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Calcitriol modulates the effects of the supernatants of bone-marrow–derived mesenchymal stem cells on neutrophil functions

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Abstract: Mesenchymal stem cells (MSCs) in bone marrow form a niche that has inevitable interactions with neutrophils. Moreover, previous documents have shown that calcitriol has an important role in regulating the cell growth of MSCs. This study set out to investigate the effects of calcitriol on the interaction between bone-marrow–derived MSCs and neutrophils. MSCs were isolated from the bone marrow of rats and pulsed with different concentrations of calcitriol (50, 100, and 200 nM) for different periods of time (24, 48, and 72 h). As the next step, the supernatants of MSCs cocultured with neutrophils for 4 h and neutrophil functions were evaluated. The results showed that the supernatants of MSCs treated with calcitriol could significantly increase the phagocytosis of *Staphylococcus aureus* by neutrophils and, conversely, decrease the respiratory burst intensity of neutrophils. Moreover, treatment of MSCs with calcitriol can cause a significant decrease in the percentage of neutrophil apoptosis. These findings were concurrent with a significant increase in IL-6 levels in the supernatant of calcitriol-treated MSCs. Consequently, the supernatant of bone-marrow–derived MSCs was pulsed with calcitriol, and the exertion of a protective role against potentially harmful reactive oxygen species production preserved phagocytosis and the survival rate of neutrophils.

Key words: Mesenchymal stem cells, calcitriol, neutrophil

1. Introduction

Complicated crosstalk between environmental factors and multiple genes determines which individuals will develop any given immune-mediated disease (Cantorna, 2010). Calcitriol [1α-25(OH)-vitamin D3] is one of the steroid hormone families and, similar to other members of these families, participates in the regulation of gene expression (Cantorna, 2010; Smyk et al., 2013). On the other hand, calcitriol may be an environmental factor that contributes to immune-mediated disease development. Environmental sources of calcitriol include diet and production in the skin following UV exposure to precursor 7-dehydrocholesterol (Namgung et al., 1994).

Bone-marrow–derived mesenchymal stem cells (MSCs) are multipotent and can give rise to mesenchymal tissues like bone, cartilage, and fat (Uccelli et al., 2008). They also have potent immunomodulatory properties and may be valuable tools for cell-based immunotherapy (Meirelles Lda et al., 2009; Ghannam et al., 2010; Zhang et al., 2013). MSCs in bone marrow and tissue form a niche that has inevitable interactions with hematopoietic cells including neutrophils (Raffaghello et al., 2008; Maqbool et al., 2011). Neutrophils are one of the major cell types that constitute innate immunity. They predominate in host tissues during acute inflammatory processes (Greenberg and Grinstein, 2002).

Recent documents have shown that calcitriol has an important role in regulating the growth of MSCs (Artaza et al., 2010; Klotz et al., 2012). The present study was carried out to investigate the effects of calcitriol on the interaction between bone-marrow–derived MSCs and neutrophils in rats.

2. Materials and methods

2.1. Materials

Propidium iodide, acridine orange, and phosphate-buffered saline (PBS) were procured from Sigma-Aldrich (St Louis, MO, USA). May–Grünwald–Giemsa stain was purchased from Merck (Darmstadt, Germany) and dextran was obtained from Fresenius Kabi (Verona, Italy). Fetal calf serum, Dulbecco's Modified Eagle Medium (DEMEM), and RPMI 1640 were purchased from GIBCO/Life Technologies Inc. (Gaithersburg, MD, USA). The enzyme-linked immunosorbent assay (ELISA) kit for interleukin (IL)-6 was purchased from Bender MedSystems (Vienna, Austria).

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2.2. Isolation and proliferation of MSCs

MSCs were isolated as described previously (Baghaban Eslaminejad et al., 2008). Briefly, bone marrow from deeply anesthetized Wistar rats was flushed out of tibias and femurs. After 2 washings by centrifugation at 1200 rpm for 5 min in PBS, cells were plated in 75-cm^2 tissue-culture flasks at concentrations of 0.3–0.4 × 10^6 cells/cm^2 in DEMEM medium supplemented with 15% fetal calf serum. Cells were incubated in humidified 5% CO_2 at 37 °C. Four days following primary culture initiation, the culture mediums were collected and centrifuged, and the pellets were replated in a fresh 75-cm^2 flask. The cultures were fed twice weekly and upon 70% confluence. The cells were removed using Trypsin-EDTA, counted, and passed at 1:3 ratios (about 1.5 × 10^6 cells/75-cm^2 flask). Cell passage was performed up to subculture 3. MSCs were then incubated with different concentrations of calcitriol (50, 100, and 200 nM) for different periods of time (24, 48, and 72 h). Supernatants of MSC cultures were collected and used for the following experiments.

