Chinese Journal of Physiology 60(6): 345-352, 2017
DOI: 10.4077/CJP.2017.BAG509

Infusion of Porcine-Derived Amniotic Fluid Stem Cells for Treatment of Experimental Colitis in Mice

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Abstract

Recently, stem cells have offered an alternative treatment for inflammatory bowel disease (IBD) or colitis to overcome the poor outcomes associated with current therapies. Amniotic fluid-derived stem cells (AFSCs) have the potential for the regeneration of impaired organs and the recovery of normal physiologic functions of damaged tissues without ethical concerns or risk of tumor formation. In this work, we aimed to examine the therapeutic effects of infusion of porcine AFSCs (pAFSCs) in dextran sulfate sodium (DSS)-induced colitis in mice. Treatment with pAFSCs was shown to inhibit the shortening of the colon after induction of colitis and dramatically ameliorated the body weight-loss induced by the DSS treatment. In addition, pAFSCs could also reduce the extent of the inflamed area represented by epithelial mesenchymal transformation in the colitis mice. The levels of the inflammatory cytokines interleukin 6 (IL-6) and interferon gamma (IFN-γ) were also reduced in colitis mice transplanted with pAFSCs. In conclusion, pAFSCs can ameliorate experimental colitis in mice, suggesting that they may be a potential treatment for IBD or colitis.

Key Words: colitis mouse model, porcine amniotic fluid-derived stem cells

Introduction

Inflammatory bowel diseases (IBDs), including ulcerative colitis (UC) and Crohn's disease (CD), are a family of relapsing and tissue-damaging diseases characterized by chronic inflammation within the gastrointestinal tract (13) caused by the dysfunction of cytokines and chemokines (10, 12). However, current therapies are ineffectiveness and also had some side-effects, like headache and diarrhea. Therefore, there is a need for novel therapeutic approaches for the treatment of IBDs.

In recent years, stem cells have been reported as a potential therapy for colitis (8, 14, 17, 24, 26). Bone marrow-derived stem cells (BMSCs) have been demonstrated to ameliorate colitis syndrome

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Received: February 13, 2017; Revised: April 28, 2017; Accepted: June 30, 2017.
 ISSN : 0304-4920. http://www.cps.org.tw

by secreting interleukin 7 (IL-7) to induce B and CD4 memory cells in the intestine (17, 24), and by secreting IL-1β and IL-10 to enhance therapeutic efficacies (8, 17). Moreover, adipocyte-, intestine- and colon-derived stem cells have also shown therapeutic effects as for BMSCs for colitis syndrome (1, 2, 15, 32, 34, 40, 42).

With recent advances in medical research, cell or organ transplantations have increased the survival rate of patients. While the numbers of donation have decreased, the demand of cell or organ transplantations has increased daily. Thus, current stem cell research has shifted to the use non-human primates for studying human diseases. However, decline in the non-primate population and difficulties in artificial breeding have led to the decreased use of non-primates. The porcine not only shares a similar anatomy and physiology with the human, it can also be easily bred artificially. Therefore, the utilization of porcine as a promising source of experimental animals can contribute to researches in stem cell transplantations.

Amniotic fluid-derived stem cells (AFSCs), shed from the conceptus into the amniotic fluid (6), can be obtained in the second trimester of a pregnant sow by amniocentesis. AFSCs are easily isolated and proliferated *in vitro* and have the potential to differentiate into cells of different lineages, including hepatocytes, myocardium cells, nerve cells, endothelial cells, osteoblasts and adipocytes (3, 6, 16, 37). In pre-clinical trials, AFSCs have been used for treatment of a variety of diseases in both the liver (21) and the kidney (28). In previous studies, mouse BMSCs and human adipose-derived mesenchymal stem cells (MSCs) were shown to contribute to protection against colitis (13, 17). However, porcine AFSCs have not yet been used to treat colitis in mouse models induced by dextran sulfate sodium (DSS). In this study, DSS was used to induce colitis in acute and chronic phases, followed by treatment with porcine AFSCs (pAFSCs). pAFSCs were shown to ameliorate weight-loss and reduce the shortening of colon length in DSS-treated mice. In addition, pAFSCs also decreased secretion of inflamed-related cytokines IL-6, interferon gamma (IFN-γ) in acute inflammation after colitis. Therefore, pAFSCs could protect colitis and be beneficial for regenerative medicine.

