Original Article

Mesenchymal stem cells administered in the early phase of tumorigenesis inhibit colorectal tumor development in rats

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(Received 2 August, 2013; Accepted 16 August, 2013; Published online 31 October, 2013)

To investigate the differences between the effects of mesenchymal stem cells (MSCs) administered in the early and late phases of tumorigenesis, MSCs were isolated from bone marrow and colorectal tumors were produced by exposing 7-week-old F344 rats to 1,2-dimethylhydrazine and dextran sulfate sodium. We evaluated tumor number and volume (week 25), MSC localization, number of aberrant crypt foci (ACF), transforming growth factor (TGF)-β1 protein levels in the rectum after administration of MSCs (week 5 or 15), and the effects of MSC-conditioned medium on ACL15 cell proliferation. Administered MSCs labeled with PKH26 were observed in the rectum. Administered MSCs in the early phase (week 5) before tumor occurrence (week 12) significantly decreased tumor number and volume (1.5 vs 4 and 21 mm^3 vs 170 mm^3; $p<0.01$), but not administered MSCs in the late phase (week 15). Administered MSCs in the early phase reduced ACF number on days 14 and 35 (1.9 vs 4.1 and 3.7 vs 7.3; $p<0.01$). Rectal TGF-β1 increased 1.3 fold on day 3, and MSC-conditioned medium containing TGF-β1 abundantly inhibited ACL15 cell proliferation. MSCs administered in the early phase but not late phase inhibited colorectal tumor development in a rat model.

Key Words: **mesenchymal stem cell, colorectal tumor, aberrant crypt foci, transforming growth factor β1**

Mesenchymal stem cells (MSCs) are non-hematopoietic stem cells that represent a small population (approximately 0.01% to 0.001%) of nucleated cells found in the bone marrow.[1] MSCs have various potentials for not only self-renewal but also multiplex differentiation. The differentiation of MSCs leads to the formation of multiple stromal lineages, such as osteoblasts, chondrocytes, adipocytes, endothelial cells, or hepatocytes.[2–4] Under different pathological conditions, MSCs also have other functions. For example, MSCs can migrate into injured tissues and function as immunosuppressive agents.[5] We have also previously reported that rat MSCs derived from the bone marrow after intravenous injection exhibit anti-inflammatory effects on injured tissues in a rat colitis model.[6] Considering these findings, MSCs can be concluded to have interesting therapeutic properties that could potentially be used in regenerative medicine, especially for the treatment of inflammatory bowel disease or graft versus host disease.[7–10]

There are various reports on MSCs in the field of oncology. MSCs have been reported to inhibit tumor growth in a Kaposi's sarcoma model and tumorigenesis in a hepatocellular carcinoma model.[11,12] In addition, an MSC-conditioned medium was observed to inhibit the proliferation and invasion of lung and esophageal cancer cells by inducing the G1 arrest of the cell cycle, resulting in the apoptosis of tumor cells.[13] MSCs have also been used in other applications. Notably, MSCs have been regarded as the most promising delivery vehicle for cell-based targeted cancer gene therapy in the future.[14] On the other hand, there are also reports that disagree with these predictions. In addition to migrating to injured tissues, MSCs can migrate into stromal tissues of tumors and interact with various cancer cells directly or indirectly via mediators. MSCs are speculated to have malignant properties as they promote tumor growth, invasion, and metastasis, which result from the enhancement of both angiogenesis and invasion as well as the inhibition of tumor cell apoptosis in breast and colon cancers.[15,16] MSCs have dual functions in cancer cells, especially with regard to tumor growth. In particular, the roles of exogenous MSCs in tumorigenesis and tumor growth in cancer cells remain controversial.

Among numerous factors, including inflammatory cytokines and growth factors, transforming growth factor (TGF)-β1, the multi-potent cytokine, has unique functions in cancer cells. TGF-β1 inhibits tumor development during the early phase of tumorigenesis, whereas TGF-β1 induces metastasis, which is mediated by the increased migration and invasion of cancer cells in the late phase of tumorigenesis.[17–19] Thus, the schedule or timing for administering exogenous agents or chemical mediators is important when evaluating their specific efficacy for tumor growth. Recently, there are numerous reports based on the interactions between MSCs and TGF-β1 (e.g., proliferation and differentiation). Therefore, unique dual effects of MSCs on tumor development may be associated with the effects of TGF-β1 derived from MSCs by themselves as well as inconsistencies in the timing of their administration.[20,21]

In the present study, we evaluated the effects of exogenous MSCs on tumor development in the early and late phases of tumorigenesis and examined their association with TGF-β1 levels in an experimental rat colorectal tumor model.

