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ORIGINAL ARTICLE

Regenerative effects of transplanting autologous mesenchymal stem cells on corticosteroid-induced osteonecrosis in rabbits

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Abstract Osteonecrosis (ON) of the femoral head is one of the most serious complications associated with steroid administration. Here, we treated corticosteroid-induced ON in the rabbit by transplanting mesenchymal cells (MCs). Rabbits were injected once with 20 mg/kg of methylprednisolone (MPSL) and divided into three groups as follows: (1) MPSL alone (no further treatment); (2) MPSL+MCs (7 days after MPSL, MCs [1×10^7/2 ml] were injected into the bone marrow cavity of the femurs); (3) MPSL+saline (7 days after MPSL, saline [2 ml] was injected into the bone marrow cavity of the femurs). Subsequently, the incidence of ON in the femurs 4 weeks after MPSL alone and MPSL+saline was 80 and 68.4%, respectively. In contrast, no ON was recorded in rabbits treated with MPSL+MCs. Vascular endothelial growth factor (VEGF) staining and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) staining was more marked in the MPSL alone and MPSL+saline groups than in the MPSL+MCs rabbits. The percentages of cells in the G1 phase in the MPSL+MCs group were significantly lower than in the other two groups. These findings suggest that the injection of autologous MCs into the femur could prevent corticosteroid-induced ON in patients treated with high-dose short-term steroid medication.

Keywords Bone marrow cells · Transplantation · Corticosteroid · Osteonecrosis · Rabbits

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Introduction

The use of corticosteroids provides great benefit for patients who have bone marrow transplantation (BMT), organ transplantation, autoimmune diseases, or spinal cord injury. However, corticosteroids have several side effects and, in particular, corticosteroid-induced osteonecrosis (ON) of the femoral head is one of the most serious [1]. The incidence of corticosteroid-induced ON in patients administered steroids for BMT, kidney transplantation, and liver transplantation were reported to be 19, 11, and 14%, respectively [2–4]. For spinal cord injury and systemic lupus erythematosus (SLE), this figure is 5% [5] and 22–44% [6, 7], respectively. Many factors such as thromboembolism, fat embolism, thrombophilia, hypofibrinolysis, vasculitis, and intramedullary hemorrhage have been related to corticosteroid-induced ON [1, 8–13]. It has also been reported that steroid increases intraosseous pressure and that this influences corticosteroid-induced ON [14, 15]. Therefore, to prevent corticosteroid-induced ON in humans, several therapies such as anti-lipid agents, anti-coagulant agents, or cyclosporin A have been suggested [16–19]. However, due to the different factors contributing to corticosteroid-induced ON, there is no established method for its prevention and treatment.

Recently, there have been reports that, in the rabbit model, a single injection of either high- or low-dose methylprednisolone (MPSL, 20 mg and 4 mg/kg, respectively) caused corticosteroid-induced ON lesions of the metaphysis and diaphysis, but not the epiphysis of the long bones [20, 21]. Furthermore, using these rabbit models, several methods such as treating with anti-lipid agents, anti-coagulant agents, cyclosporin A, and the release of intraosseous pressure have been tested for the prevention of corticosteroid-induced ON [22–24]. Recently, autologous bone marrow cell (BMC) transplantation into the femoral

head at an early stage (non-collapsing stage) was reported to be effective in preventing the progression of ON in humans [25, 26].

BMCs contain not only hematopoietic stem cells (HSCs), but also mesenchymal stem cells (MSCs). The latter, which differentiate not only into osteoblasts, but also endothelial cells, play a crucial role in regulating osteogenesis and angiogenesis [27]. We have previously found that transplanting mesenchymal cells (MCs) into the femurs of rabbits administered steroids can treat corticosteroid-induced ON [28]. In the present study, we analyze the mechanism underlying the regeneration of corticosteroid-induced ON after this treatment and show the etiopathogenesis of corticosteroid-induced ON.

Materials and methods

Rabbits

Adult male Japanese white rabbits ranging in age from 28 to 32 weeks and weighing from 3 to 4 kg (defined as animals with closed growth plates) were obtained from Shimizu Laboratory Supplies (Kyoto, Japan). The rabbits were kept in single cages and fed a standard diet. The study protocol was approved by the Animal Experimentation Committee, Kansai Medical University (Osaka, Japan).

