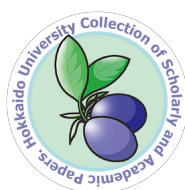




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Original Article

Therapeutic effects of human amnion-derived mesenchymal stem cell transplantation and conditioned medium enema in rats with trinitrobenzene sulfonic acid-induced colitis

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Abstract: Cell therapy with mesenchymal stem cells (MSCs) is expected to provide a new strategy for the treatment of inflammatory bowel disease (IBD). Large amounts of MSCs can be obtained from human amnion. Therefore, we investigated the effect of transplantation of human amnion-derived MSCs (hAMSCs) or enema of conditioned medium (CM) from hAMSCs into rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. In the first experiment, 10-week-old male Sprague-Dawley rats were intravenously injected with hAMSCs (1×10^6 cells) 3 h after rectal administration of TNBS (45 mg/kg). In the second experiment, rats with TNBS-induced colitis received CM by enema into the colon for 3 days. Colitis was investigated by endoscopy, histology, immunohistochemistry, and by measuring mRNA expression of inflammatory mediators. Administration of hAMSCs or CM enema significantly improved the endoscopic score. In addition, these two interventions resulted in significantly decreased infiltration of neutrophils and monocytes/macrophages and decreased expression levels of TNF- α , CXCL1, and CCL2. In conclusion, transplantation of hAMSCs and CM enema provided significant improvement in rats with TNBS-induced colitis. CM from hAMSCs and hAMSCs may be new strategies for the treatment of IBD.

Keywords: Mesenchymal stem cells, amnion, conditioned medium, trinitrobenzene sulfonic acid, colitis

Introduction

Crohn's disease (CD) is a chronic, relapsing, progressive inflammatory disorder of the gastrointestinal tract [1]. Prevalence of CD is generally high in Europe and North America, and the number of Crohn's patients in Asia is rapidly increasing [2, 3]. Currently, treatments for CD focus on suppressing inflammation with medications. Anti-inflammatory agents, such as 5-aminosalicylates, corticosteroids, and immunomodulators, have been widely used [4], and anti-tumor necrosis factor (TNF)- α biological agents have dramatically changed the strategy to control mucosal inflammation [5-7]. However, anti-inflammatory treatments have limited therapeutic efficacy because of the loss of response and side effects [8], and complicated cases

may require surgery, such as bowel resection and ileostomy [4]. Therefore, the development of alternative treatment is needed.

Cell therapy with mesenchymal stem cells (MSCs) is expected to make a new therapeutic strategy. MSCs are multipotent cells that are present in adult tissue and that can differentiate into a variety of lineages, including bone, cartilage, or fat [9]. It has been reported that MSCs have the potential to improve damaged tissues by the secretion of a variety of growth factors and anti-inflammatory molecules [10, 11].

The amnion has been found to be a rich source of MSCs and has the added benefit of accessibility because it is generally discarded as medi-

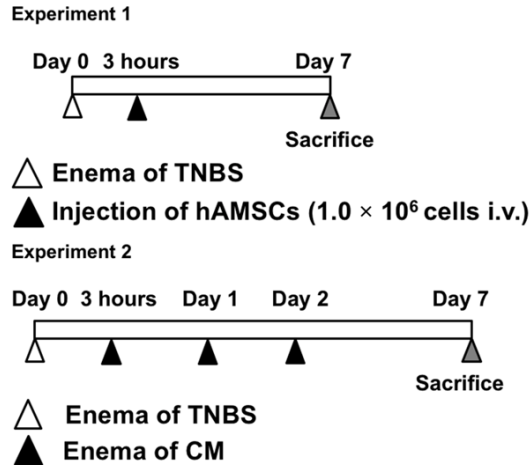


Figure 1. Experimental protocol for 2,4,6-trinitrobenzene sulfonic acid (TNBS)-colitis model. Experiment 1: protocol for the human amnion-derived mesenchymal stem cell (hAMSC) transplantation model. Rats received enemas of TNBS on day 0 and 1×10^6 hAMSCs were intravenously infused 3 h later. Experiment 2: protocol for the enema of conditioned medium (CM) model. Rats received enemas of TNBS on day 0 and CM was intrarectally infused 3, 24, and 48 h later.

cal waste and can be obtained without the need of an invasive procedure [12, 13]. We have previously demonstrated the efficacy of human amnion-derived MSCs (hAMSCs) for several inflammatory diseases in animal models, such as dextran sulfate sodium (DSS)-induced severe colitis [14], radiation proctitis [15], acute and chronic pancreatitis [16], and carbon tetrachloride-induced liver fibrosis [17]. In addition, we have shown that conditioned medium (CM) obtained from hAMSCs downregulates the activity of cultured inflammatory cells and has the capacity to protect cells from radiation injury [14-17]. DSS-induced colitis model is widely used as a model for ulcerative colitis (UC), and 2,4,6-trinitrobenzene sulfonic acid (TNBS) is widely used as a model for CD because the pathology of TNBS-induced colitis resembles human CD [18, 19]. However, the therapeutic efficacy of hAMSCs against TNBS-induced colitis has not been reported. We thereby investigated whether the intravenous administration of hAMSCs or CM enema could improve TNBS-induced colitis in rats.

Materials and methods

Animals

The experimental protocol was approved by the Animal Care and Use Committees of Hokkaido

University. Ten-week-old male Sprague-Dawley rats were procured from Japan SLC (Hama-matsu, Japan); three rats were housed per cage in a temperature-controlled room (24°C) on a 12 h light/12 h dark cycle. All rats had access to standard pellets *ad libitum*.

Induction of colitis

A 2-mm diameter rubber tube was inserted into the rectum approximately 8 cm from the anus. Colitis was induced by intrarectal administration of 200 μ L TNBS (45 mg/kg, Wako Pure Chemical Industries, Osaka, Japan) and 30% ethanol under pentobarbital anesthesia (200 μ L, Kyoritsu-seiyaku Corporation, Tokyo, Japan) on day 0 (**Figure 1**).