Materials and Methods

Animals. Seven-week-old F344 rats were purchased from Charles River Japan (Osaka, Japan). All the rats were allowed free access to standard pellet chow and water. The rats were randomly assigned to various groups. All the experimental protocols were approved by the Animal Care and Use Committees of Osaka City University.

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doi: 10.3164/jcbn.13-68

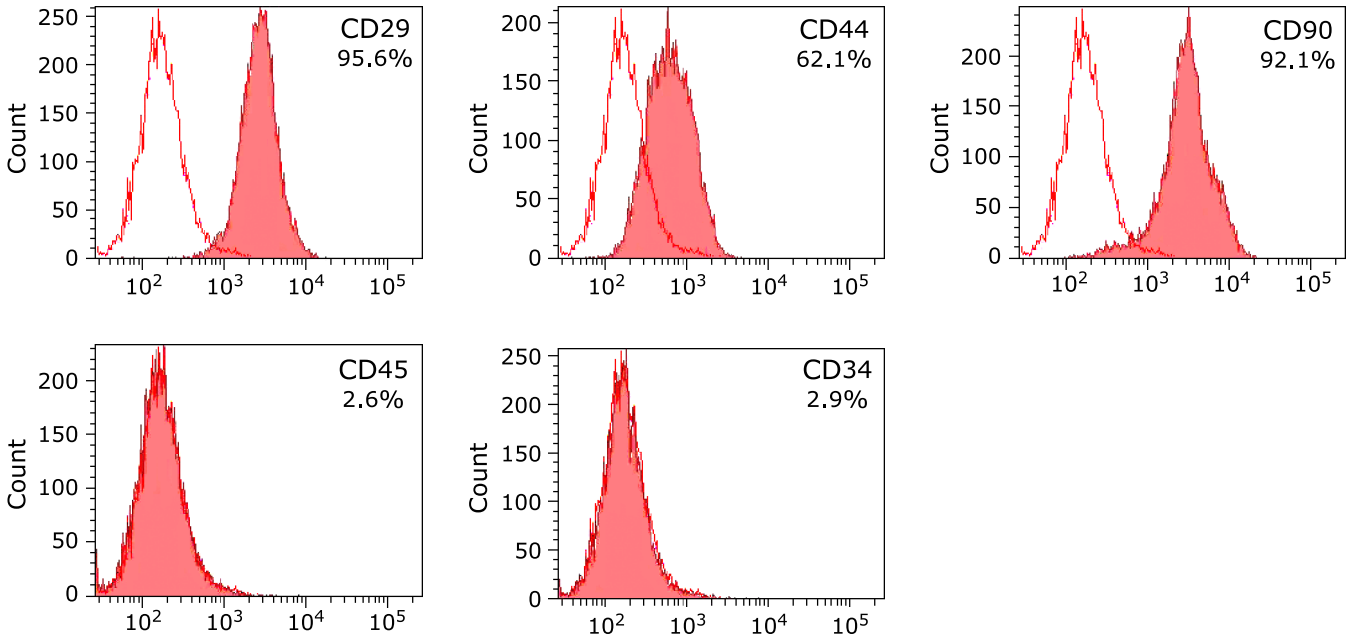

Fig. 1. Characterization of MSCs. Characterization of MSCs was identified by flow cytometry using antibodies against CD29, CD34, CD44, CD45, and CD90. MSC cells were positive for CD29, CD44, and CD90, but negative for CD34 and CD45.