Preparation of MCs and injection into the bone marrow cavity

The rabbits were anesthetized using Fluothane® (Takeda Pharmaceutical Company Ltd., Osaka, Japan). An 18-gauge needle connected to a syringe was inserted into the center of the left femur and the bone marrow fluid was aspirated (Fig. 1a). To harvest the MCs, BMCs were cultured in DMEM (Invitrogen Corp., Grand Island, NY) with 10% FBS (Invitrogen) supplemented with antibiotics in a humidified atmosphere of 5% CO_2 at 37°C for 5 days. Non-adherent cells were discarded and adherent cells (MCs) were harvested from the surface of the flask using 0.05% trypsin (Invitrogen).

We first confirmed that 2 ml of Indian ink injected into the center of the femur was sufficient for diffusion into the femoral bone marrow cavity and to completely stain the femur from end to end (Fig. 1b, c). For the experiments reported here, therefore, an 18-gauge needle was inserted into the center of the right femur and 1×10^7 cultured MCs were injected into the bone marrow cavity in 2 ml of medium.

Protocol

Rabbits with corticosteroid-induced ON were prepared according to the method described previously [20]. In brief,

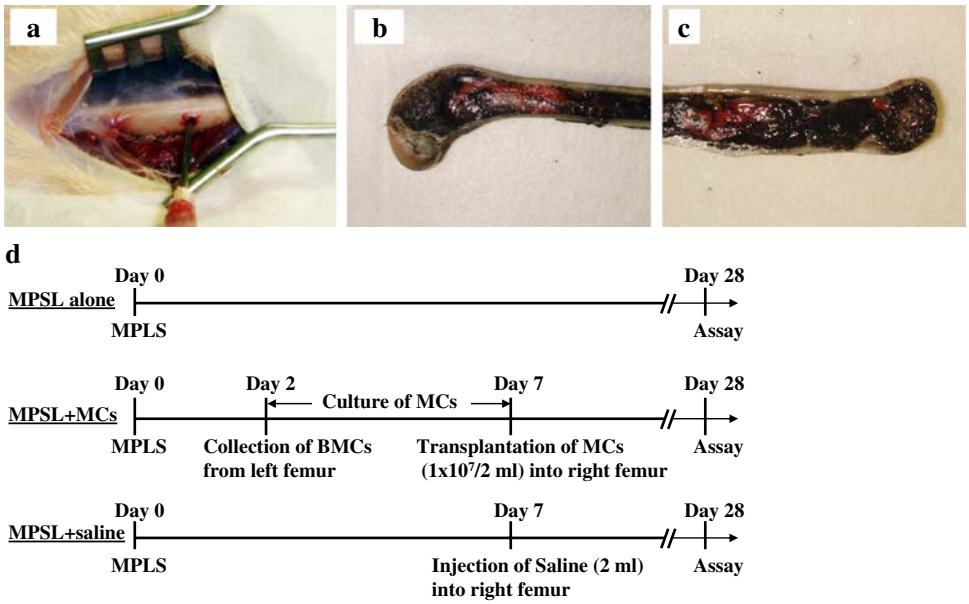

Fig. 1 Experimental protocol. **a** Bone marrow fluid aspirated from the femur. **b**, **c** Indian ink (2 ml) injected from the center of the femur showing diffusion to both proximal (**b**) and distal sites (**c**). **d** Rabbits injected once with 20 mg/kg of methylprednisolone (MPSL) into the gluteus medius muscle were divided into three groups: (1) MPSL alone—rabbits received no further treatment; (2) MPSL+MCs—7 days after the injection of MPSL, mesenchymal cells (MCs; 1×10^7/2 ml), precultured for 5 days in vitro, were directly injected into the bone marrow cavity of the right femur; (3) MPSL+saline—7 days after the injection of MPSL, saline (2 ml) only was directly injected into the bone marrow cavity

rabbits were injected once with 20 mg/kg of MPSL (Pfizer Japan Inc., Tokyo, Japan) into the gluteus medius muscle. Thereafter, the animals were divided into three groups as follows (Fig. 1d): (1) MPSL alone, in which the rabbits received no further treatment; (2) MPSL+MCs, in which, 7 days after the injection of MPSL, MCs (1×10^7/2 ml) were directly injected into the bone marrow cavity of the right femurs; and (3) MPSL+saline, in which, 7 days after the injection of MPSL, saline (2 ml) was directly injected into the bone marrow cavity of the right femur.