Materials and Methods

Animals

The ICR mice were obtained from the Laboratory Animal Center of National Taiwan University College of Medicine, Taipei, Taiwan, and they were fed with Laboratory Rodent Diet 5001 (PMI Nutrition Int., St. Louis, MO, USA) and maintained indoor at 21-25°C with 50-70% humidity. The daily light cycle was 5 am to 7 pm. All experimental procedures on the animals were approved by the Institutional Animal Care and Use Committee (National Taiwan University).

Collection of pAFSCs

pAFSCs were collected as described in a previous report (27). Briefly, amniotic fluid was obtained from sows that were pregnant for 70 days, filtered through 70-μm mesh (MiltenyiBiotec, Aubum, CA, USA), and centrifuged at 12,000 rpm for 10 min. Amniotic fluid pellets were acquired, re-suspended and cultured in a 10-cm dish (TPP, Trasadingen, Switzerland) at a density of 2×10^5 cells/cm^2. After confluence, the supernatant was discarded and the cells were re-plated in a 1:2 ratio.

Establishing the DSS-Treated Experimental Colitis Mouse Model

Acute colitis was induced in 8-week-old ICR mice by administering 2% DSS (International Laboratory, San Francisco, CA, USA) in drinking water from day 0 to day 7. The mice with induced colitis were then divided into four groups, and each group had 4 mice. pAFSCs (6×10^6 cells in 0.3 ml phosphate buffer saline, PBS) were injected *via* the intraperitoneal. Passage 9 to 10 cultures of MSCs were used for the experiments. Group 1 (normal) received no DSS or pAFSC treatment, Group 2 (pAFSC) did not receive DSS but received pAFSC treatment daily from day 1 to day 7, Group 3 (DSS) received DSS and 0.3 ml PBS until day 7, and Group 4 (DSS+AFSC) received both DSS and pAFSC treatment from day 1 to day 7.

Histopathological Analysis

After treatment for 7 days, the mice were anesthetized with 2, 2, 2-tribromoethanol and were sacrificed by cervical dislocation. Whole colons were collected, soaked and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 3 days. The tissues were then embedded in methylmethacrylate and sectioned longitudinally in 5-μm thick slices. Histopathologic analysis was performed in a double-blind fashion by two pathologists using a scoring system (25). The histologic score was the sum of the two parameters tallied as follows: For epithelium (E), “0” means normal morphology and “1” indicates the loss of goblet cells, “2” represents loss of goblet cells in large areas, “3” shows loss of crypts and a score of “4” means loss of crypts in large areas. For infiltration (I), “0” means no infiltrates, “1” indicates

infiltrates around the crypt bases, "2" represents infiltrates reaching the lamina muscularis mucosa, "3" shows extensive infiltration reaching the lamina muscularis mucosa and thickening of the mucosa with abundant edema, and a score of "4" means infiltration of the lamina submucosa. The histologic score was the sum of the two parameter, *i.e.* total score = E + I.

Enzyme-Linked Immunosorbent Assay (ELISA) Detection of Serum Pro-Inflammatory Cytokines

Before sacrificing the mice, blood were collected from the orbital area and centrifuged to obtain the serum, which was then stored at -80°C. The levels of IFN-γ and IL-6 were determined by a mouse cytokine ELISA kit (BD Cytometric Bead Array Mouse Inflammation Kit, Catalog No. 552364, BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol.

Immunohistochemistry Analysis

Hematoxylin and eosin (H&E) stain protocol was modified from previous study (11). For immunohistochemistry, tissue sections were blocked by 5% milk for 1 h, and stained with primary antibody anti-green fluorescent protein (GFP) (Abcam, Cambridge, MA, USA) at 4°C overnight, followed by secondary antibody (Dako, Santa Clara, CA, USA) for 1 h at room temperature (RT) and 3,3'-diaminobenzidine (DAB) enhancer (Dako).