Isolation and culture of MSCs. MSCs were harvested and cultured as described previously.[7] Briefly, 7-week-old male F344 rats were sacrificed by cervical dislocation, and bone marrow was obtained from the tibia and femur of rats. MSCs were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM: Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic (GIBCO, Carlsbad, CA). All cells were incubated at 37°C in a 5% CO_2 humidified cell culture incubator. Non-adherent cells were removed by changing the medium at 72 h every 4 days. MSCs morphologically appeared to be a homogenous population of spindle-shaped cells such as fibroblasts. These cells were used in subsequent experiments after the fifth passage. To evaluate the lineage, MSCs were washed and fixed and permeabilized by a fixation buffer and permeabilization buffer, and then stained with anti-CD29 (GenWay Biotech Inc, San Diego, CA), anti-CD44 (Acris Antibodies, Hiddenhausen, Germany), anti-CD90 (AbD Serotec, Oxford, UK), anti-CD34 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-CD45 (Acris Antibodies) for 30 min at 4°C.[22] The cells were washed, and a LSR II flow cytometer (BD Biosciences, San Jose, CA) was used to confirm that these cells expressed CD29, CD44, and CD90 and not CD34 or CD45 (Fig. 1).

Preparation of MCS-conditioned medium. Fifth-passaged MSCs were incubated with DMEM without phenol red and were supplemented with 10% FBS in 75 mm^3 culture flasks at 37°C in a 5% CO_2 humidified cell culture incubator for 1 week, and MSC-conditioned medium was collected and filtered using an Amicon-Ultra-15 (50k) centrifugal filter unit (Millipore, Billerica, MA) to remove albumin. Similarly, DMEM without phenol red supplemented with 10% FBS was also filtered using an Amicon-Ultra-15 (50k) centrifugal filter unit. Each medium was subsequently supplemented with 2% bovine serum albumin (BSA).

Experimental protocol for colorectal tumor model and detection of aberrant crypt foci. The experimental protocol for the colorectal tumor model was performed using a previously described protocol shown in Fig. 2A.[23] Briefly, 1,2-dimethylhydrazine (DMH; 40 mg/kg body weight; Wako Pure Chem. Ind. Ltd., Osaka, Japan) was subcutaneously injected 3 times in the first week. Rats were then provided drinking water containing 1.0% dextran sulfate sodium (DSS) (Wako Pure Chem. Ind. Ltd.) every day during the second week. On week 5 or 15, the MSCs (4×10^6 cells/body with 1 ml saline) or saline alone (control) were administered to rats through the tail vein, and rats were sacrificed at week 25. The appropriate number of MSCs in the present study was determined according to the previous our report.[6] We evaluated tumor number and their total volume at week 25. Incidence of tumor was defined as a ratio per rat of which one or more nodules over than 1 mm in diameter was macroscopically recognized throughout the entire colon. The development of aberrant crypt foci (ACF), which are presumed to be the earliest identifiable preneoplastic lesions in the colorectal tumor model,[24–26] was also evaluated on days 0, 14, and 35 after the administration of MSCs on week 5 (Fig. 3A). Excised colonic tissues were cut open along the longitudinal median axis, fixed flat between 2 sheets of filter paper with 10% formalin (Wako Pure Chem. Ind. Ltd.), and then stained with 0.05% methylene blue for 6 min. The number of ACF per colon was counted under a light microscope at 40× magnification.

Localization of exogenously administered MSCs. Before the MSCs were administered, they were labeled with PKH26 to detect their localization in the colonic tissues. The MINI26 cell linker kit (Sigma) was used according to the manufacturer's instructions. Colonic tissues were also analyzed using a fluorescence microscope and the same protocol.

TGF-β1 protein levels in the rectum and MSC-conditioned medium. Rectal samples were obtained on days 3, 14, and 35 after the MSCs administration. Rectal tissues were homogenized and lysed on ice in buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM ethylenediaminetetraacetic acid, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, 2 μg/ml of leupeptin, 2 μg/ml of aprotinin, and 2 μg/ml of pepstatin. The protein content of the lysate was measured with a modified bicinchoninic acid method (BCA) protein assay reagent kit (Pierce, Rockford, IL). We measured TGF-β1 protein levels in the rectum and MSC-conditioned medium by performing enzyme-linked immunosorbent assay (ELISA). The sample was quantified using Quantikine Mouse/Rat/Porcine/Canine TGF-β1 immunoassay (R&D Systems, Minneapolis, MN), according to the manufacturer's