Measurement of the viability of cultured MCs

To evaluate whether MPSL influenced the viability of cultured MCs, we measured the amount of bone-type alkaline phosphates (BAP), an osteogenesis marker, in the culture medium, according to the scheme shown in Fig. 2a. In the "MPSL+MCs cultured for 1 day" group, 1×10^7 BMCs were collected from the rabbits 2 days after the injection of MPSL and were cultured in DMEM with 10% FBS at 37°C for 1 day. Non-adherent cells were discarded and adherent cells (MCs) were collected; 1×10^6 of these were incubated for 1 day and the BAP was quantified in the supernatants using an enzyme immunoassay method (Metra BAP EIA kit, Quidel Corp., San Diego, CA) according to the manufacturer's protocol. In the "MPSL+MCs cultured for 3 days" and the "MPSL+MCs cultured for 5 days" groups, 1×10^7 BMCs were collected from the rabbits 2 days after the injection of MPSL and were cultured for 3 and 5 days, respectively. The BAP was then quantified as above. The controls consisted of 1×10^7 BMCs collected from normal rabbits and cultured for 5 days. The BAP was quantified in the same way.

Pathological examination and evaluation of corticosteroid-induced ON

The rabbits were sacrificed and their femurs fixed for 1 week in 10% formalin. Bone samples were then decalcified with bone-type alkaline phosphates (EDTA; Wako Pure Chemical Industries Ltd. Osaka, Japan) for 14 days. The specimens were embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin (HE). Corticosteroid-induced ON was determined 1 and 4 weeks

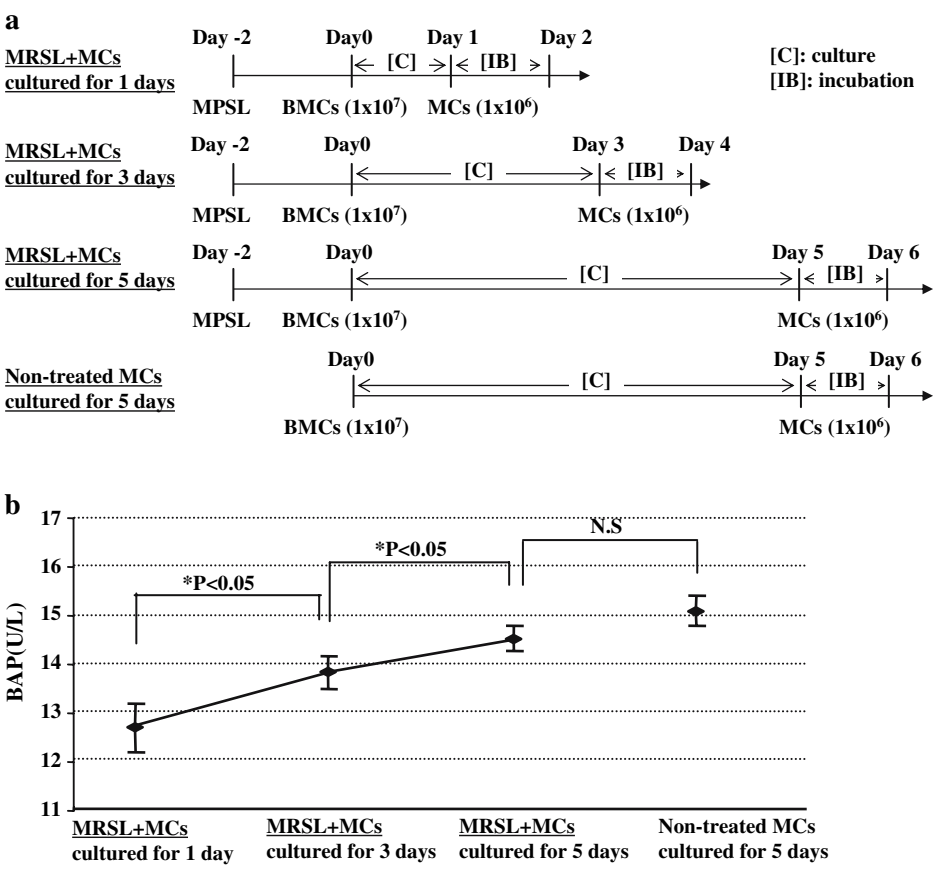

Fig. 2 Measurement of the viability of cultured MCs. **a** Bone marrow cells (BMCs) collected from rabbits 2 days after the injection of MPSL. 1×10^7 BMCs were cultured for 1, 3, and 5 days, non-adherent cells discarded, MCs collected, and 1×10^6 MCs were incubated for 1 day. The amount of bone-type alkaline phosphates (BAP) in the supernatants was then quantified. Controls: BMCs from normal rabbits with the same protocol cultured for 5 days. **b** BAP from the four groups. The results are mean±SD of six femurs. Statistical analyses were carried out by Student's *t*-test: $^*P < 0.05$