Isolation and expansion of hAMSCs

The Medical Ethical Committee of Hokkaido University Graduate School of Medicine, Sapporo, Japan approved this research, and one pregnant woman gave written informed consent. The human fetal membrane was obtained during Cesarean delivery, and the amnion was manually peeled from the chorion. hAMSCs were isolated and expanded by digestion with brightase (Nippi, Tokyo, Japan) and dispase (Wako Pure Chemical Industries, Osaka, Japan), followed by seeding in uncoated plastic dishes with minimal essential medium (MEM) α (Life Technologies, Carlsbad, LA, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 100 U/mL of penicillin, and 100 μ g/mL of streptomycin (Wako Pure Chemical Industries, Osaka, Japan). Cell cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. After 3-4 days in culture, non-adherent cells were removed and adherent cells were maintained in culture until they reached 80% confluence. The passage was performed using 0.5% trypsin/EDTA (Life Technologies).

Preparation of CM gel from hAMSC culture

hAMSCs were cultured in a complete medium until the cells reached a sub-confluent state. After washing with phosphate-buffered saline (PBS, Life Technologies) three times, cells were further cultured with serum-free MEM α for 48 h. Next, CM was collected and centrifuged at 400 $\times$ g for 5 min; the supernatant was stored at -80°C until use. CM gel was made by mixing CM with 2% carboxymethyl cellulose (CMC, Wako Pure Chemical Industries). Serum-free MEM α mixed with CMC was used as a standard medium (SM) gel.

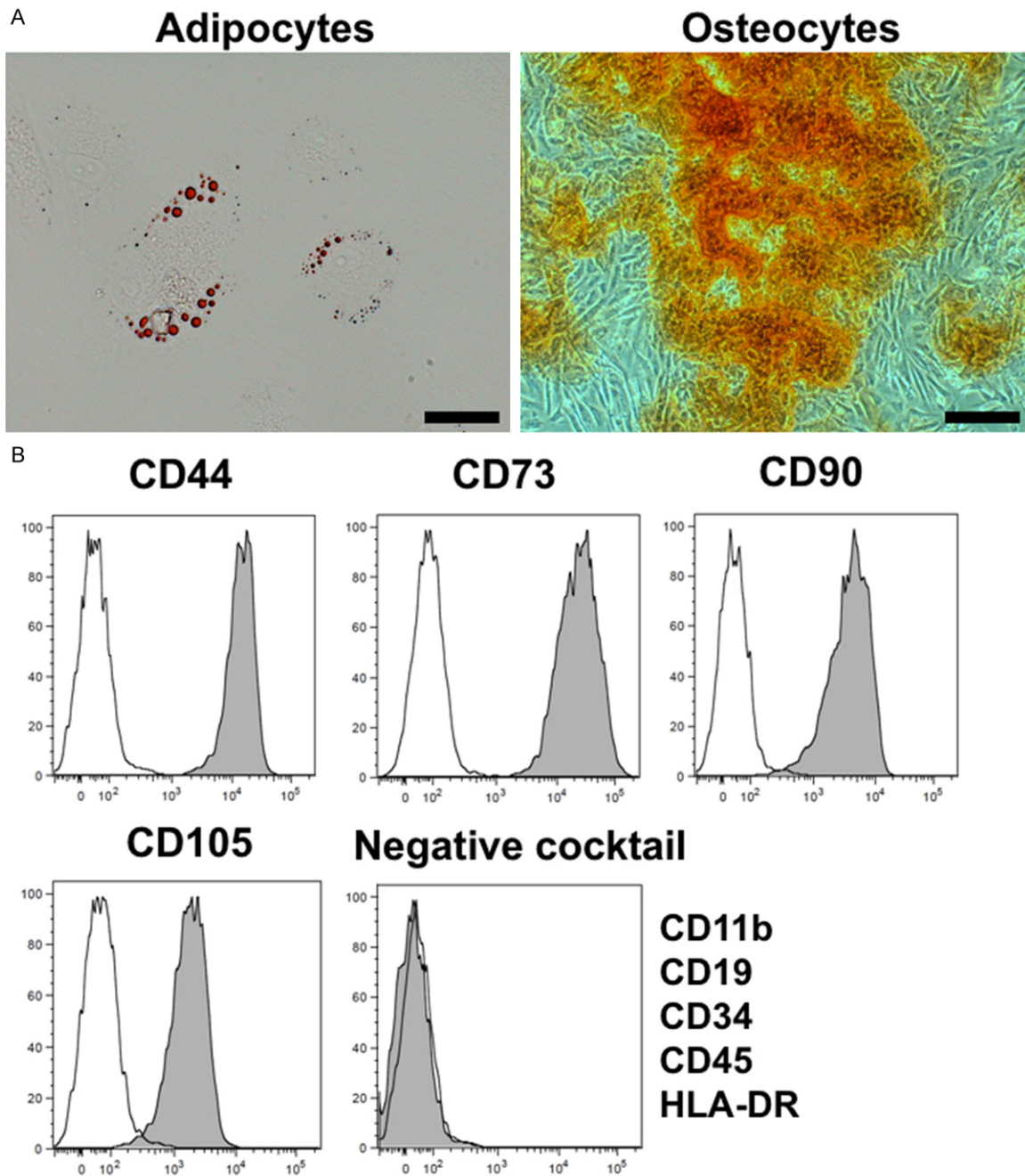


Figure 2. Characterization of cultured human amnion-derived mesenchymal stem cells (hAMSCs). A. Multipotency of hAMSCs. Differentiation into adipocytes was confirmed by the presence of lipid vesicles stained with oil red O (left panel). Scale bars, 50 μ m. Differentiation into osteocytes was confirmed by the existence of mineral nodule deposition stained with alizarin red S (right panel). Scale bars, 200 μ m. B. Flow cytometry of hAMSCs. The negative cocktail contained antibodies against CD11b, CD19, CD34, CD45, and HLA-DR. Closed areas indicate staining with a specific antibody, whereas open areas represent staining with isotype control antibodies.