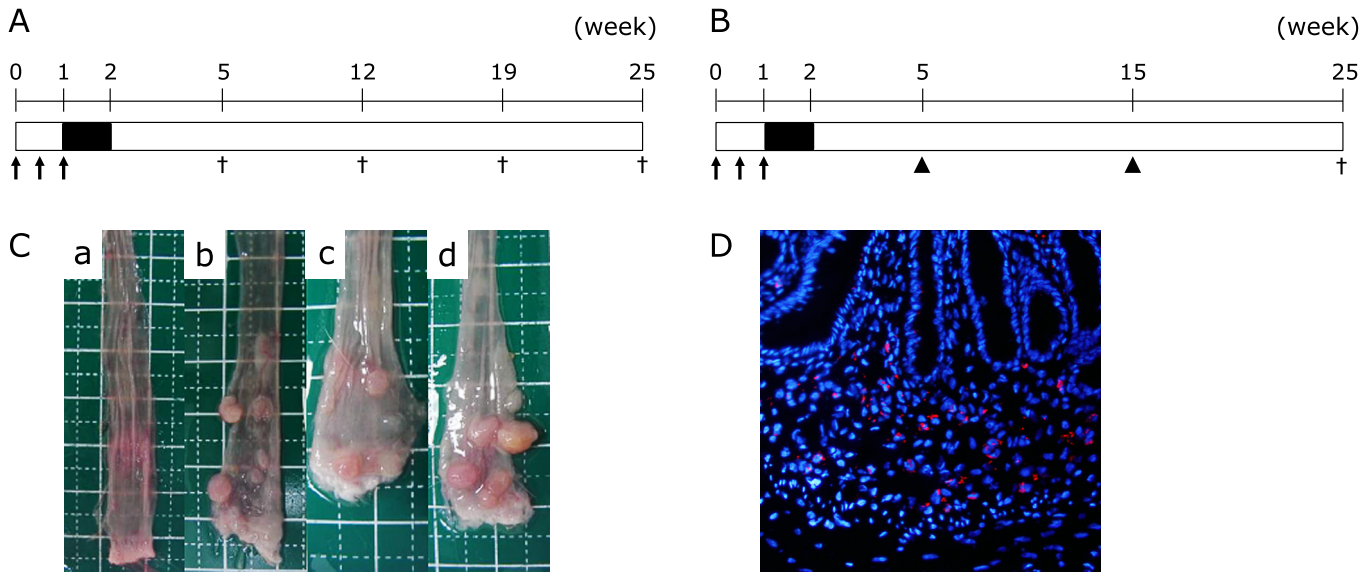

Fig. 2. Experimental protocols, macroscopic images of a rat colorectal tumor model and localization of exogenously administered MSCs. (A) Experimental protocol to confirm the period of formation and location of tumor formation. (B) Experimental protocol to confirm the effects of administering MSCs on colorectal tumors between early and late phases of tumorigenesis. ↑, subcutaneous injection of 1,2-dimethylhydrazine (DMH; 40 mg/kg); ■, duration of the free drinking of water containing 1.0% dextran sulfate sodium (DSS) during the second week; ▲, the intravenous injection of MSCs (4×10^6 cells/body), and †, sacrificed rats. (C) Tumors at week 25. (a) normal group; (b) control group; (c) MSCs group (administered at week 5); and (d) MSCs group (administered at week 15). (D) Exogenously administered MSCs with PKH26 (red) was localized the lamina propria, particularly near the bottom of the crypt, on day 3 after the administration of MSCs (blue: DAPI staining) (40× magnification).

instructions. The mean value was expressed as the unit (pg/μg protein) of TGF-β1 protein levels (pg) per total protein levels (μg protein) to correct for the difference in the excised rectal tissue volume. These experiments were performed in duplicate.

Cell proliferation assay. Cell proliferation was determined by the MTT assay using the Cell Proliferation Kit I (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. ACL15 cells (1.2×10^4 cells/well) and rat colorectal cancer cells, which were created by administering DMH to F344 rats, were seeded on 96-well plates for 24 h in RPMI 1640 without phenol red supplemented with 10% FBS. After 24 h, ACL15 cells were harvested in RPMI 1640 without FBS. After this preconditioning, the cells were incubated in DMEM without phenol red and were supplemented with 2% BSA or MSC-conditioned medium supplemented with 2% BSA for 72 h. The absorbance was measured at 570 nm using a microplate reader (CORONA, MTP-500, Ibaraki, Japan). ACL15 cells were provided by RIKEN BRC through the National Bio-Resource Project of MEXT (Japan) and were maintained in RPMI 1640 (Sigma) supplemented with 10% FBS and 1% Antibiotic-Antimycotic in 5% CO_2 humidified atmosphere and 95% air at 37°C.

