Effect of mesenchymal stem cells on hypoxia-induced desensitization of β2-adrenergic receptors in rat osteosarcoma cells

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Abstract. The β2-adrenergic receptor (β2AR) mediates the effects of chronic stress in several neoplasms, however, β2AR signaling is impaired by hypoxia in various tissues. While hypoxia is a common feature significant in the progression of solid tumors, little is known about the effect of hypoxia on β2AR signaling in the tumor microenvironment. Previously, it has been reported that the systemic administration of mesenchymal stem cells (MSCs) increased the engraftment and metastatic colonization of rat osteosarcoma (OS) cells. In the current study, the effect of MSCs on the hypoxia-induced desensitization of the β2AR in OS cells was investigated. Epinephrine, norepinephrine and isoproterenol increased the cellular proliferation of the rat OS cell line COS1NR and rat MSCs in a dose-dependent and β2AR antagonist-sensitive manner. While isoproterenol had significant proliferative effects on MSCs under normoxic and hypoxic conditions, COS1NR cells did not respond under hypoxic conditions. A sensitivity assay for the β2AR revealed that hypoxia impaired the sensitivity of COS1NR cells, whereas hypoxia did not affect MSCs. An immunoassay revealed no significant change in the expression of hypoxia-inducible factor-1α (HIF1α) in COS1NR cells, whilst an immunoassay demonstrated a 15% increase in MSCs following isoproterenol stimulation. In COS1NR cells co-cultured with MSCs under hypoxic conditions, isoproterenol caused a significant increase in proliferation and this effect was inhibited by an anti-interleukin (IL)-6 antibody. A tumor formation assay in syngeneic rats revealed that the systemic administration of MSCs enhances the growth of OS and the effect of MSCs was inhibited by IL-6 neutralization.

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In conclusion, MSCs are resistant to the hypoxia-induced desensitization to β2AR. Hypoxia caused a siginificant desensitization of the β2AR in COS1NR cells alone, whereas MSCs may support tumor progression through cellular interactions.

Introduction

Solid tumors are composed of a heterogeneous cell population, including a variety of tumor stromal cells. These cells create the tumor microenvironment, which is significant in tumor progression, through cellular interactions (1). Mesenchymal stem cells (MSCs) are bone marrow-derived multipotent cells, that are capable of differentiating into a variety of tissues, including fat, cartilage, bone and possibly muscle (2). Being part of the tumor microenvironment, MSCs are considered to create niches and facilitate tumor growth and metastasis (2). We have previously reported that MSCs promoted engraftment and metastatic colonization in a rat osteosarcoma (OS) model (3). Pathway analysis from a gene expression profile identified that the genes involved in adhesion, cytokine-cytokine receptors and extracellular matrix pathways were highly expressed in the MSCs (3). In accordance with our data, Bian *et al* have previously reported a critical role of interleukin (IL)-6 in the interaction between OS cells and MSCs (4).

β2-adrenergic receptors (β2ARs) mediate osteogenesis (5). β2AR signaling induces *c-fos* gene expression in OS cell lines (5), modulates bone turn-over in osteoblasts (6) and affects the osteogenesis of MSCs via the cyclic AMP (cAMP)/PKA pathway (7). In addition, β2AR activation leads to the upregulation of hypoxia-inducible factor-1α (HIF-1α) via Akt and ERK1/2 signaling (8), that may be a mechanism by which β2AR signaling accelerates tumor growth in several types of cancer (9-13). Conversely, hypoxia impairs β2AR signaling in a variety of tissues (14-16). Baloğlu *et al* demonstrated that *in vitro* hypoxia increased the sensitivity of β2AR to desensitization, owing to an increase in $G^{i/0}$ protein activity (14). Although hypoxia is a common feature significant in the progression of solid tumors, little is known about the effect of β2AR signaling in the tumor microenvironment.

The present study investigated the effect of hypoxia on β2AR signaling in OS cells and MSCs derived from the

bone marrow of syngeneic rats. Cellular interactions were examined using *in vitro* and *in vivo* approaches. Co-culture experiments revealed that hypoxia caused significant desensitization of β2AR on OS cells but not MSCs and that MSCs affected the response of OS cells to β2AR agonists. Systemic administration of MSCs resulted in the enhanced growth of subcutaneously transplanted OS in response to β2AR agonists.

