations of 0.3×10^6 to 0.4×10^6 cells/cm^2 in DEMEM medium supplemented with 15% fetal calf serum. Cells were incubated in a humidified incubator with 5% CO_2 at 37 ℃. Four days following primary culture initiation, the culture mediums were collected, centrifuged and cells were seeded in a 75-cm^2 flask. Upon 70% confluence, cells were tripsinased using Tripsin/EDTA, counted and passed at 1:3 ratios (about 1.5×10^6 cell/75-cm^2 flask). A cell suspension of the third generational passage was collected. Then, MSCs were incubated with different concentrations of calcitriol (50, 100 and 200 nM) for different time periods (24, 48 and 72 hr). Then, MSCs cultures were trypsinized, collected and used for following experiments.

Macrophage isolation

Resident macrophages were obtained from the peritoneal cavity of rats by injection of PBS. Cells were washed twice in PBS and suspended in RPMI-1640 medium containing 10% heat-inactivated FCS, 10,000 U/ml penicillin G sodium, 10 mg/ml streptomycin sulfate, and 2 mM glutamine. Cells were counted using Neuber's chamber and the viability of the cells was determined by Trypan blue dye exclusion. Then, 100 μl of live cell suspension (2×10^6 cell/ml) were pre-incubated in 96-well microplates for 40 min at 37 ℃ in a humidified atmosphere of 5% CO_2. This process promoted macrophage adherence to the plate. Non-adherent cells were removed by washing the plate twice with PBS.

Incubation of macrophage with MSCs

Here, 100 μl of MSC suspension (2×10^5 cell/ml) was added to each well of 96-well microplates containing macrophages and incubated for 4 hr at 37 ℃ in a humidified atmosphere of 5% CO_2.

Phagocytic assay by neutral red

The basic phagocytic ability of macrophage (opsonin independent phagocytosis) was measured by neutral red uptake. MSCs were co-cultured with macrophages for 4 hr. Then, 200 μl neutral red solutions (dissolved in 10 mmol/l PBS at the concentration of 0.075%) were added and cells were incubated for 1 hr. Supernatant was discarded and

cells were washed twice in PBS to remove the neutral red that was not phagocytized by macrophages. Then, cell lysate solution (ethanol and 0.01 % acetic acid at the ratio of 1:1, 200 μl/well) was added to lysed cells. Next, cells were incubated at 4 ℃, overnight. The optical density was measured at 490 nm by a microplate reader.

Phagocytosis assay by opsonized heat-killed baker's yeast

This assay was performed as previously described with some modifications (16). Briefly, after stationary incubation of the macrophages with the heat-killed opsonized yeast at 37 ℃ for 1 hr, macrophages were detached from the bottom of microplate by incubation on ice for 5 min. Cells were washed, cytocentrifuged onto glass slides, and fixed in methanol. Next, the slides were stained with May–Grunwald–Giemsa staining. Bacterial ingestion was assayed by light microscopy. Phagocytosis activities of macrophages were expressed as percentage of neutrophils containing at least one yeast.

Respiratory burst

Intracellular generation of reactive oxygen species (ROS) was measured by NBT reduction as described previously (17, 18). In brief, cells were incubated for 30 min at 37 ℃, then cells were incubated with NBT solution (1 mg/ml) for 1 hr at 37 ℃. The unused NBT was washed and the reduced dye was measured at 520 nm.

Evaluation of macrophage viability

Macrophage viability was evaluated by MTT assay as described earlier (18, 19). Briefly, after the co-culture of macrophage and MSC, cultures were treated with 20 μl of the MTT solution (5 mg/ml) for 4 hr at 37 ℃. Then, 150 ml DMSO was added and the mixture was shaken vigorously to dissolve formazan crystal. The optical density (OD) was measured at 550 nm using microplate reader (Dynatech, Denkendorf, Germany). The experiments were done in triplicate.

Statistical analysis

Data were analyzed using the one-way ANOVA followed by Dunnett's *post-hoc* test and presented as means±SD. *P*-values of less than 0.05 were considered statistically significant.