2.3. Neutrophil isolation

Blood samples were collected under ether anesthesia by cardiac puncture in sodium citrate (0.129 M; pH 6.5; 9:1, v/v). The samples were centrifuged at 2000 rpm for 20 min, and the buffy coat was subjected to dextran sedimentation (1% w/v) followed by centrifugation (400 × *g*, 30 min) on a Ficoll–Hypaque density gradient, as previously described (Ottonello et al., 1999). The plasma and the mononuclear cell layer were discarded, and contaminant erythrocytes were removed by hypotonic lysis. The cells were washed and suspended in RPMI 1640 (Ottonello et al., 1999). Neutrophils were counted in a Neuber chamber, and the viability of the cells was determined by Trypan blue dye exclusion. Purity of neutrophils was 95% following this procedure.

2.4. Incubation of neutrophils with supernatants of MSCs

The bottom chambers of 24-well flat-bottomed plates were loaded with supernatants of MSCs. Afterwards, 5 × 10^5 neutrophils in 200 µL of RPMI 1640 supplemented with 15% fetal calf serum were added and incubated for 4 h. Following incubation, the neutrophils were isolated and used for the next experiments.

2.5. Evaluation of neutrophil apoptosis

Neutrophil apoptosis was evaluated by fluorescence microscopy. In brief, the staining solution was prepared by adding 100 µL of 1 mg/mL propidium iodide and 100 µL of 1 mg/mL acridine orange to 10 mL of PBS. Neutrophil suspensions were mixed 1:1 with the staining solution in microtiter wells (Turina et al., 2005). The percent of apoptotic cells was determined in an improved Neubauer rhodium hemocytometer under fluorescent microscopy.

2.6. Phagocytosis assay

To evaluate phagocytosis activity of neutrophils against the heat-killed *S. aureus*, ATCC 25923 stain at a concentration of 10^8 cells/mL was applied. Neutrophils were mixed with *S. aureus* at a ratio of 1:10 in U-bottom plates with a final volume of 0.2 mL and incubated for 0.5 h at 37 °C, and then the slides were stained with May–Grünwald–Giemsa staining. Bacterial ingestion was assayed by light microscopy under oil immersion. Phagocytosis activities of neutrophils were expressed as percentage of neutrophils that internalized at least one *S. aureus* (Hamaliaka and Novikova, 2010).

2.7. Respiratory burst

NBT reduction test was performed as described previously with some modifications (Müller et al., 1981; Nabi et al., 2005; Hamaliaka and Novikova, 2010). In brief, 200 µL of neutrophil suspension (25 × 10^5 cell/mL) was mixed with 200 µL/mL of *S. aureus* suspension (10^8 cells/mL) and 200 µL of 0.1% NBT in PBS (pH 7.4). The mixture was incubated at room temperature for 15 min and subsequently kept at 37 °C for an additional 15 min. The reduced dye was extracted in dioxane and quantitated at 520 nm.

2.8. IL-6 assay

Supernatants from MSC cultures were checked for levels of IL-6 using the ELISA kit according to the manufacturer's instructions.

2.9. Statistical analysis

Data were analyzed using one-way ANOVA plus Dunnett's post-hoc test and are presented as means ± SDs. P-values of less than 0.05 were considered statistically significant.

3. Results

Circulating neutrophils have a short life span of 6–10 h, after which the cells undergo apoptosis (Coxon et al., 1999). The effects of the supernatants of calcitriol-pulsed MSCs on neutrophil survival were assessed by propidium iodide/acridine orange staining. Through this method, neutrophils were classified by color and chromatin morphology. In cell populations, the green cells (excluding propidium iodide) are viable with diffused chromatin, and those with condensed chromatin are apoptotic. The red cells (including propidium iodide) with noncondensed chromatin are necrotic (Figure 1A) (Salti et al., 2000). A significant reduction in apoptosis was observed in neutrophils cocultured with supernatants of MSCs treated with 100 and 200 nM of calcitriol for 48 h and/or 72 h compared with supernatants of the control group (supernatants of MSCs that were not pulsed with calcitriol) (Figure 1B).