Statistical analysis. All values represent the mean ± SE. Significant differences were assessed by the Mann-Whitney *U* test or one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test. Probability values less than 0.05 were considered to indicate statistical significance.

Results

Development of colorectal tumors in the experimental model. Experimental colorectal tumors were developed in accordance with a previous report.[23] At first, we macroscopically examined the entire colon using an experimental protocol (weeks 5, 12, 19, or 25) to confirm the period and location of tumor formation. No tumor formation was observed on week 5, and tumor nodules began to develop mostly in the rectum on week 12. One or more colorectal tumors formed in every rat on week 19 and 25 (Table 1). Average tumor size was 3.5 ± 0.9, 3.6 ± 0.5, and 3.8 ± 0.2 mm in diameter at weeks 12, 19, and 25, respectively, and there was no differences in it among these periods. Based on these findings, we defined weeks 5 and 15 as being the early and late phases of this experimental tumorigenesis (Fig. 2B).

Effects of administering MSCs on experimental colorectal tumorigenesis and localization of exogenously administered MSCs in the rectum. At week 25, we evaluated the tumor number and volume in each group. Administering exogenous MSCs in the early phase (week 5) significantly reduced the tumor number and volume, unlike that observed in the control (1.5 vs 4 and 21 mm^3 vs 170 mm^3, respectively; $p<0.01$). In contrast, administering MSCs in the late phase (week 15) had no effect on these variables (Fig. 2C, Table 2). To examine the presence of administering MSCs in the rectum, we evaluated its localization in the rectum. MSCs labeled with PKH26 (red) were localized in the lamina propria, particularly near the bottom of the crypt, on day 3 after the administration of MSCs (Fig. 2D).

Effects of administering MSCs on the occurrence of ACF. On week 5 (the early phase), no obvious tumor nodules were observed throughout the entire colon. However, the occurrence of ACF was confirmed at that time (Fig. 3B), and its mean number was 1.3 ± 0.6. The mean number of ACF in the group that received MSCs was lower than that in the control on days 14 (1.9 vs 4.1; $p<0.01$) and 35 (3.7 vs 7.3; $p<0.01$), respectively (Fig. 3C).

TGF-β1 protein levels in the rectum and MSC-conditioned medium. The protein levels of TGF-β1 in the rectum increased by 1.3 fold on day 3 after the administration of MSCs. However, there was no increase in TGF-β1 protein levels on days 14 and 35 (Fig. 4A). To examine whether TGF-β1 derived from exogenously administered MSCs may affect colonic tumorigenesis in the rectum, we measured TGF-β1 protein levels in the MSC-conditioned medium. TGF-β1 protein levels increased in the MSC-conditioned medium by approximately 2.2 fold, unlike that observed in the control medium ($p<0.01$) (Fig. 4B).