Differentiation of hAMSCs into adipocytes and osteocytes

The hAMSCs were seeded into 6-well plates, and differentiation into adipocytes and osteo-

cytes was induced when the hAMSCs were 80%-90% confluent. To induce differentiation into adipocytes, hAMSCs were cultured with hMSC adipogenic differentiation medium (Lonza, Basel, Switzerland), according to the

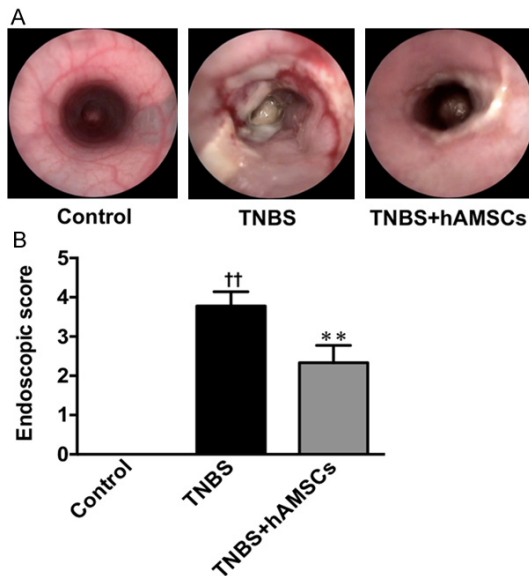


Figure 3. Effect of human amnion-derived mesenchymal stem cell (hAMSC) transplantation in rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. A. Endoscopic imaging. B. Endoscopic score. The following parameters were analyzed and graded as 0 (absent) or 1 (present): changes in the vascular pattern; mucosal granularity; strictures; bleeding; and ulcers (total score ranging therefore from 0 to 5) [20]. Values are expressed as means $\pm$ SEM (N = 6-9 animals/group). ††P < 0.01 vs. the control group. **P < 0.01 vs. the TNBS group.

manufacturer's instructions. After 3 weeks of differentiation, cells were stained with oil red O (Sigma-Aldrich, St. Louis, MO, USA) to confirm differentiation. To induce differentiation into osteocytes, hAMSCs were cultured in hMSC osteogenic differentiation medium (Lonza), according to the manufacturer's instructions. After 2 weeks of differentiation, cells were stained with alizarin red S (Sigma-Aldrich) to confirm differentiation.

Flow cytometry

Cultured hAMSCs were harvested with 0.5% trypsin/EDTA and stained using the Human MSC Analysis Kit (Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA), which included phycoerythrin (PE)-conjugated anti-CD44, allophycocyanin (APC)-conjugated anti-CD73, fluorescein-isothiocyanate (FITC)-conjugated anti-CD90, and PerCP-Cy5.5-conjugated anti-CD105 antibodies, as well as a negative cocktail (PE-conjugated anti-CD11b, anti-CD19, anti-CD34, anti-CD45, and anti-HLA-DR antibodies), according to the manufacturer's

instructions. Cells were analyzed using a FACS Canto II flow cytometer (BD).

Transplantation of hAMSCs

In experiment 1, 1×10^6 hAMSCs suspended in 200 μ L of PBS were intravenously transplanted through the penile vein 3 h after administration of TNBS on day 0 (N = 9) (Figure 1); 200 μ L of PBS was injected into the Control (N = 6) and TNBS (N = 9) groups.

Enema of hAMSC-CM

In experiment 2, 400 μ L of the hAMSC-CM gel was injected intrarectally 3, 24, and 48 h after administration of TNBS in the TNBS+CM gel group (N = 9, Figure 1); 400 μ L of SM gel was injected in the TNBS group (N = 9).