Materials and methods

Cell lines. The COS1NR cell line was established from 4-(hydroxyamino)-quinoline 1-oxide-induced transplantable OS in male Fischer 344 (F344) rats (17,18). The cells were cultured in Eagle's Modified Essential Medium (MEM) supplemented with 10% FBS (JRH Biosciences, Lot no. 1E0666, Lenexa, KS, USA). Rat MSCs were isolated and maintained in primary culture as previously described (19,20). Briefly, bone marrow cells were obtained from the femoral bone shaft of 7-week-old male F344 rats and seeded into 75 cm^2 flasks (T-75 flasks, Corning Costar, Cambridge, MA, USA) containing 15 ml of standard medium consisting of MEM supplemented with 15% FBS and a mixture of antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Sigma-Aldrich, St. Louis, MO, USA). Cell cultures were maintained in a humidified atmosphere of 95% air and 5% CO$_2$ at 37°C. After reaching confluency (~10 days), the cells were released from the substratum using a 0.25% trypsin-EDTA solution and inoculated into 12- and 6-well plates (Falcon, Franklin Lakes, NJ, USA) for biochemical analyses and staining, respectively, at a density of 1x10^4 cells/cm^2.

Gene expression profiling of COS1NR and MSCs. The gene expression profiling was performed by Agilent array analysis (Agilent Technologies, Böblingen, Germany). Total RNA was isolated from COS1NR cells and MSCs and underwent a quality assessment by Agilent 2100 Bioanalyzer. Total RNA (500 ng) was processed by Agilent expression array analysis using a Quick Amp Labeling kit and Gene Expression Hybridization kit. Data analysis was carried out using Agilent Feature Extraction software, analyzing pathways that possibly interact in OS cells and MSCs.

Hypoxic conditions. For hypoxic stimulation, the culture plates were placed in a MCO-175M multi-gas incubator (Sanyo Electric Co., Ltd., Tokyo, Japan) at 37°C and flushed with a gas mixture of 5% CO$_2$/95% N$_2$. The oxygen concentration was maintained at 2% in the chamber using an oxygen regulator (Sanyo Electric Co., Ltd.).

Cell viability assessment. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. A single-cell suspension was plated in 96-well plates at 2.0x10^3 cells/well and allowed to attach to the plates at 37°C for 4 h. The cells were then cultured for 48 h under hypoxic or normoxic conditions. The cells were left either untreated or treated with neurotransmitters for 10 min, 8 h after the start of culture. Cell viability was measured using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay Reagent (Promega Corp., Madison, WI, USA). Cell proliferation was measured at an

emission wavelength of 492 nm. All experiments were carried out in quadruplicate and viability was expressed as the ratio of the number of viable cells with treatment to the number of viable cells without treatment.

cAMP enzyme immunoassay (EIA) and total HIF-1α protein quantification. β2AR sensitivity was determined using a cAMP EIA kit (Item no. 581001, Cayman Chemical Company, Ann Arbor, MI, USA), according to the manufacturer's instructions. Total HIF-1α protein quantification was performed using the human/mouse total HIF-1α immunoassay kit (Item no. KCB1935, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Cells were cultured in 12-well plates for cAMP measurements and in 96-well plates for HIF-1α protein quantification. Seeding density was 200,000-300,000 cells/cm^2. Confluent cells were exposed to hypoxia for 48 h. Following 8 h of hypoxia, the cells were treated with 10 μM of isoproterenol for 10 min. Following 48 h of hypoxia, cell culture extracts and formaldehyde-fixed cells were used for the cAMP assay and HIF-1α immunoassay, respectively.

Co-culture experiment. The co-culture of COS1NR cells and MSCs was performed using a 6-well format cell culture insert with a 1.0-μm pore size polyethylene terephthalate track-etched membrane (Becton-Dickinson, Franklin Lakes, NJ, USA) in 6-well flat-bottomed multi-well tissue culture plates. MSCs were placed in the upper chambers. The number of cells and the volume of culture medium in the dividing chamber culture were identical to those in the control. Co-cultured cells were subjected to the same treatment as the isolated culture described above. For the neutralization of IL-6 in bioassays, cells were cultured with a 1:400 dilution of an IL-6 antibody for 3 days (ab6672, Abcam, Cambridge, UK).