The NBT reduction assay was used to measure the reactive oxygen species (ROS) activity in neutrophils (Hamaliaka and Novikova, 2010). Our findings showed that supernatants of MSCs pulsed with 50 nM calcitriol for 72 h and supernatants of MSCs treated with 100 and 200 nM calcitriol for 48 h and/or 72 h significantly diminished

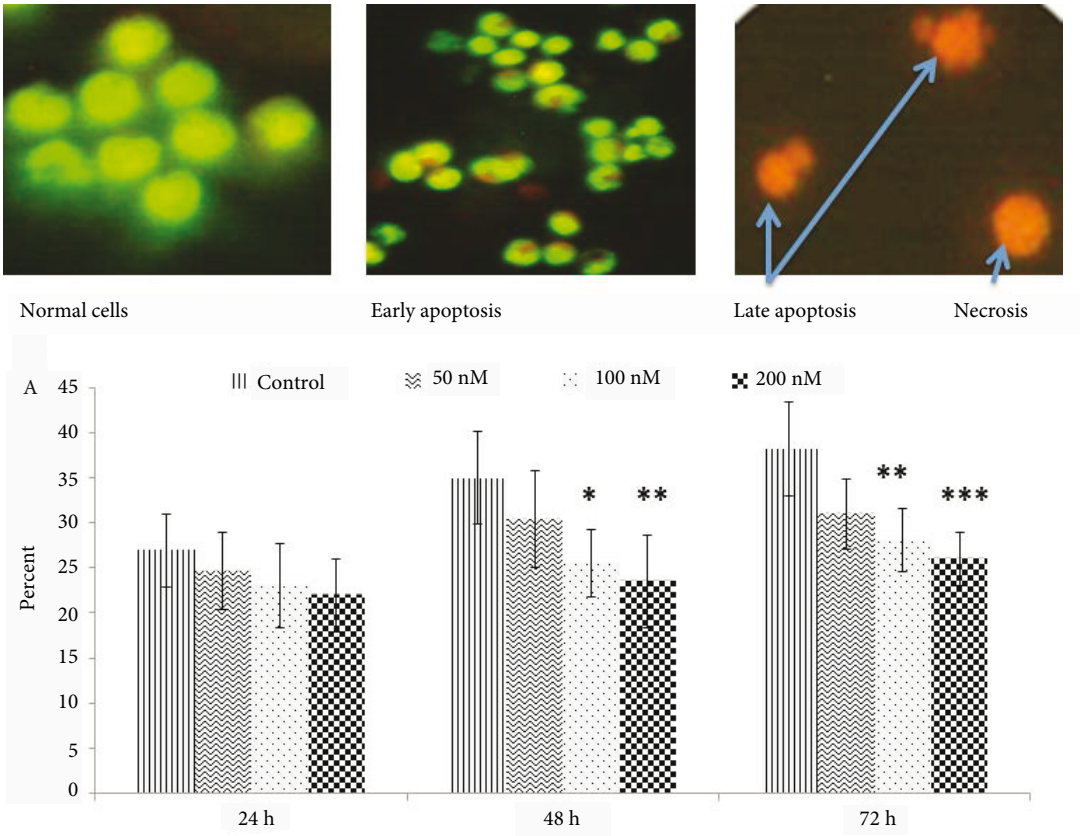

Figure 1. Evaluation of neutrophil apoptosis after 4 h of coculture with supernatants of MSCs treated with calcitriol. A) Neutrophil apoptosis was assessed by propidium iodide/acridine orange staining. The green cells with diffused chromatin are viable, and those with condensed chromatin are apoptotic. The red cells with noncondensed chromatin are necrotic. B) Supernatants of MSC treated for at least 48 h with calcitriol at a 100 nM concentration had a positive effect on neutrophil survival (*: $P < 0.05$, **: $P < 0.001$, ***: $P < 0.0001$ versus control group).

the rate of respiratory burst of cocultured neutrophils compared with supernatants of the control group (Figure 2).

Phagocytosis is an essential function of neutrophils (Greenberg and Grinstein, 2002). Phagocytosis activity of neutrophils significantly increased following coculture with supernatants of MSCs treated with at least 50 nM calcitriol for 48 h and/or 72 h compared with supernatants of the control group (Figure 3).