Endoscopic assessment

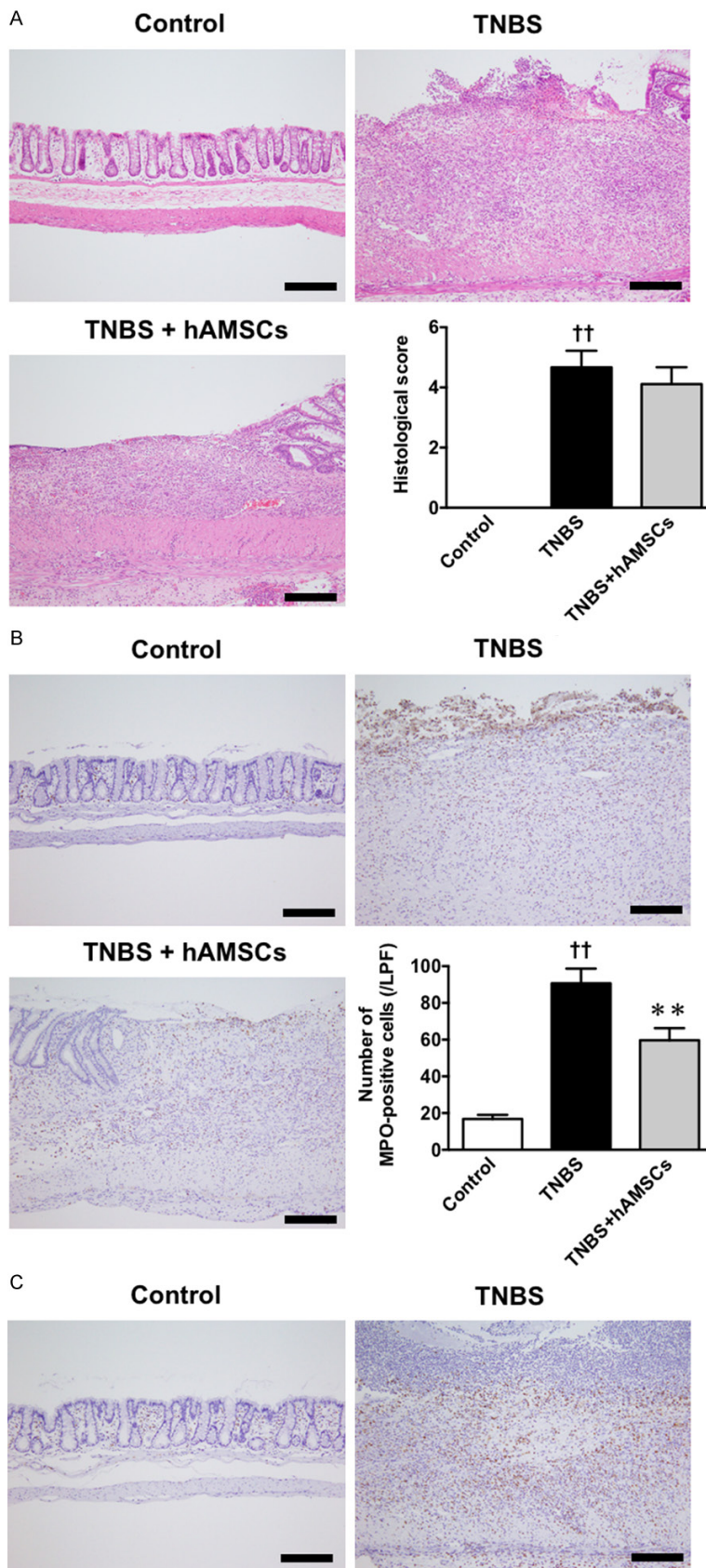
The colons of rats were examined using a fiberoptic (1.6 mm in diameter; Tesala, AVS, Tokyo, Japan) on day 7. Colon injury was scored using an adapted endoscopic index of colitis [20]. The following parameters were analyzed and graded as 0 (absent) or 1 (present): changes in the vascular pattern; mucosal granularity; strictures; bleeding; and ulcers (total score ranging therefore from 0 to 5).

Histological examination

All rats were sacrificed on day 7. The abdomens of rats were opened under anesthesia, and an endoscope was inserted from the anus. The main lesions were observed by the endoscope and marked by crystal violet (Wako Pure Chemical Industries). A 1.5-cm section proximal and distal to the main lesion of colon was removed, fixed in 40 g/L of formaldehyde saline (Wako Pure Chemical Industries), embedded in paraffin, and cut into 5- μ m sections. Tissue sections were stained with hematoxylin and eosin (H&E; Wako Pure Chemical Industries) and microscopically examined by an independent observer. The tissues were scored in a blinded fashion by the independent observer, and colonic inflammation was assessed using the histopathological grading scale as described previously [21].

Immunohistochemical examination

Tissue sections were stained with anti-myeloperoxidase (MPO) antibody (dilution, 1:300;



Thermo Scientific, Waltham, MA, USA), anti-rat CD68 monoclonal antibody (dilution, 1:50; AbD Serotec, Kidlington, UK), and anti-rat CD3 antibody (dilution, 1:50; BD) for 40 min. Ten random fields per section from each rat were photographed, and the number of MPO-, CD68-, and CD3-positive cells in the low-powered field were counted.

RNA isolation and quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNAs of the rat rectum were extracted using the RN-easy Mini Kit (Qiagen, Hilden, Germany). 1 µg of the total RNA was reverse-transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). PCR amplification was performed using a 25-µL reaction mixture that contained 1 µL of cDNA and 12.5 µL of Platinum SYBR Green PCR Mix (Life Technologies). β-actin mRNA amplified from the same samples served as an internal control. After initial denaturation at 95°C for 2 min, a two-step cycle procedure was used (denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min) for 40 cycles in a 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Gene expression levels were determined using the comparative threshold cycle (ddCt) method, using β-actin as an internal control [22]. Data were analyzed using Sequence Detection Systems software (Applied Biosystems).