In vivo tumor formation assay in syngeneic rats
Experiment I. Cells (5x10^6) of COS1NR in 100 μl of phosphate-buffered saline (PBS) were inoculated into the subcutaneous tissue in the posteriors of F344 rats (groups 1 and 2). Each group consisted of 4 rats and the growth rate of the tumor was evaluated every week. The size of the tumors was calculated using the formula volume = 0.2618 x L x W x (L + W) (21). For β2AR stimulation, the Alzet osmotic minipumps were inserted (DURECT Corporation, Cupertino, CA, USA) containing isoprotelenol (3 mg/kg/day, group 1) or PBS (group 2) in the nape of the neck at week 4. At week 7, the rats were sacrificed and samples from subcutaneous tumors in each group were fixed in 3.7% formaldehyde neutral-buffered solution and then processed routinely for histology, stained with hematoxylin-eosin and examined under light microscopy. On the day of minipump implantation, intratumoral oxygen pressure was measured using the needle probe technique (KIMOC-6650, Eppendorf, Germany). The measurement was performed in quadruplicate at a depth of 5 mm from the tumor surface.

Experiment II. cells (5x10^6) of COS1NR in 100 μl of PBS were inoculated into the subcutaneous tissue in the posteriors of F344 rats. Subsequently, the same number of MSCs in 100 μl of PBS were directly injected into the circulation through the tail vein twice at weeks 1 and 3 (groups 1 and 2).
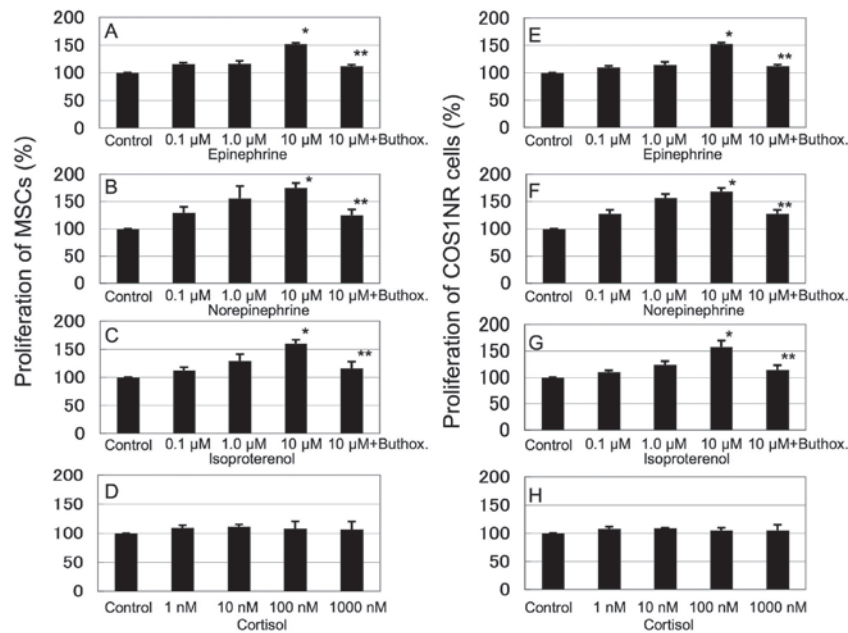
Figure 1. Effect of stress-related neurotransmitters/hormones on cellular proliferation of (A-D) MSCs and (E-H) COS1NR cells. (A and E) Epinephrine, (B and F) norepinephrine or (C and G) isoproterenol increased cellular proliferation in a dose-dependent manner, this effect was inhibited by a β2AR-selective inhibitor. *P<0.05 compared with the control group. **P<0.05 compared with the 10 μM group. MSCs, mesenchymal stem cells; β2AR, β2-adrenergic receptor.
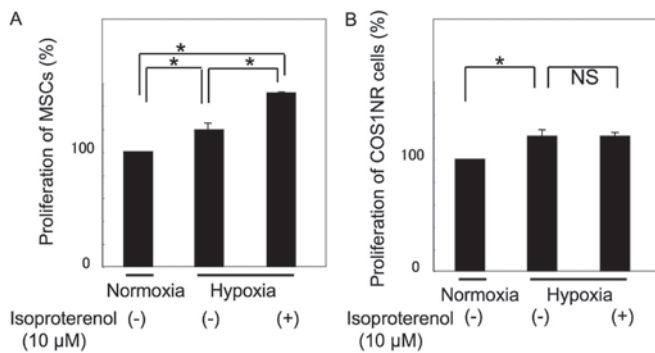

Figure 2. Effect of isoproterenol on the proliferation of (A) MSCs and (B) COS1NR cells under normoxic and hypoxic conditions. Isoproterenol had significant proliferative effects on MSCs under both conditions. COS1NR cells were affected under normoxic, but not under hypoxic conditions. *P<0.05; NS, not significant; MSCs, mesenchymal stem cells.