doi: 10.3164/jcbn.13-68

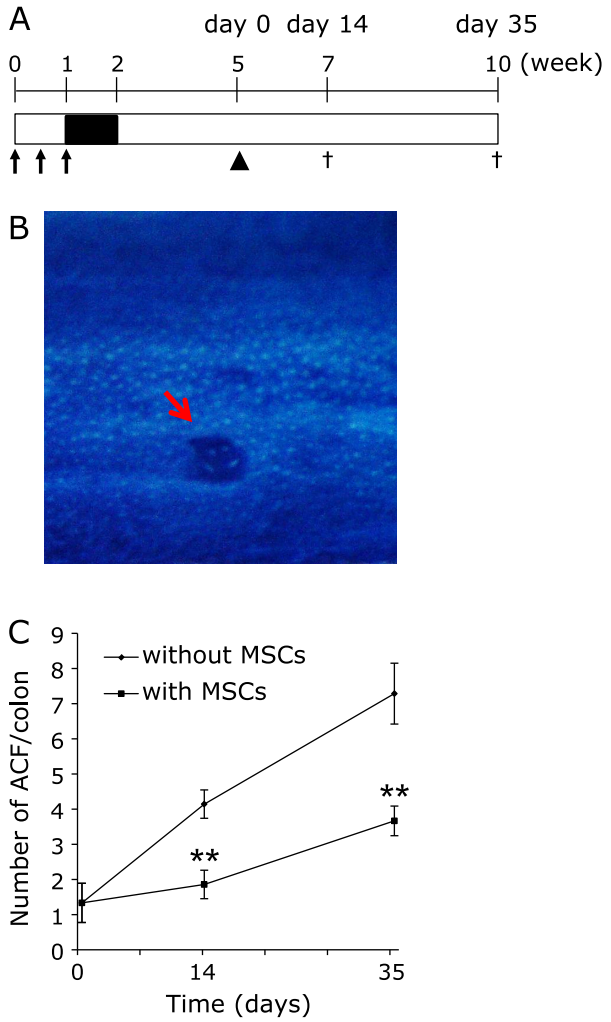

Fig. 3. Experimental protocol, macroscopic images, and ACF occurrence after administering exogenous MSCs. (A) Experimental protocol to confirm the effects of exogenously administered MSCs on the occurrence of ACF. ↑, subcutaneous injection of 1,2-dimethylhydrazine (DMH, 40 mg/kg); ■, duration of the free drinking of water containing 1.0% dextran sulfate sodium (DSS) during the second week; ▲, intravenous injection of MSCs (4×10^6 cells/body), and †, sacrificed rats. (B) Methylene blue staining of an excised rat colon. ACF are shown at arrows (40× magnification). (C) The incidence of ACF with or without the administration of MSCs on days 0, 14, or 35. Each value represents the mean ± SEM ($n = 7$). $^{**}p<0.01$ compared to group that was not administered MSCs.

Proliferation assay for ACL15 cells by MSC-conditioned medium. To examine the direct effects of the MSC-conditioned medium on cell proliferation, we performed the MTT assay using ACL15 cells. The MSC-conditioned medium significantly inhibited the proliferation of ACL15 cells in a concentration-dependent manner (maximum inhibition rate, 52%; $p<0.01$) (Fig. 4C).

Discussion

The results of the present study revealed that exogenously administered MSCs in the early phase inhibited ACF formation (the earliest identifiable preneoplastic lesions) and tumor development associated with increased TGF-β1 protein levels in a rat colorectal tumor model.

With regard to tumorigenesis, recruited or coexisting MSCs exhibit different functions, such as the growth or inhibition of tumors, corresponding to the tumor conditions.[11–13,27] In other words, MSCs may become either malignant agents or inhibitors against tumors. Thus, it is very important to determine the phase of tumorigenesis. Most reports showing the effects of MSCs on tumor growth have been based on the evaluation of the late phase of tumorigenesis, and there are no reports about the effects of exogenously administered MSCs in the early phase on tumor development. To confirm tumor development and the tumorigenic phase of this experimental model, we examined tumor development over a time course (weeks 5, 12, 19, or 25) using previously reported methods. Tumor development began from week 12, and one or more tumors existed in every rat on week 19 and thereafter. The majority of tumors occurred in the rectum. During the early phase (week 5), ACF occurred and there were no obvious tumor nodules, and the results regarding the stage and location of tumors were consistent with previously reported findings.[20] Based on these findings, weeks 5 and 15 were defined as the early and late phases of tumorigenesis, respectively. We used this definition to evaluate the effects of exogenous MSCs on tumor development in accordance with the respective phases of tumorigenesis in subsequent experiments. Our results show that exogenously administered MSCs in the early phase of tumorigenesis inhibit tumor development in *in vivo* experiments. Interestingly, MSCs administered in the late phase of tumorigenesis had no effects on tumor development over the course of 10 weeks. Furthermore, our preliminary studies showed that the sizes and growth rates of subcutaneous xenograft tumors were larger and faster in the mixture group (ACL 15 cells and MSCs), respectively, than those in the control group (ACL 15 cells alone) (data not shown). In fact, MSCs had different functions for tumor development model. In addition, the present findings that exogenously administered MSCs especially in the early phases of tumorigenesis inhibited tumors development in *in vivo* experiment might have been firstly demonstrated. Our results on the effects of MSCs administered in the early phase on the ACF appearance (the earliest identifiable preneoplastic lesions in the colorectal tumor model) may support the inhibitory effects of MSCs on tumor development and an aspect of their inhibitory mechanism(s). Different effects of exogenous MSCs on tumor development were observed according to the time points between the early and late phases of tumorigenesis at which they were administered, although a longer follow

Table 1. Incidence of colorectal tumors in rat model

	Incidence of tumor (%)	Average tumor number (/rat)	Average tumor size (mm)
week 5	0 (0/3)	0	0
week 12	67 (2/3)	1.0 ± 0.6	3.5 ± 0.9
week 19	100 (3/3)	3.0 ± 1.2	3.6 ± 0.5
week 25	100 (6/6)	3.5 ± 0.8	3.8 ± 0.2

Incidence of tumor was defined as a ratio of rat of which one or more tumors over than 1 mm in diameter was recognized throughout the entire colon. Average tumor size was calculated by total sum of respective tumor size of all rats.