Statistical analysis

Data are expressed as mean ± SEM. Parameters were com-

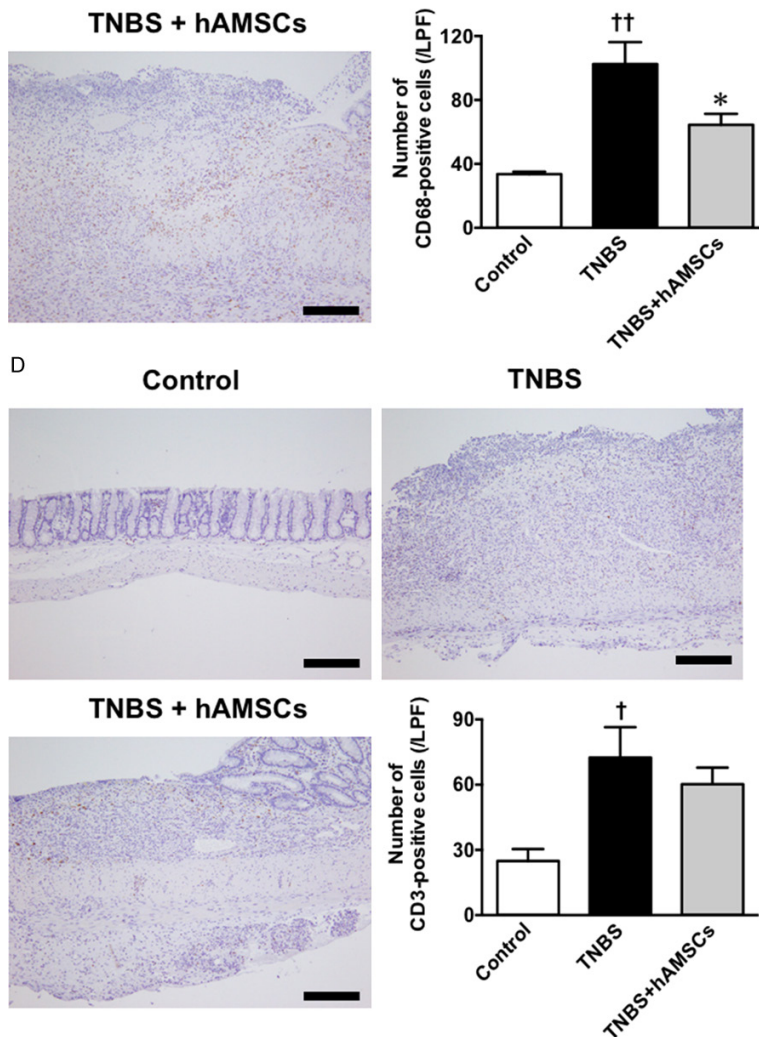


Figure 4. Effect of human amnion-derived mesenchymal stem cell (hAMSC) transplantation on the histological score and infiltration of inflammatory cells. A. Hematoxylin and eosin (H&E) staining and histological score. B. Myeloperoxidase (MPO) staining. C. CD68 staining. D. CD3 staining. The number of positive cells was counted in 10 sections/sample in each low-power field. Scale bars, 200 μ m. Values are expressed as means $\pm$ SEM (N = 6-9 animals/group). $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. the control group. $^*P < 0.05$, $^{**}P < 0.01$ vs. the 2,4,6-trinitrobenzene sulfonic acid (TNBS) group.

pared between three groups by one-way ANOVA followed by the Newman-Keuls test. Differences were considered statistically significant where $P < 0.05$.

Results

Characterization of hAMSCs

To evaluate the multipotency of hAMSCs, we induced differentiation of cultured hAMSCs into adipocytes and osteocytes. hAMSCs differenti-

ated into adipocytes and osteocytes, as demonstrated by oil red O and alizarin red S staining, respectively (**Figure 2A**). Flow cytometry of cultured hAMSCs demonstrated that these cells expressed CD44, CD73, CD90, and CD-105 but not CD11b, CD19, CD34, CD45, or HLA-DR, which is characteristic of MSCs (**Figure 2B**) [23].

Effect of hAMSC transplantation on endoscopic parameters in rats with TNBS-induced colitis

Deep ulcers, bleeding, mucosal granularity, and strictures were detected in the TNBS group; however, ulcers were shallow and the strictures were not observed in the TNBS+hAMSCs group (**Figure 3A**). hAMSC transplantation significantly improved the endoscopic score (**Figure 3B**).

Histological changes after hAMSC transplantation in rats with TNBS-induced colitis

We next investigated histological changes after hAMSC transplantation. H&E staining demonstrated destruction of the ductal structures and large numbers of inflammatory cells in the TNBS group. However, these changes were attenuated by hAMSC trans-

plantation on day 7. The histological score in the TNBS+hAMSCs group tended to be lower than in the TNBS group (**Figure 4A**). Immunohistological examination demonstrated that the numbers of infiltrated MPO-positive neutrophils, CD68-positive monocytes/macrophages, and CD3-positive T lymphocytes were significantly higher in the TNBS group than in control animals (**Figure 4B-D**). hAMSC transplantation significantly decreased the number of neutrophils and monocytes/macrophages (**Figure 4B and 4C**) and appeared to decrease

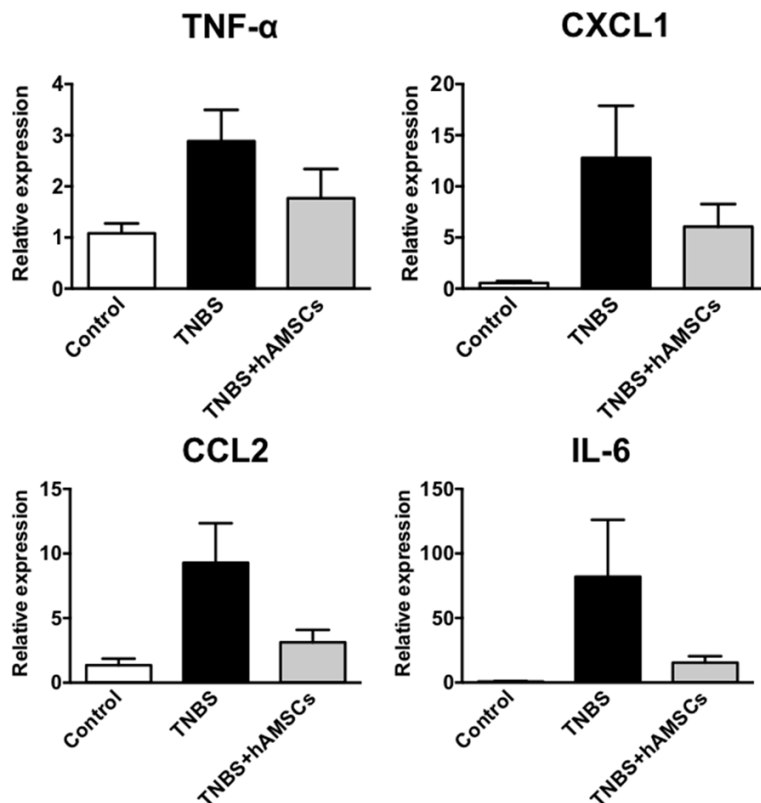


Figure 5. Effect of human amnion-derived mesenchymal stem cell (hAMSC) transplantation on mRNA expression of inflammatory mediators in the colon of rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with tumor necrosis factor (TNF)- α , chemokine (C-X-C motif) ligand (CXCL) 1, chemokine (C-C motif) ligand (CCL) 2, and interleukin (IL)-6. Values are expressed as means $\pm$ SEM (N = 6-9 animals/group).

in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats. A. Endoscopic imaging. B. Endoscopic score. The following parameters were analyzed and graded as 0 (absent) or 1 (present): changes in the vascular pattern; mucosal granularity; strictures; bleeding; and ulcers (total score ranging therefore from 0 to 5) [20]. Values are expressed as means $\pm$ SEM (N = 6-9 animals/group). $^{**}P < 0.01$ vs. the Control group, $^{*}P < 0.01$ vs. the TNBS+ standard medium (SM) gel group.

the number of T cells (Figure 4D).