In group 2, the injected MSCs were neutralized with anti-IL-6 antibodies as described above. Each group consisted of 4 rats. The growth rate of the tumors was evaluated every week and compared with that of the control group without the injection of MSCs (group 3). For β2AR stimulation, the Alzet osmotic minipumps containing isoprotelenol (3 mg/kg/day, all groups) were inserted in the nape of neck at week 4. The growth rate was evaluated as described above. Samples from subcutaneous tumors in each group were fixed in 3.7% formaldehyde neutral-buffered solution and then processed routinely for histology, stained with hematoxylin-eosin and examined under light microscopy.

Statistical analysis. Statistical analyses of cellular proliferation, intracellular cAMP and total HIF-1α expression were performed with Student's t-tests using Stata 8 (StataCorp, College City Station, TX, USA).

Results

Effect of stress-related neurotransmitters/hormones on COS1NR cells and MSCs. Fig. 1 shows the *in vitro* response of COS1NR cells and MSCs treated with epinephrine (0.1-10 μM), norepinephrine (0.1-10 μM), isoproterenol (0.1-10 μM) and cortisol (1-1000 nM). Stimulation with epinephrine, norepinephrine or isoproterenol increased the cellular proliferation in a dose-dependent manner. Treatment with 10 μM epinephrine, norepinephrine or isoproterenol resulted in significantly increased proliferation in MSCs (136±3.9, 152±8.8 and 133±4.9% of the control, respectively, P<0.05) and in COS1NR cells (131±9.1, 150±4.2 and 138±7.7% of the control, respectively, P<0.05, Fig. 1A-C, E-G). These effects were significantly inhibited by 100 μM buthoxamine, a β2AR-selective inhibitor (P<0.05, Fig. 1A-C, E-G). Cortisol did not affect cellular proliferation significantly. These results suggest that β2AR signaling mediates the response to stress-related hormones and neurotransmitters in the OS microenvironment (Fig. 1D and H).

Effect of hypoxic stimulation on COS1NR cells and MSCs. The cellular proliferation of COS1NR cells and MSCs under hypoxic conditions was then examined. Following 48 h of culturing under 2% O₂ conditions, proliferation increased to 118±7.8% in MSCs and 121±6.3% in COS1NR cells, compared with the normoxic control (P<0.05, Fig. 2). Under hypoxic conditions, MSCs treated with 10 μM isoproterenol demonstrated an increase in proliferation to 127±2.1% of the hypoxic control (P<0.05), whereas COS1NR cells demonstrated no significant effect, suggesting that β2AR signaling-induced proliferation is impaired by hypoxic stimulation in COS1NR cells.

β2AR sensitivity and HIF-1α expression. Assays for β2AR sensitivity and HIF-1α expression were performed to clarify the mechanism of impairment. Fig. 3 shows that β2AR sensi-

Table I. Expression of IL-6 and IL-6R in COS1NR cells and MSCs.

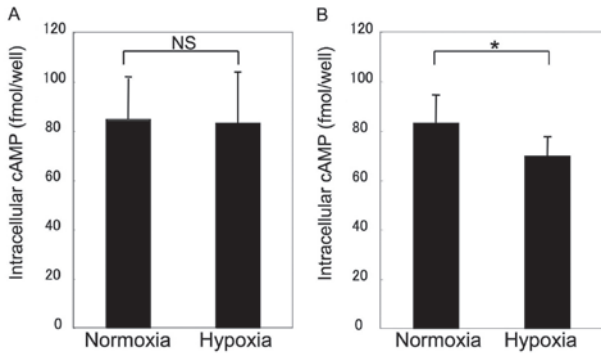| Gene	MSC	COS1NR
IL6	1078.80584	3100.5327
IL6R	400.46093	908.59117

IL, interleukin; MSC, mesenchymal stem cell.