after MPSL injection by HE staining. As described by Yamamoto et al. [20], the diagnosis of ON was blindly made by three investigators independently (TK, HM, TA) on the basis of the diffuse presence of empty lacunae or pyknotic nuclei of osteocytes in the bone trabeculae, accompanied by surrounding BMC necrosis. Only BMC necrosis in both hematopoietic and fat cells with no bone trabecula included was assessed as corticosteroid-induced ON; lesions composed of only a few empty lacunae within the normal bone trabecula and/or fat-cell necrosis alone were excluded from the diagnosis of corticosteroid-induced ON.

Immunological staining

The sections were immuno-stained for vascular endothelial growth factor (VEGF) using an anti-VEGF monoclonal antibody, clone JH121 (Upstate Biotechnology, Lake Placid, NY). In brief, sections were incubated with anti-VEGF monoclonal antibody overnight. After washing with 0.05 M phosphate buffer (pH 7.6), the sections were labeled with peroxidase and goat anti-mouse Ig antibody (Histofine Mousestain Kit; Nichirei Biosciences Inc., Tokyo, Japan). Following washing with 0.05 M phosphate buffer (pH 7.6), the reaction product was developed using 3-3′-Diaminobenzidine (DAB). The sections were then counter-stained with Mayer's hematoxylin to show the nuclei. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) staining was also carried out to detect apoptosis, as previously described [29].

Analysis of the cell cycle

To evaluate the activation of the BMCs from the proximal and distal sides of the femurs, we analyzed the cell cycle using flow cytometry. In brief, the cells were treated with propidium iodide (PI; Sigma-Aldrich Co., St. Louis, MO) and ribonuclease A (RNase; Sigma-Aldrich Co.), and the stained cells were analyzed using a FACScan (Becton, Dickinson and Company, Franklin Lakes, NJ).

Statistical analyses

The numbers of proximal and distal femurs with ON lesions were compared using the χ^2 test with Bonferroni correction for multiple comparisons. Analyses of BAP and the percentages of cells in the G1 phase of the cell cycle were compared using Student's *t*-test. Differences were considered as statistically significant when the *P* value was less than 0.05.

Results

Analysis of the activity of MCs treated with MPSL

To evaluate whether MPSL influenced cultured MCs, we compared the amount of BAP in the medium of 1-, 3-, and 5-day MC cultures. As shown in Fig. 2b, the amounts of BAP from the "MPSL+MCs cultured for 5 days" group was significantly greater than the "MPSL+MCs cultured for 3 days" group and was almost the same as the amount of BAP from the control "non-treated MCs cultured for 5 days." These findings suggest that the functionality of MCs from femurs treated with MPSL was restored to almost normal levels by culturing them for 5 days.

Incidence of ON and histological findings seven days after MPSL

The proximal and distal sides of the femurs from rabbits treated with MPSL alone, but not from normal rabbits, showed empty lacunae and osteocyte pyknotic nuclei within the bone trabeculae (Fig. 3a, b compared to 3c, d). The incidence of ON one week after the injection of MPSL was 50% (5 of 10).

Incidence of ON and histological findings 28 days after MPSL

In this study, to examine the effects of transplanting MCs into the bone marrow cavity on corticosteroid-induced ON, we analyzed the histology of the proximal and distal sides of femurs by HE staining. Femurs from rabbits treated with MPSL alone or with MPSL+saline showed empty lacunae and pyknotic nuclei of osteocytes within the bone trabeculae (Fig. 4a–d). In contrast, the proximal and distal sides of the femurs from rabbits treated with MPSL+BMT did not show any signs of ON, except for a few empty lacunae within the normal bone trabeculae or fat-cell necrosis (Fig. 4e, f). As shown in Table 1, the incidence of corticosteroid-induced ON in the proximal femurs of rabbits treated with MPSL alone, MPSL+saline, or MPSL+MCs was 53.3% (8 of 15), 47.4% (9 of 19), and 0.0% (0 of 15), respectively. Its incidence in the distal femurs of rabbits treated with MPSL alone, MPSL+saline, or MPSL+MCs was 53.3% (8 of 15), 47.4% (9 of 19), and 0.0% (0 of 15), respectively. Furthermore, corticosteroid-induced ON in all femurs from rabbits treated with MPSL alone, MPSL+saline, or MPSL+MCs was 80.0% (12 of 15), 68.4% (13 of 19), and 0.0% (0 of 15), respectively. These findings indicate that transplanting MCs significantly protects from corticosteroid-induced ON.