Statistical Analysis

The experimental data were analyzed by using the Statistical Package for the Social Sciences (SPSS) version 15 (SPSS Inc., Chicago, IL, USA), Prism 6.0 (Graphpad Software, La Jolla, CA, USA) and Microsoft EXCEL version 2003 (Microsoft, Redmond, WA, USA). The data are presented as mean ± standard error of the mean (SEM). The non-parametric Mann-Whitney U test was used to examine the cytokine results, while changes in bodyweights were measured by using the Wilcoxon matched-pair signed-rank test. Changes in the colon length and the histological severity score were analyzed by Student's unpaired two-tailed *t*-test. Statistical significance was set at $P < 0.05$.

Results

Therapeutic Effects of pAFSCs in DSS-Treated Colitis Mice

To examine whether pAFSCs could ameliorate colitis, ICR mice were treated with 2% DSS for 7 days. All of the DSS-treated mice showed similar signs of UCs, for example, the percentage in the bodyweight of the DSS-administered mice was lower compared with the solvent control, and the DSS-treated mice also showed shorter colon length (Figs. 1A, 1B & 1C). The results showed that the experimental colitis mouse model was successfully established. The effects of pAFSCs in the DSS-treated colitis were next explored by infusing pAFSCs to DSS-induced colitis mice. After pAFSC infusion for 7 days, the mice restored the the body weight and reversed the shortening of the colon length (Figs. 1A, 1B & 1C). Histological examination of the colonic sections from the DSS-induced colitis mice showed severe inflammatory areas in the acute phase when compared to the healthy Group 1 animals. On the other hand, the histological severity score was improved in the pAFSC-treated mice (Fig. 2). Taken together, the data indicated that pAFSCs showed therapeutic effects in the DSS-treated mice.

Histopathological Analysis of pAFSC-Treated Colons in Colitis Mice

Recent studies demonstrated that epithelial-mesenchymal transition (EMT) contributes to the pathogenesis of IBD (33), especially to the loss of intestinal epithelial cells (IECs), increasing intestinal permeability and decreasing epithelial barrier functions (18, 22). By histological analysis, the intact crypt and villi structure were observed in normal colon (Figs. 3A & 3B); in contrast, the DSS-treated group demonstrated damages in the crypts of the colon compared to healthy animals; the distal colon also displayed more severe damages than the proximal colon (Figs. 3C & 3D). Interestingly, pAFSC infusion ameliorated the extent of the less inflammatory areas and exhibited epithelial mesenchymal transformation compared to the DSS-treated group (Figs. 3E & 3F). The results indicated that the DSS-treated intestinal lesion sites had recovered the original normal anatomical conformation and physiologic functions.

pAFSC Infusion Suppresses Serum Levels of Proinflammatory Cytokines

In previous studies, proinflammatory cytokines were shown to play important roles in DSS-induced colitis (36). In addition, MSCs have been proven to posses anti-inflammatory abilities (23, 31). Thus, we next investigated whether the prevention of pAFSCs in DSS-induced colitis was through regulation of the expression of proinflammatory cytokines. In the DSS-treated colitis mice, the serum levels of IFN-γ and IL-6 were significantly increased during acute colitis