Results

Phagocytosis is an essential function of macrophages (20). Neutral red (NR) is a vital stain that is engulfed by viable macrophages and accumulated in their lysosomes (21). The data showed that following the co-culture with calcitriol-treated MSCs, opsonin independent phagocytosis of macrophages was increased significantly in a dose-dependent manner (Figure 1). As shown in Figure 2,
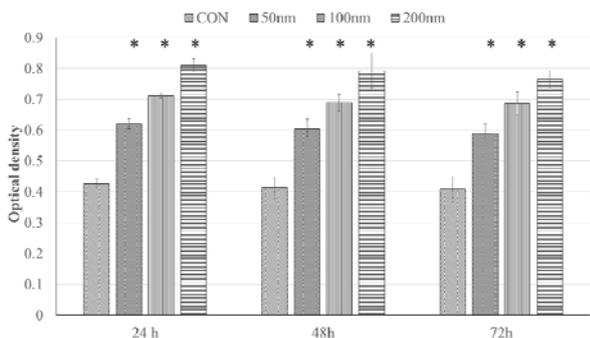
Figure 1. Evaluation of neutral red uptake by macrophage. Macrophages were co-cultured with MSCs pulsed with calcitriol and/or non-treated MSC for 4hr.. The results showed that neutral red uptake ability of macrophage was significantly increased following co-culture with calcitriol-treated MSCs in a dose-dependent manner (*$P < 0. 001$ vs control group)

Figure 2. Phagocytic activity of macrophages that were co-cultured with MSCs against opsonized yeast Compared to control group, calcitriol-treated MSCs significantly increased phagocytic activity of macrophages (* $P< 0.01$, ** $P< 0. 001$ vs control group)

opsonin dependent phagocytosis of macrophages that were co-cultured with calcitriol-treated MSCs, was significantly increased. However, this change was not in a dose-dependent manner.

NBT reduction assay showed that calcitriol-treated MSCs significantly diminished the rate of respiratory bust of co-cultured macrophages in a dose-dependent manner, as compared to the control group (Figure 3).

Macrophage metabolic activity that was measured by MTT assay was used to evaluate survivability of macrophages. Our findings indicated that, in a dose-dependent manner, the survival rate of macrophages that were co-cultured with calcitriol-treated MSCs, was significantly greater than that of macrophages co-cultured with non-treated MSCs (Figure 4).

Discussion

Macrophages have critical roles in the initiation and expression of innate and adaptive immune responses (22). Based on their ability to remove pathogens and instruct other immune cells, these cells play an essential role in protecting the host
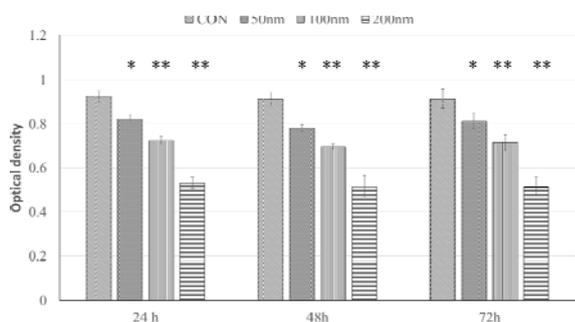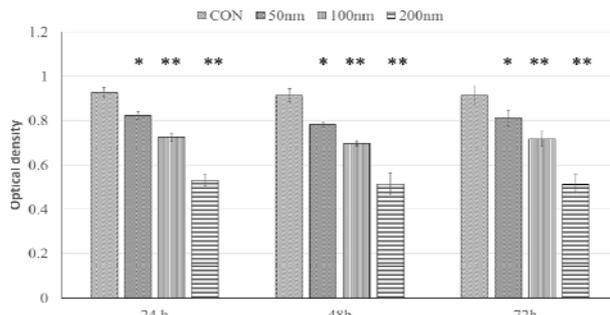
And participate in the pathogenesis of inflammatory and degenerative diseases (23, 24). Moreover, macrophages have a crucial function in later phases of tissue homeostasis and repair, such as removal of cellular debris and clearance of apoptotic cells (25). Macrophages are remarkably plastic and according to the environmental factors, they can change their functional phenotype (23-25). Classically-activated macrophages or M1 macrophages show potent anti-microbial and inflammatory properties while alternatively-activated macrophages or M2 macro-phages produce less pro-inflammatory cytokines and play a role in resolution of inflammation through trophic factor secretion and high phagocytic activities (24, 25).