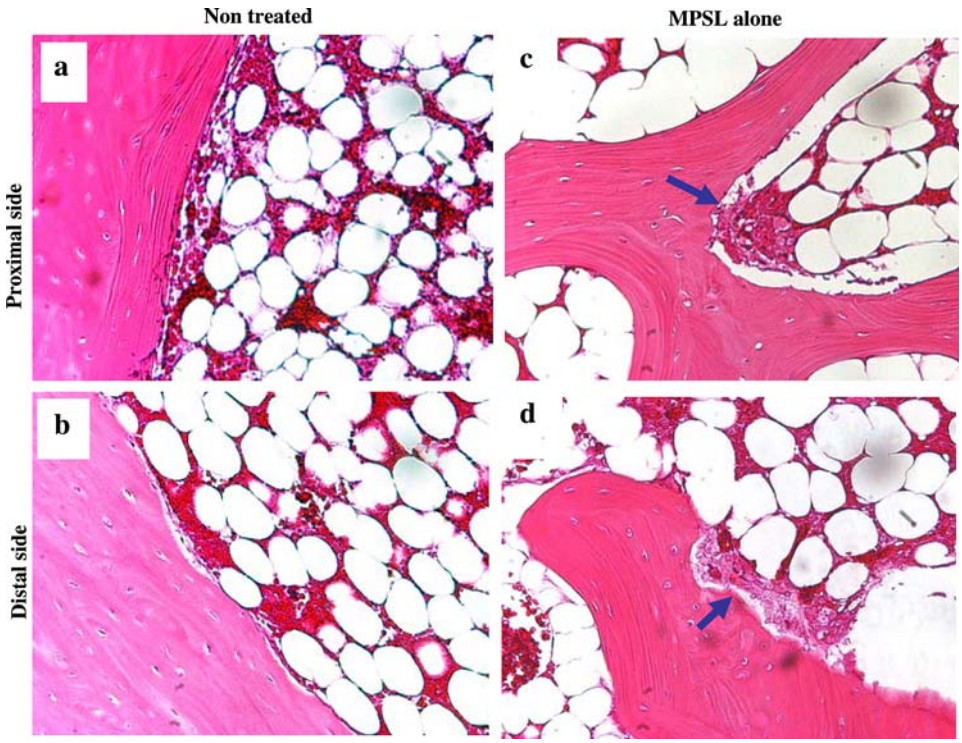

Fig. 3 Histological findings one week after MPSL injection. The histology of the proximal and distal sides of the femurs in normal rabbits is shown (**a**, **b**). The proximal and distal sides of the femurs one week after MPSL show diffuse presence of empty lacunae, pyknotic nuclei of osteocytes within the bone trabeculae, and bone marrow necrosis (**c**, **d**). ×400