Table 2. Incidence of colorectal tumors with administering MSCs in a rat model

	Incidence of tumor (%)	Average tumor number (/rat)	Average tumor volume (mm3/rat)
Control	100 (6/6)	4.0 ± 0.5	170 ± 40
with MSCs (5W)	67 (4/6)	1.5 ± 0.6**	21 ± 9**
with MSCs (15W)	100 (6/6)	3.5 ± 0.6	214 ± 59

Tumor volume = 1/2 (length × width × width). $^{**}p<0.01$ compared to group that was not administered MSCs.

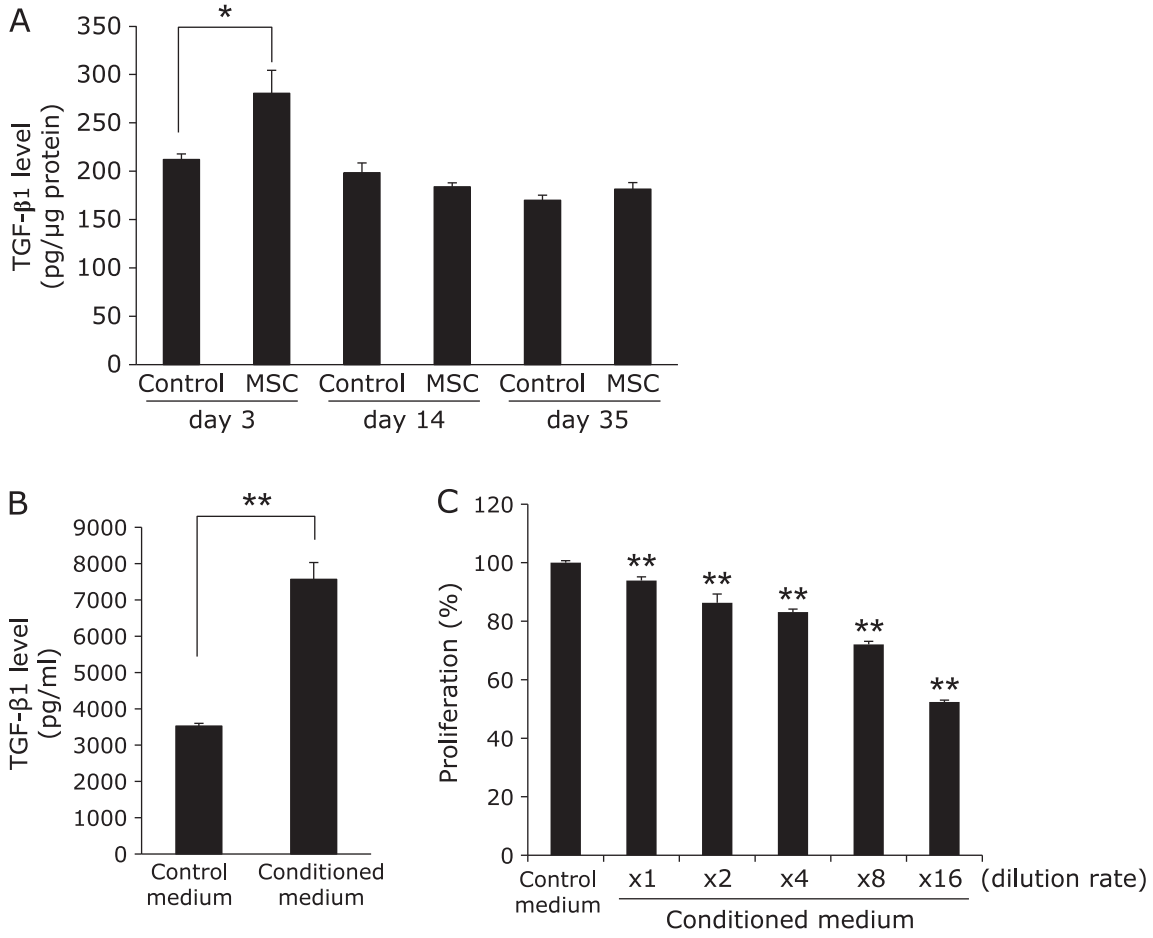

Fig. 4. TGF-β1 protein levels in the rectum and MSC-conditioned medium and proliferation assay for ACL15 cells by MSC-conditioned medium. (A) TGF-β1 protein levels in the rectum on days 3, 14, and 35 after administering MSCs. Each value represents the mean ± SEM (n = 5). *p<0.05 compared to the group that was not administered MSCs. (B) Protein levels of TGF-β1 in the MSC-conditioned medium. Each value represents the mean ± SEM (n = 5). **p<0.01 compared to the controls. (C) The effects of MSC-conditioned medium on the proliferation rate of ACL15 cells compared to those of the control medium (100%). Each value represents the mean ± SEM (n = 5). **p<0.01 compared to the control medium.

up period after the administration of MSCs may potentially exhibit different effects, such as promoting tumor growth.

The localization of exogenously administered MSCs lasted at least until day 35. Therefore, their inhibition of tumor development must be a result of having local effects. MSCs have other characteristic properties such as the production and secretion of various mediators (e.g., TGF-β1, platelet-derived growth factor, and vascular endothelial growth factor) in cultured medium.[3,20] This finding suggests that these mediators must affect tumor development. Among them, TGF-β1, the multi-potent cytokine, is an important regulator of several critical functions, such as cell cycle, differentiation, inflammation, and the apoptosis of cancer cells.[19,28] TGF-β1 induces metastasis, which is mediated by increasing the migration and invasion of cancer cells in the late phase of tumorigenesis,[17–19] whereas it also inhibits tumor development during the early phase of tumorigenesis by inducing GI arrest of tumor cells.[17] In the early phase of tumorigenesis, tumor cells remain sensitive to TGF-β1 signalling, resulting in the suppression of tumor growth. In the late phase of tumorigenesis, tumor cells exhibit the opposite response to TGF-β1 stimulation for tumor growth.