To determine the mechanism of calcitriol treatment, we measured the level of IL-6 in supernatants of MSCs. A significant increase in IL-6 production was observed in supernatants of the MSCs pulsed with at least 50 nM calcitriol for 72 h and at least 100 nM for 48 h compared with supernatants of the control group (Figure 4).

4. Discussion

Mature neutrophils leave the bone marrow compartment and move towards the blood. Therefore, a direct relationship between MSCs in the bone marrow compartment and mature neutrophils could not be exactly explained. However, some findings have suggested that tissue-resident MSCs localized in perivascular and periendothelial areas produce a place for neutrophil and MSC interaction (Crisan et al., 2008; Brandau et al., 2010). Cultured perivascular cells derived from various tissues exhibited a phenotype similar to that of bone-marrow–derived MSCs (Crisan et al., 2008). MSC-mediated immunomodulation displays a principal defense mechanism against harmful immune reaction at the interface between the mesenchymal compartment and blood in vivo (Rasmusson, 2006).

Immunomodulatory effects of MSCs require preliminary activation of the MSCs by immune cells such as neutrophils via secretion of the proinflammatory cytokines including TNFα and IL-1 (Ren et al., 2008; Ghannam et al., 2010). After activation, MSCs mediate immunosuppression through the secretion of soluble mediators (such as nitric oxide, prostaglandin E2, indoleamine 2,3-dioxygenase, and IL-6) and up-regulation of modulatory molecules (including galectins, PDL1,

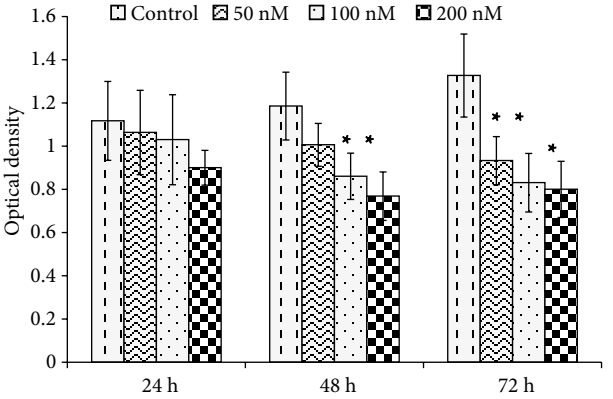

Figure 2. Modulation of neutrophil respiratory burst by supernatants of calcitriol-treated MSCs. Compared with supernatants of the control group, the supernatants of MSCs pulsed with 50 nM calcitriol for 72 h and supernatants of MSCs treated with at least 100 nM calcitriol for 48 h and/or 72 h significantly diminished the rate of respiratory burst of cocultured neutrophils (*: $P < 0.0001$ versus control group).

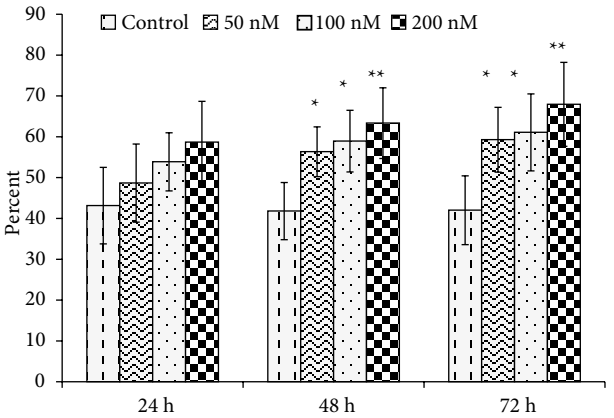

Figure 3. Evaluation of phagocytosis ability of neutrophils after coculture with MSC supernatants pulsed with calcitriol. Phagocytosis activity of neutrophils significantly increased after coculture with supernatants of MSCs treated with at least 50 nM calcitriol for 48 h and/or 72 h compared with supernatants of control group (*: $P < 0.05$, **: $P < 0.001$ versus control group).

TGF-β, and HLA-G) (Meisel et al., 2004; Maby-El Hajjami et al., 2009; Ghannam et al., 2010). Recent evidence has demonstrated that calcitriol inhibits MSC-proliferation–induced cell cycle arrest and promotes accumulation of MSCs in the G0/G1 phase without inducing apoptosis (Artaza et al., 2010; Klotz et al., 2012). These effects were associated 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 increased (Artaza et al., 2010).