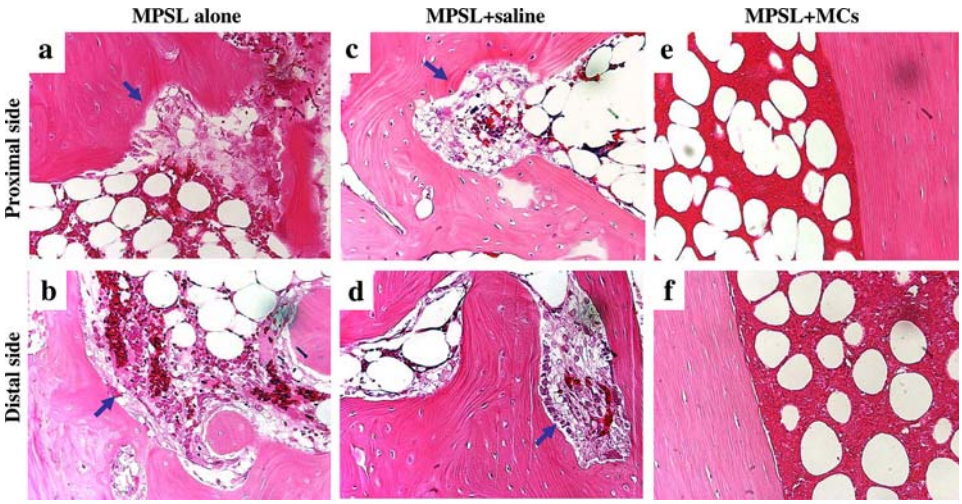

Fig. 4 Histological findings 4 weeks after MPSL injection. The proximal and distal sides of femurs from rabbits treated with MPSL alone show diffuse presence of empty lacunae, pyknotic nuclei of osteocytes within the bone trabeculae, and bone marrow necrosis (**a**, **b**). Both sides of the MPSL+saline femurs also show necrosis (**c**, **d**). Both sides of the femurs in the MPSL+MCs-treated group show an absence of necrosis (**e**, **f**). ×400

Table 1 Incidence of osteonecrosis (ON) in femurs 4 weeks after the injection of methylprednisolone (MPSL)

Group	Number of femora	Number of femora with ON		
		Proximal side	Distal side	Total
MPSL alone	15	8 (53.3%)*	8 (53.3%)*	12 (80.0%)*
MPSL+saline	19	9 (47.4%)*	9 (47.4%)*	13 (68.4%)*
MPSL+MCs	15	0 (0.0%)	0 (0.0%)	0 (0.0%)

The *asterisks* represent the *P* values of MPSL+MCs versus MPSL alone or MPSL+saline

* $P < 0.05$

Ischemic and apoptotic findings in the femurs 28 days after MPSL

VEGF, a promoter of angiogenesis, is rapidly induced in response to a hypoxic environment. To assess the relationship between the development of ON and local ischemia, we analyzed the expression of VEGF. Femurs from rabbits treated with MPSL alone or MPSL+saline showed many VEGF-positive regions in the bone marrow (Fig. 5a, b). On the other hand, femurs from rabbits treated with MPSL+MCs showed only a few positive regions (Fig. 5c).

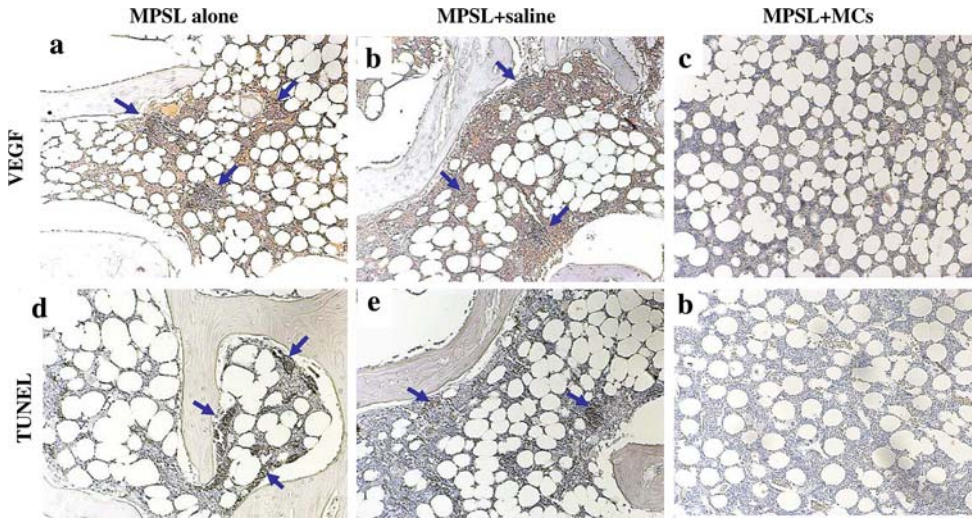

Fig. 5 Ischemic and apoptotic changes. Femurs from rabbits treated with MPSL alone show many vascular endothelial growth factor (VEGF)-positive cells (**a**). Femurs in the MPSL+saline group also show VEGF-positive cells (**b**). The MPSL+MCs group shows very few VEGF-positive cells (**c**). Femurs from rabbits treated with MPSL alone show many cells with chromatin condensation and nuclear fragmentation by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) (**d**). The MPSL+saline group, also shows TUNEL-positive cells (**e**). The MPSL+BMT group shows only a few cells with chromatin condensation (**f**). ×400

Additionally, in order to assess the relationship between the development of corticosteroid-induced ON and the level of apoptosis, we analyzed apoptotic cells using TUNEL staining. Femurs from rabbits treated with MPSL alone or MPSL+saline showed many TUNEL-positive regions in the bone marrow (Fig. 5d, e), but those from rabbits treated with MPSL+MCs showed only a few positive regions (Fig. 5f). These findings suggest that corticosteroids induce ischemic changes in the bone marrow of femurs, causing apoptosis, and that transplanting MCs prevents such changes.