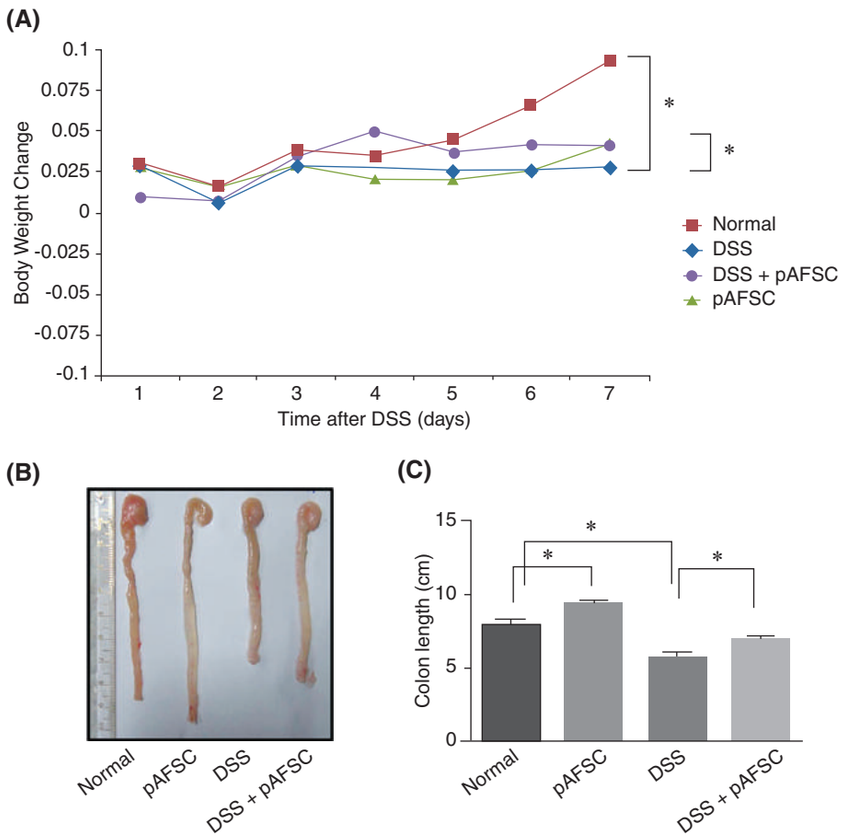

Fig. 1. pAFSCs ameliorate DSS-induced colitis. Mice were given 2% DSS in water for seven days. After DSS-induced colitis, mice were infused with pAFSCs, or PBS as a control, on day 1. (A) Body weight changes. Mice were evaluated by daily changes in bodyweight and the results were represented as percentage of the body weight on day 0. (B, C) Changes in the length of the colon. After seven days, colons were harvested for length measurement. Panel (C) is the quantification of the colonic lengths (panel B) obtained. The results are presented as means ± SEM (n = 4 mice per group). *$P < 0.05$.

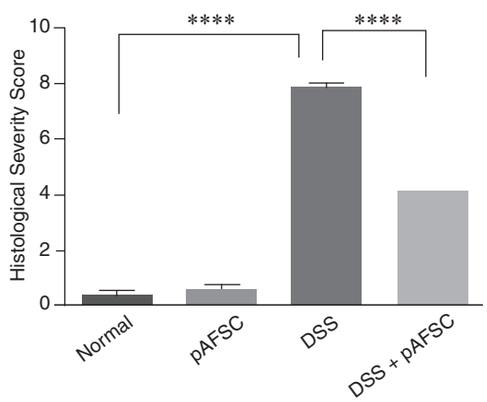

Fig. 2. Therapeutic efficacy of pAFSCs on the colitis. The histologic severity scores of the pAFSC-treated mice were compared to the DSS-treated control group. The results are analyzed from four independent experiments. Values are mean ± SEM (n = 4 mice per group). ****$P < .00001$.

compared to the normal group; concurrently, pAFSCs suppressed the elevated expression levels of IFN-γ and IL-6 (Figs. 4A & 4B). These results indicate that the infusion of pAFSCs had an inhibitory effect on the expression of proinflammatory cytokines in DSS-induced colitis (Fig. 4).

Discussion

Mesenchymal stem cells are multipotent cells, which could be isolated from various tissues, that are able to differentiate into cells of different lineages. In addition, MSCs also have immunomodulatory properties that can express IL-6, IL-10, monocyte chemoattractant protein-1 (MCP-1) and MCP-2 to modulate the immune system (23, 31). Hence, MSCs can be used for allogenic or xenogenic transplantation without immune rejection. Recently, amniotic fluid may be used as an alternative source of MSCs. The amniotic fluid-derived stem cells