Effect of hAMSC transplantation on colonic mRNA expression levels of inflammatory mediators in rats with TNBS-induced colitis

In the TNBS group, mRNA expression levels of several inflammatory cytokines in the colon were increased, including TNF- α , chemokine (C-X-C motif) ligand (CXCL) 1, chemokine (C-C motif) ligand (CCL) 2 and interleukin (IL)-6 (Figure 5). hAMSC transplantation also tended to decrease the expression levels of TNF- α , CXCL1, CCL2, and IL-6, although this was not statistically significant.

Effect of hAMSC-CM gel enema on endoscopic parameters in rats with TNBS-induced colitis

We next investigated the effect of hAMSC-CM gel in rats with TNBS-induced colitis. Deep ulcers, bleeding, mucosal granularity, and strictures were detected in the TNBS+SM gel group; however, in the TNBS+CM gel group, ulcers were shallow and bleeding was not detected (Figure 6A). An enema of hAMSC-CM gel significantly improved the endoscopic score (Figure 6B).

Histological changes by hAMSC-CM gel enema in rats with TNBS-induced colitis

H&E staining demonstrated destruction of the ductal structures and large numbers of inflam-

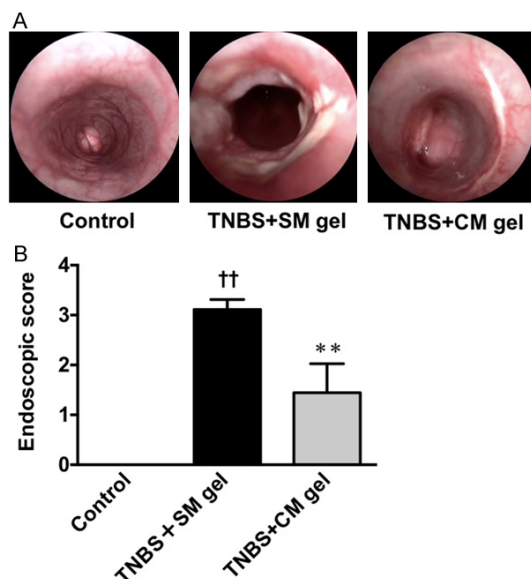
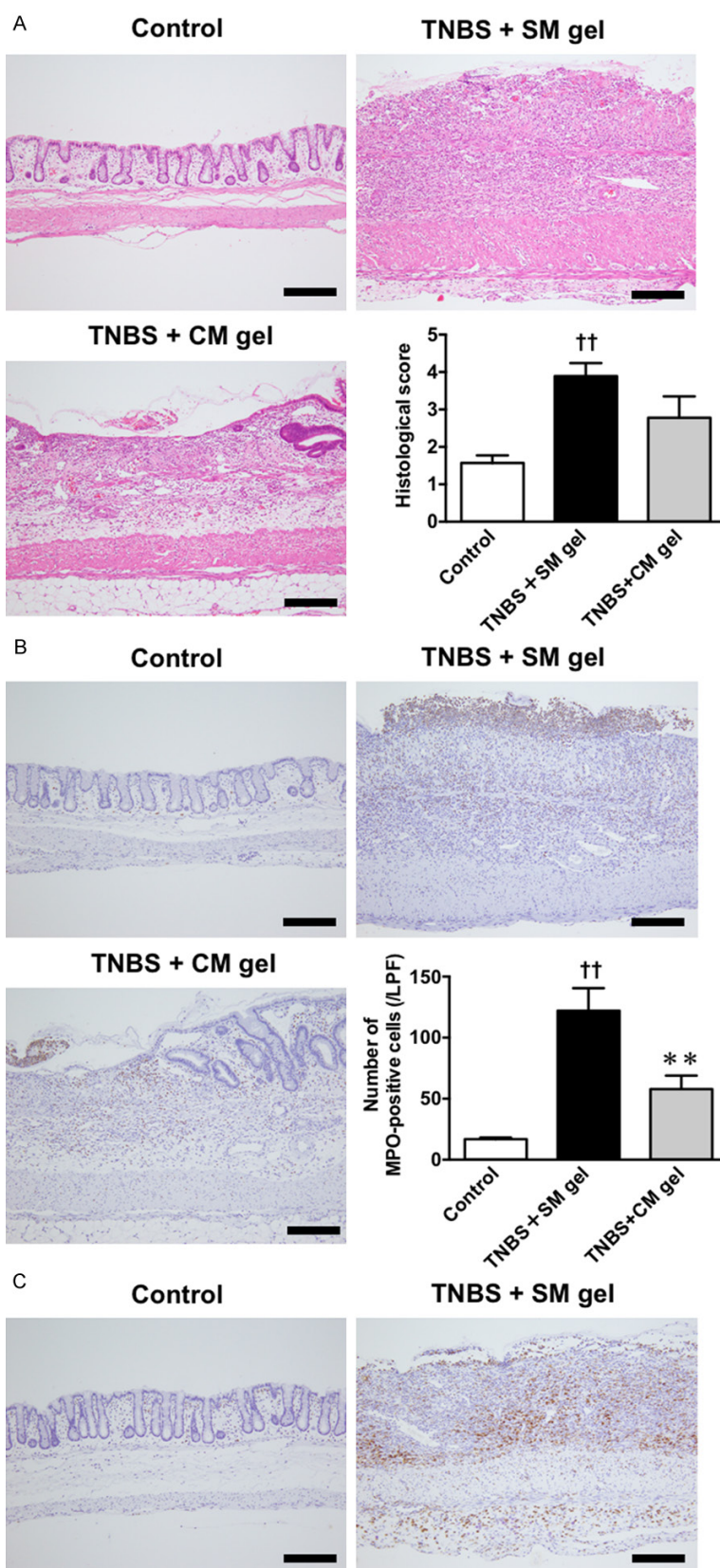