Analyses of the cell cycle

To examine the activation of BMCs in the proximal and distal sides of the femurs, we analyzed the cell cycle using flow cytometry. As shown in Fig. 6, the percentages of cells in the G1 phase of the cell cycle in the femurs of rabbits treated with MPSL alone or MPSL+saline were high compared to untreated rabbits. These findings suggest that steroids induced cell-cycle arrest of the BMCs in the femurs of MPSL-treated rabbits. On the other hand, the percentages of cells in the G1 phase after treatment with MPSL+MCs were significantly decreased compared to MPSL alone or MPSL+saline and they approached normal levels. Based on these findings, we suggest that transplanting MCs suppresses steroid-induced cell-cycle arrest and normalizes the cell cycle of BMCs in the femurs.

Discussion

Steroids are indispensable for the treatment of spinal cord injury, rejection of transplanted organs, and autoimmune diseases [1–7]. However, they not only have great benefits but also several side effects. In particular, corticosteroid-induced ON of the femoral head is one of the most serious complications, characterized by variable areas of dead trabecular bone and bone marrow, extending to, but excluding, the subchondral plate. A prospective study on corticosteroid-induced ON of the femoral head using magnetic resonance imaging (MRI) detected signs thereof 2 months after the administration of steroids [30]. Recently, in human studies, Hernigou and Beaujean [25] reported that, after grafting autologous BMCs into the femoral head, hip replacement was necessary in only 9 of 145 hips (6.2%) at the non-collapse stage (stage I and stage II), whereas 25 of 44 (56.8%) replacements were required at the collapse stage (stage III and stage IV) at 5–10 years. Furthermore, Gangji et al. [26] reported that only 1 of 10 hips (10%) at stages I and II progressed to the collapse stage 2 years after grafting autologous BMCs into the femoral head, whereas, when left untreated, 5 of 8 (62.5%) had progressed from the non-collapse to the collapse stage by that time.

Steroids have been reported to stimulate the differentiation of pluripotential mesenchymal cells into adipocytes [31] and to inhibit osteoblastogenesis and promote the apoptosis of osteoblasts and osteocytes [32]. Furthermore, it has been reported that, during the early stages of steroid therapy, the lifespan of osteoblasts and osteocytes

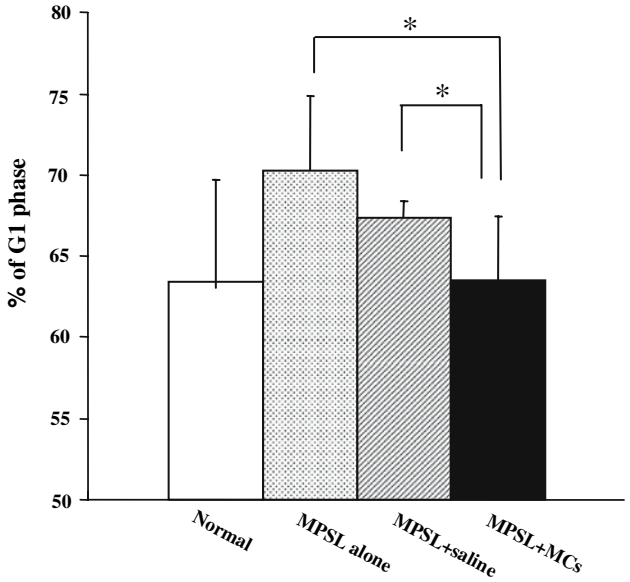

Fig. 6 Analyses of cell cycle in BMCs. BMCs from the four groups (normal, MPSL alone, MPSL+saline, and MPSL+MCs) 4 weeks after the injection of MPSL were stained with propidium iodide (PI) and the percentages of cells in the G1 phase were analyzed. The results are expressed as the mean ± SD of six femurs. Statistical analyses were carried out by Student's *t*-test: $*P < 0.05$