[29,30] These characteristic effects of TGF-β1 on tumor cell proliferation were similar to variables affected by exogenous MSCs. In addition, the local contents of TGF-β1 in the rectum also increased for a few days after the administration of MSCs. The changes in content of TGF-β1 were in accordance with the results observed after the administration of exogenous MSCs. These findings indicated that different effects of MSCs between early and late phases on colonic tumorigenesis may reflect on the properties of TGF-β1 for colonic cell proliferation. Moreover, the secretion of TGF-β1 from MSCs is necessary to directly inhibit proliferation of immature colonic epithelial cells consist of ACF. As well as a significant increase in TGF-β1 in the MSC-conditioned medium, the results of an *in vitro* study showed that the MSC-conditioned medium unlike the control medium inhibited the proliferation of ACL15 cells. These results suggest that administered MSCs may affect colonic tumorigenesis via the biological activity of TGF-β1. However, this point is a major limitation of this study, because we could not prove the direct effect of TGF-β1 on tumorigenesis in this experimental model. It is generally indicated that systemic administration of TGF-β1 affect various immune systems including the colonic physiology. In addition, local injection of TGF-β1 or its neutralizing antibody directly to the rectum or a focal increase in its levels is thought to be methodologically difficult in the present tumorigenesis model. Furthermore, a possible mechanism involved in the other inflammatory cytokines (e.g., tumor necrosis factor-α, inteleukin-1β, -10 and inducible nitric oxide synthase) and a detailed mechanism(s) for the migration of MSCs into the rectum are still unclear. Therefore, more detailed and other mechanism(s) and factors associated with the observations for this experimental model need

doi: 10.3164/jcbn.13-68

to be investigated.

In summary, MSCs administered in the early phase of tumorigenesis inhibits colorectal tumor development mediated by a local increase in TGF-β1 protein levels in a rat model of colorectal tumors.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (K.T.).

Abbreviations

ACF	aberrant crypt foci
DSS	dextran sulfate sodium
MSC	mesenchymal stem cell
TGF	transforming growth factor

Conflict of Interest

No potential conflicts of interest were disclosed.

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