Figure 6. Effect of human amnion-derived mesenchymal stem cell conditioned medium (hAMSC-CM)



matory cells in the TNBS+SM gel group. However, these changes were attenuated by hAMSC-CM gel enema on day 7 (**Figure 7A**). The histological score in the TNBS+CM gel group tended to be lower than that of the TNBS+SM gel group. Immunohistological examination demonstrated that the numbers of infiltrated neutrophils, monocytes/macrophages, and T lymphocytes were significantly higher in the TNBS+SM gel group. These elevated immune cell populations were significantly decreased following treatment with an enema of hAMSC-CM gel (**Figure 7B-D**).

Effect of hAMSC-CM gel enema on colonic mRNA expression levels of inflammatory mediators in rats with TNBS-induced colitis

In the TNBS+SM gel group, mRNA expression levels of TNF- α , CXCL1, CCL2 and IL-6 were increased (**Figure 8**). An enema of CM gel tended to decrease the expression levels of TNF- α , CXCL1 and CCL2, although this was not statistically significant. The expression level of IL-6 was not attenuated by enema of CM gel.

Discussion

This is the first study to investigate the therapeutic potential of hAMSC transplantation and hAMSC-CM gel enemas in rats with TNBS-induced colitis. We found that both hAMSC transplantation and hAMSC-CM gel enema provided significant improvement in colitis.

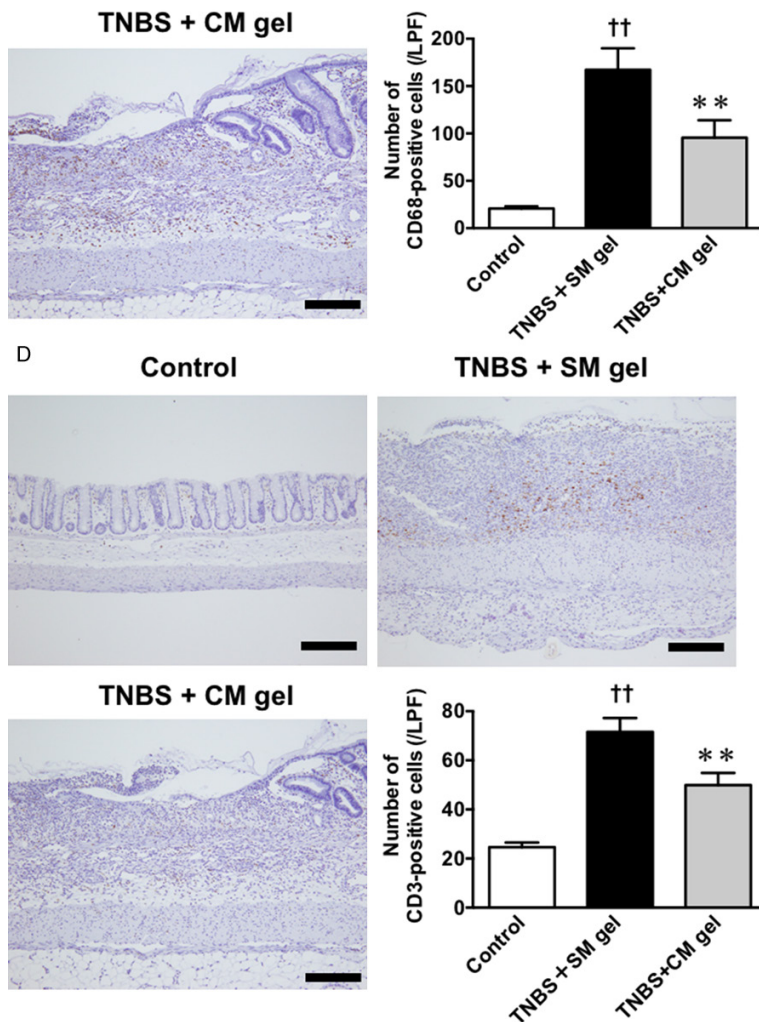


Figure 7. Effect of conditioned medium obtained from human amnion-derived mesenchymal stem cell culture (hAMSC-CM) on the histological score and infiltration of inflammatory cells. A. Hematoxylin and eosin (H&E) staining and histological score. B. Myeloperoxidase (MPO) staining. C. CD68 staining. D. CD3 staining. The number of positive cells was counted in 10 sections per sample in each low-power field. Scale bars, 200 μ m. Values are expressed as means $\pm$ SEM (N = 6-9 animals/group). ^{††}P < 0.01 vs. the control group, ^{**}P < 0.01 vs. the 2,4,6-trinitrobenzene sulfonic acid (TNBS) + standard medium (SM) gel group.

In the present study, hAMSC transplantation attenuated acute inflammation, decreased the infiltration of neutrophils, monocytes/macrophages, and T cells, and suppressed the expression levels of inflammatory mediators in the colons of rats. The efficacy of bone marrow-derived MSCs (BM-MSCs), umbilical cord-derived MSCs (UC-MSCs) and adipose-derived MSCs (AD-MSCs) has been recently reported in small animal models of CD induced by TNBS [24-26]. Stavelly *et al.* demonstrated that BM-MSCs were more effective for the treatment of enteric neuropathy and plexitis than

AD-MSCs in a guinea pig model of TNBS-induced acute colitis [25]. Yanfen *et al.* reported that the Wnt and Notch signaling pathways were suppressed by the transplantation of BM-MSCs in rats with TNBS-induced colitis [27].