Neutrophil homoeostasis and turnover are highly regulated in the body by apoptosis. MSCs significantly protect neutrophils from apoptosis (Brandau et al., 2010; Maqbool et al., 2011). In this study, treatment of mesenchymal stem cells with calcitriol enhanced neutrophil viability due to a reduction in apoptosis. In addition, the incubation of MSC supernatant reduces the rate of apoptosis in LPS-stimulated neutrophils by secretion of IL-8 and macrophage inhibitory factor (Brandau et al., 2010). Moreover, the IL-6 present in MSC culture supernatants is an essential factor for neutrophil rescue from apoptosis (Raffaghello et al., 2008; Maqbool et al., 2011). Further studies indicated that MSCs decrease the mitochondrial proapoptotic protein Bax through IL-6 signaling and increase the mitochondrial antiapoptotic protein MCL-1 (Raffaghello et al., 2008). Interestingly, our findings indicated that the level of IL-6 in supernatants of MSCs treated with calcitriol significantly increased compared with supernatants of the control group. A novel study showed that cocultivation of rat-bone-marrow–derived MSCs with pancreatic islet cells and/or streptozotocin-damaged pancreatic islet cells significantly protected the islet cells from apoptosis. These data were concurrent with increased secretion of IL-6 and TGF-β1 into the cocultured medium in comparison with monoculture of MSCs, islet cells, and streptozotocin-damaged islet cells (Karaoz et al., 2010).

It seems that MSCs have no effect on neutrophil phagocytosis, expression of adhesion molecules, and chemotaxis in response to IL-8, f-MLP, or C5a (Raffaghello et al., 2008). Nevertheless, we demonstrated that the supernatant of bone-marrow–derived mesenchymal stem cells pulsed with calcitriol may cause a significant increase in the phagocytic ability of neutrophils.

ROS are required agents for the elimination of invading microbes by neutrophils (Hamaliaka and Novikova,

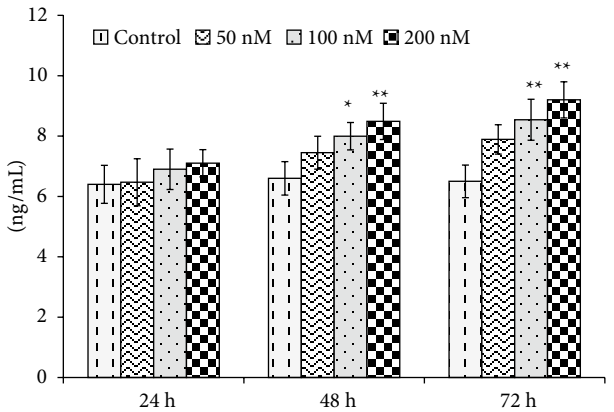

Figure 4. Effect of calcitriol on the level of IL-6 in MSC supernatants. Compared with supernatants of the control group, treatment of MSCs with calcitriol may increase the level of IL-6 (*: $P < 0.001$, **: $P < 0.0001$ versus control group).

2010). On the other hand, when the production of ROS is excessive or inappropriate, ROS are involved in severe host tissue injury and immunopathological conditions (Babior, 2000). Supernatants of MSCs were shown to inhibit basal and f-MLP–stimulated production of ROS by neutrophils through an IL-6-mediated mechanism (Raffaghello et al., 2008). In this study, concurrent with an increasing IL-6 level, the supernatant of the calcitriol-treated MSCs profoundly increased the respiratory burst of neutrophils compared with supernatants of the MSCs.

Gene expression is a time-consuming process. Therefore, we propose that the minor effects on neutrophil activity of MSC supernatant pulsed with calcitriol for 24 h, compared with other treatment groups, may be due to limitations on the time required for gene expression, including the IL-6 gene.

As a result, our findings suggest that the supernatant of bone-marrow–derived MSCs pulsed with calcitriol, while exerting a protective role against potentially harmful ROS production, preserves phagocytosis, an essential neutrophil effective function, and the survival rate of neutrophils. These results may be due to the significant increase in IL-6 levels in the supernatant of calcitriol-treated MSCs. However, this survey is a preliminary study, and the precise mechanisms involved in these effects remain to be clarified.

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