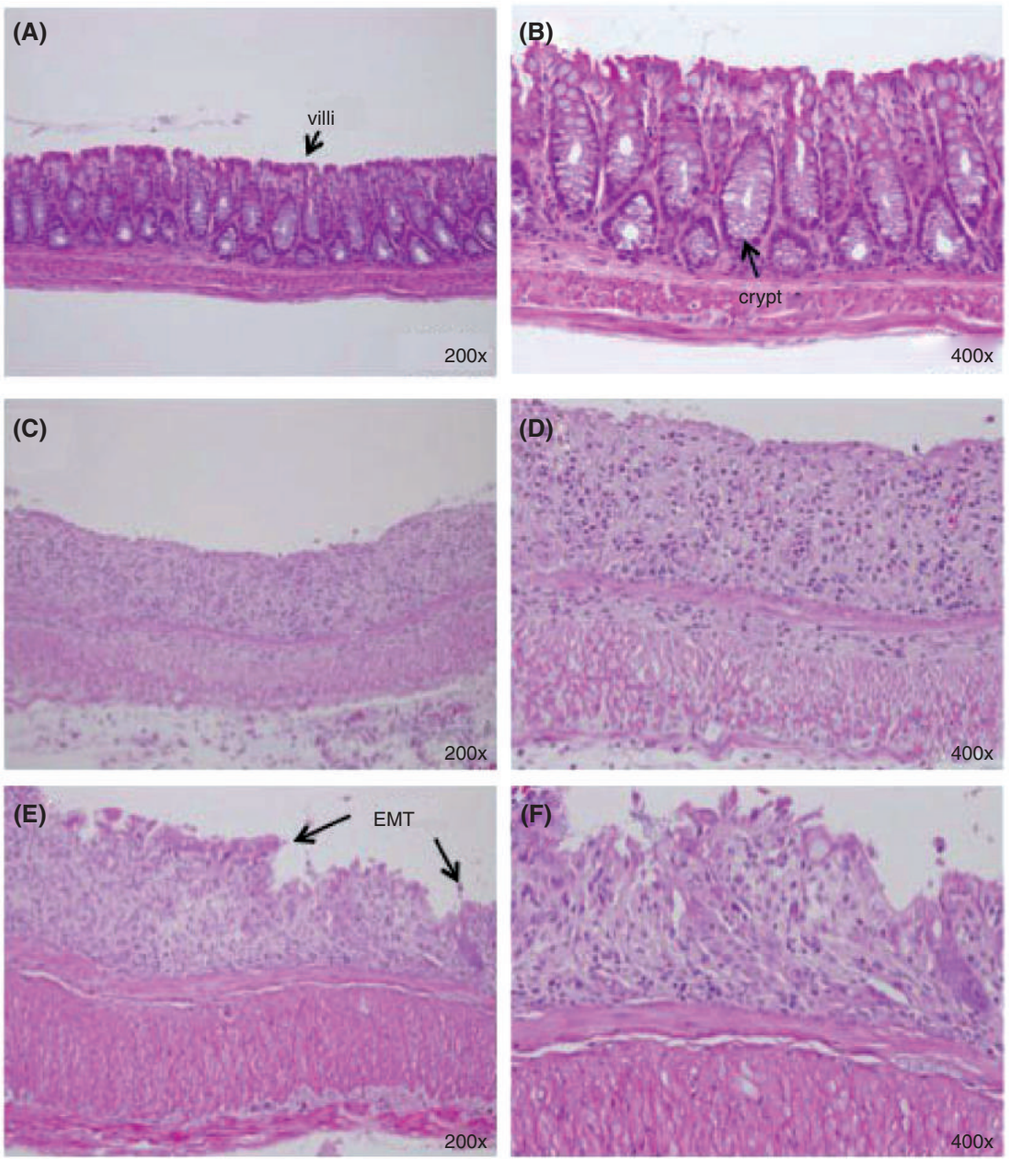

Fig. 3. Histological changes in the distal colon post-pAFSC infusion in the DSS-induced colitis mice during the acute phase. Histological sections of the colon were stained with H&E demonstrated normal villi and crypts at x200 (A) and x400 (B) magnifications. The DSS-treated group revealed intestinal gland destruction and crypt damage with infiltration of inflammatory cells in the lamina propria at x200 (C) and x400 (D) magnifications. The pAFSC-treated group demonstrated smaller inflamed area represented by EMT at x200 (E) and x400 (F) magnifications. The paraffin sections are representative of three separate experiments.