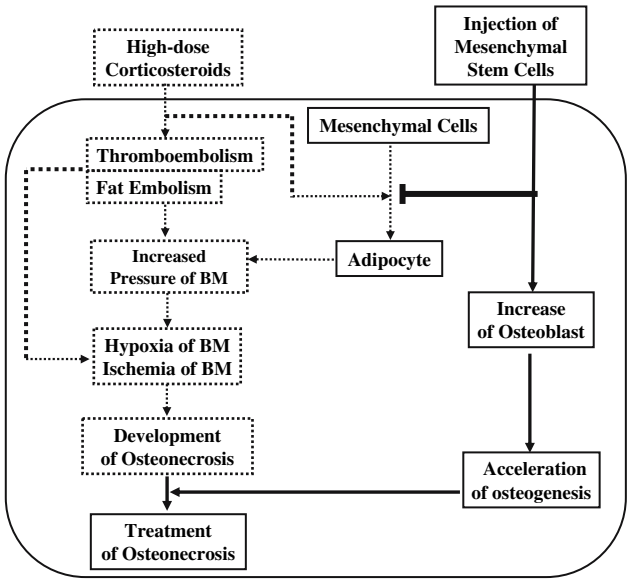

Fig. 7 Mechanisms underlying the treatment of osteonecrosis by transplanting MCs. We hypothesize that transplanting MCs may prevent the differentiation of MCs into adipocytes and lead to the acceleration of osteogenesis. As a result, hypoxia and ischemic changes in the femurs are normalized. The *dotted arrows* show the cascade of events, leading to osteonecrosis induced by high-dose corticosteroids. The *solid arrows* show the effects of transplanting MCs

decreased, and that osteoclast numbers transiently increased but decreased subsequently [33]. In addition, a high dose of steroids has been reported to induce cell-cycle arrest and the apoptosis of endothelial cells, which are differentiated from MSCs [34]. Based on these findings, steroids inhibit not only osteoblastogenesis, but also angiogenesis in the femoral bone marrow microenvironment. Here, we found that the functionality of MPSL-treated MCs cultured for 3 days was significantly reduced compared to the 5-day cultures, by which time, MC activity had recovered to almost normal levels. Thus, MPSL-treated MCs are able to recover on culture in vitro for 5 days.

Miyanishi et al. [24] found that larger doses of steroids increased fat-cell size and reported that drilling a hole (1.0-mm diameter) in the femurs of rabbits administered with steroids could decrease the intraosseous pressure. In the present study, the incidence of corticosteroid-induced ON 1 week after injecting MPSL was 50%, increasing to 80% later on. The incidence of ON in the femurs of rabbits treated with MPSL+saline was 68.4%, a slight decrease compared to MPSL alone. These findings suggest that the injection procedure itself may help to relieve intraosseous pressure. However, the incidence of ON in the femurs of rabbits treated with MPSL+saline remained high. In contrast, its occurrence in rabbits treated with MPSL+MCs was 0%. In immunological staining, many TUNEL-positive regions, indicating apoptosis, were detected in the MPSL alone and MPSL+saline groups. Moreover, many VEGF-positive regions, reflecting a response to local hypoxia, were observed in the femurs of rabbits treated with MPSL alone or MPSL+saline, but only a few TUNEL-positive and VEGF-positive regions were found in the femurs of rabbits treated with MPSL+MCs. Based on these findings, we suggest that corticosteroid-induced ischemic changes play a crucial role in apoptotic cell death within the medullary space, resulting in the induction of ON, and we further suggest that transplanting MCs facilitates regenerative effects to counteract corticosteroid-induced ON.

The percentages of cells in the G0/1 phase of the cell-cycle increase in inactivated cells, the proliferation of which is slow or not apparent, but are low in activated cells. Here, the percentages of cells in the G1 phase in the femurs from the MPSL alone and MPSL+saline groups were significantly increased compared to normal femurs. In contrast, the fraction of cells in the G1 phase in femurs from rabbits treated with MPSL+MCs was significantly lower than MPSL alone, and was, in fact, restored to normal levels. We suggest that transplanting MCs effectively accelerates the proliferation of BMCs within the corticosteroid-treated femurs.

On the basis of the present findings, we hypothesize that transplanting MCs after preculture in vitro can prevent the differentiation of adipocytes from BMCs. As a result, the intraosseous pressure decreases and the subsequent ischemic and apoptotic changes in the femurs are prevented. Furthermore, accelerating osteogenesis in femurs by transplanting MCs leads to the regeneration and prevention of corticosteroid-induced ON (Fig. 7). Therefore, we

recommend the direct injection of MCs into the femurs to abrogate corticosteroid-induced ON in patients treated with high-dose and short-term steroid medication as a therapy for acute spinal cord injury.

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Conflict of interest All of the authors confirm that they have no conflicts of interest with regard to this work.

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