One of the suggested mechanisms of wound healing by MSCs is through their production of a variety of humoral factors. It has been considered that humoral factors from MSCs could contribute to the improvement of colitis [28, 29]. Gholamrezanezhad *et al.* reported that BM-MSCs labeled with ¹¹¹In-oxine accumulated in the lungs, then gradually in the liver and spleen after intravenous administration [30]. We have recently demonstrated that intravenously-administered hAMSCs were distributed in the lung, but not in the damaged rectum in the colitis model [15]. Therefore, secretory factors produced from MSCs could contribute to the improvement of colitis. Recently, prostaglandin E2 (PGE2) has been receiving attention for being a humoral factor secreted from MSCs. PGE2 is well known as a major modulator of the MSC-induced anti-inflammatory response, and can repolarize pro-inflammatory M1 macrophages into

anti-inflammatory M2 macrophages [31]. M2 macrophages promote the resolution of inflammation and tissue repair by releasing the immunoregulatory cytokine IL-10. It has been reported that BM-MSCs and UC-MSCs produce PGE2 [32, 33]. Our group has also reported that the concentration of PGE2 in hAMSC-CM was markedly higher than in chorion MSC-CM [34]. Although the concentration of PGE2 in the CM from different sources of MSCs has not been compared in a single study, it is conceivable that hAMSCs can produce significant levels of PGE2.

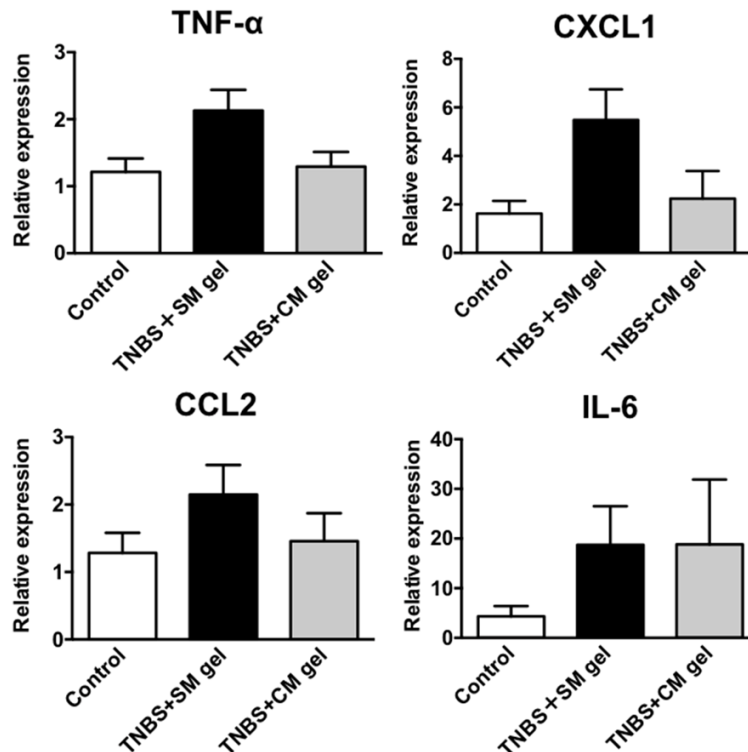


Figure 8. Effect of conditioned medium obtained from human amnion-derived mesenchymal stem cell culture (hAMSC-CM) on mRNA expression of inflammatory mediators in the colon of rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with tumor necrosis factor (TNF)- α , chemokine (C-X-C motif) ligand (CXCL) 1, chemokine (C-C motif) ligand (CCL) 2, and interleukin (IL)-6. Values are expressed as means $\pm$ SEM (N = 6-9 animals/group).

The present study demonstrates that treatment with an enema of hAMSC-CM gel can also attenuate acute inflammation in rats with TNBS-induced colitis. Robinson *et al.* demonstrated that single administration of BM-MSC-CM enema was effective for the treatment of TNBS-induced colitis in guinea pigs [35]. The administration of BM-MSC-CM maintained the number of myenteric neurons in the colon, prevented enteric neuropathy and alleviated colonic dysfunction associated with colitis. In addition, Watanabe *et al.* demonstrated that three intraperitoneal administrations of BM-MSC-CM provided an improvement in rats with TNBS-induced colitis [28]. Although the optimal route for administration of MSC-CM remains to be investigated, enemas may be a practical choice for administration of MSC-CM. It has been reported that vascular endothelial growth factor (VEGF) and CCL2 are secreted from cultured BM-MSCs [28]. VEGF has been shown to promote the regeneration of damaged colon [36],

and CCL2 has been shown to promote re-epithelialization [37]. In addition, it has been reported that hAMSC-CM contains CCL2, VEGF, and other cytokines such as basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) [38, 39]. bFGF has been shown to enhance wound repair, and HGF might promote tissue regeneration and wound-healing [40, 41].

Amnion is an attractive cell source for MSCs since a large quantity of hAMSCs can be obtained from the human fetal membrane (FM) without invasive procedure; over 10^6 cells per gram of the amnion ($1.9 \pm 0.2 \times 10^6/\text{g}$) and more than 10^9 or 10^{10} hAMSCs may be obtained at the third passage of cells, within one month (our unpublished data). Furthermore, a first-in-human clinical trial of FM-MSC transplantation for nine patients with steroid-refractory acute GVHD has been reported [42].

FM-MSCs appeared safe for intravenous infusion, and the overall response rate in severe refractory acute GVHD was similar to that observed with transplantation of BM-MSCs.

In conclusion, transplantation of hAMSCs and hAMSC-CM gel enema provide significant improvement in colitis pathologies in rats with TNBS-induced colitis. Since FM is routinely discarded as medical waste and can be obtained without invasive procedure, hAMSCs or CM from hAMSCs may make new therapeutic strategies against inflammatory bowel disease.

Acknowledgements

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Disclosure of conflict of interest

None.

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