have the same multilineage potential abilities to be differentiated into adipocyte, bone, chondrocyte, or neuron-like cells as MSCs do. However, the advantage of AFSCs is that they have a higher expansion potency than MSCs derived from the umbilical cord or the adipose in the first passage (39). In addition, human AFSCs express octamer-binding transcription factor 4 (OCT4), sex determining region Y-box 2 (SOX2), Nanog, zinc finger protein 42 (Rex1) and cyclin A (29), and are, therefore, considered as multipotent stem cells sharing characteristics of both embryonic and adult stem cells. Interestingly, AFSCs could be reprogrammed into induced pluripotent stem cells (iPSCs) by using the four Yamanaka factors, OCT4, SOX2, KLF4 and c-MYC (7, 20), or the expression of the only OCT4 (30). Hence, AFSCs may be more

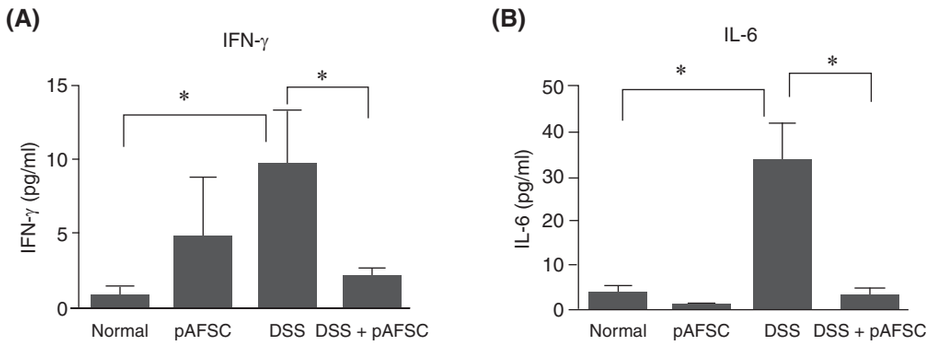

Fig. 4. Reduced expression of inflammatory cytokines by pAFSCs infusion. The serum levels of IFN-γ (A) and IL-6 (B) were analyzed by ELISA on day 7 after DSS-induced colitis. Values are mean ± SEM (n = 4 mice per group). *$P < 0.05$.

easily applied for cell therapy.

Until now, most AFSCs were derived from the human, mice and rats, and the AFSCs from these sources possess anti-inflammation properties for treatment of several diseases. For examples, rat AFSCs could improve survival and enhance repair of damaged intestine in necrotising enterocolitis (41); murine AFSCs attenuated hyperoxia-induced acute lung injury (38), and human AFSCs alleviated neuropathic pain in a chronic constrictive injury nerve model (4). Recently, human AFSCs were demonstrated to be effective in the treatment of colitis through secreted several molecules, including anti-inflammatory molecules IL-10 or IL-13 (19). However, results of this work showed that the reduction in the fold of severity score when porcine AFSCs used was much better than the human AFSC condition medium. Therefore, we suggest that pAFSCs have other paracrine effects for colitis disease. In the present study, we demonstrated that pAFSCs were capable of repressing colon shortening and body weight loss, reduced the severity score and attenuated expression of proinflammatory genes after colitis induction.

The integrity of the monolayer of intestinal epithelial cell (5) is important to maintains the protection from external pathogens in the intestine (9); disrupted IEC barrier contributes to loss of epithelial cells leading to IBD. The EMT is the process in which the morphology of the epithelial cells is transformed into mesenchymal cells, and studies have illustrated that up-regulation of EMT is implicated in UC (35). In our study, the loss of epithelial cells was revealed by histological analysis in DSS-treated colitis mice; on the other hand, pAFSCs infusion reversed the anatomical conformation on the top of villi in the intestine.

In summary, our finding is the first study to provide evidence that AFSCs from porcine had the therapeutic ability for DSS-induced colitis mice through suppressing proinflammatory cytokines in the acute phase. Moreover, pAFSCs could improve the damaged crypt and villi of the colon *via* EMT. These findings suggest the feasibility of pAFSC treatment for IBD and may provide a cell source for clinical applications.

Acknowledgments

This study was supported by the National Science Council (NSC 101-2313-B-002-017-MY3) and Ministry of Science and Technology (MOST 105-2313-B-002-039-MY3).

Conflict of Interests

The authors declare that there are no conflicts of interests.

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