As mentioned above, MSCs in tissue and bone marrow are interconnected with macrophages (23, 25); therefore, they may participate in determination of macrophages phenotype in microenvironments milieu. Pervious work showed that human macrophages co-cultured with bone marrow-drived MSCs, expressed high level expression of CD206, a marker of M2 macrophages and produced high levels
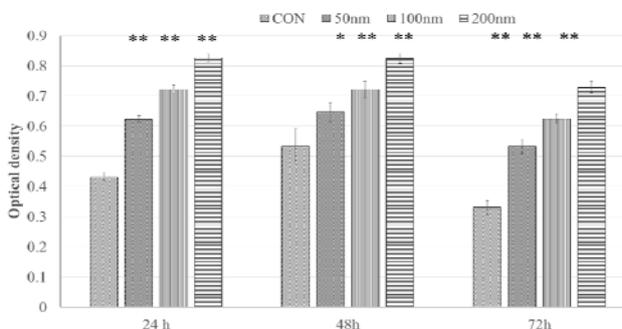

Figure 3. Evaluation of modulation of macrophage respiratory burst by calcitriol-treated MSCs. Compared to control group, calcitriol-treated MSCs diminished the rate of respiratory bust of macrophages in a dose-dependent manner (* $P< 0.01$, ** $P< 0. 001$ vs control group)

Figure 4. Effect of calcitriol on modulation of macrophage viability by MSCs. As compared to control group, calcitriol-treated MSCs significantly increased the viability of macrophage in a dose-dependent manner, (*$P< 0. 01$, ** $P< 0. 001$ vs control group)

of IL-6 and IL-10 and low levels of IL-12 and TNF-α as compared to controls (25). Thefore, these macrophages showed typical M2 phenotype. Calcitriol could be considered as an environmental factor which can alter the interaction between MSC and macrophages. It has been demonstrated that calcitriol inhibits MSC proliferation, induces cell cycle arrest and promotes accumulation of MSCs in G0/G1 phase without inducing apoptosis (4, 5). These effects were concurrent with a decrease in the GTPase Rho and the atypical Rho family GTPase Rhou/Wrch-1 expression without inducing Wnt-1 expression. The expression of survivin was also dysregulated (5).

It was demonstrated that MSCs co-cultured with macrophages, caused an increase in phagocytic activity of macrophages againt *Escherichia coli* (25). Higher phagocytic activity is a character of M2 macrophages (23, 24). Our data showed that bone marrow-derived MSCs that were treated with calcitriol, may cause a significant increase in uptake of neutral red and phagocytic activity of opsonized heat-killed baker's yeast. Interestingly, a new survey by our group showed that the supernatant of calcitriol-treated MSCs could significantly increase the phagocytosis of *Staphylococcus aureus* by neutrophils (12).

Reactive oxygen species (ROS) play essential role in removal of invading microrganisms by phagocytes (26). Nevertheless, when the production of ROS is excessive or inappropriate, ROS is involved in severe host tissue injury and participate in immuno-pathological conditions (27). M2 macrophages have remarkable anti-inflammatory properties and produce a diminished level of ROS (23, 24). In this study, calcitriol-treated MSCs profoundly increased the respiratory burst of macrophages compared to macrophages that were co-cultured with MSCs alone. Also, previous work indicated that supernatant of calcitriol-treated MSCs could significantly decrease respiratory burst intensity in neutrophils (12).

Here, MTT assay also showed that treatment of MSCs with calcitriol, enhanced macrophage viability. Similarly, calcitriol-treated MSCs caused a significant decrease in the percentage of neutrophils apoptosis (12).

Conclusion

Overall, these findings suggest that while bone marrow-derived MSCs that were pulsed with calcitriol, exert a protective role against potentially harmful ROS production, they preserve phagocytosis, an essential macrophage function, and the survival rate of macrophages. Therefore, calcitriol may accelerate and potentiate anti-inflammatory M2 macrophage polarization by MSCs. This may explain a new mechanism for immunodulatoy properties of calcitriol.

Acknowledgment

Conflict of interests

The authors declare that they have no conflicts of interest. All authors read and approved the final manuscript.

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