Figure 3. β2AR sensitivity of (A) MSCs and (B) COS1NR cells under normoxic and hypoxic conditions. β2AR sensitivity was not affected in MSCs and was significantly decreased in COS1NR cells. *P<0.05; NS, not significant; β2AR, β2-adrenergic receptor; MSCs, mesenchymal stem cells; cAMP, cyclic AMP.
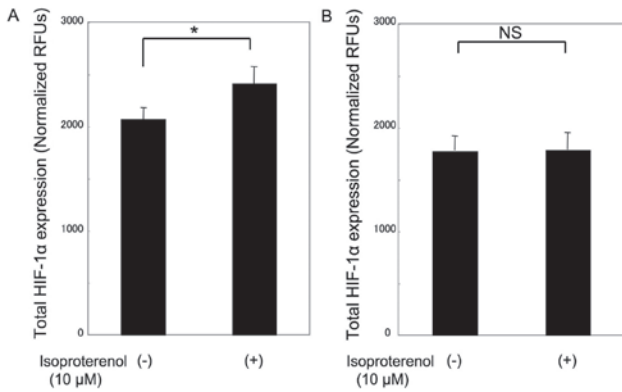

Figure 4. Effects of isoproterenol on total HIF-1α expression in (A) MSCs and (B) COS1NR cells under hypoxic conditions. HIF-1α expression in MSCs was 115% of that in untreated cells but was not significantly increased in COS1NR cells. *P<0.05; NS, not significant. HIF-1α, hypoxia-inducible factor-1α; MSCs, mesenchymal stem cells; RFU, relative fluorescence units.
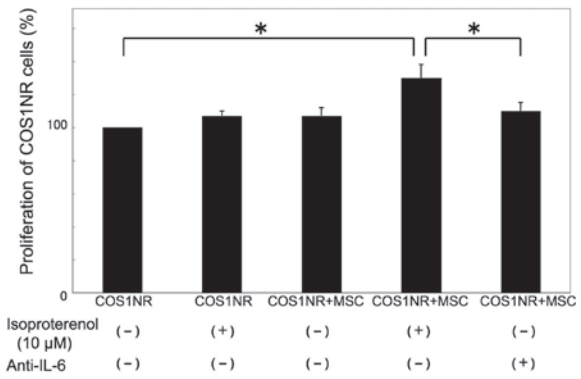

Figure 5. Cellular proliferation of COS1NR cells co-cultured with MSCs under hypoxic conditions. Isoproterenol did not affect the proliferation of COS1NR cells alone. However, when COS1NR cells were co-cultured with MSCs, a significant increase in proliferation occurred, this was inhibited by an anti-IL-6 antibody. *P<0.05. MSCs, mesenchymal stem cells; IL, interleukin.
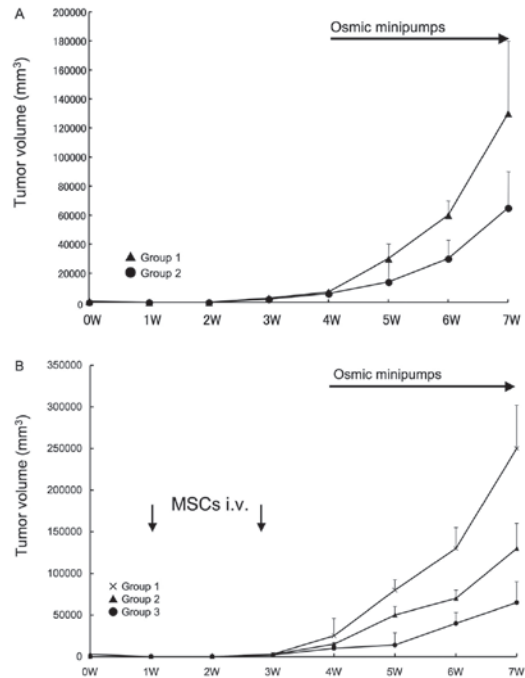

Figure 6. (A) Growth curve of subcutaneous tumors in experiment I. Group 1, COS1NR (5x10^{6} cells); Group 2, COS1NR (5x10^{6} cells) receiving isoproterenol (3 mg/kg/day from week 4). (B) Growth curve of subcutaneous tumors in experiment II. Group 1, COS1NR+MSCs (5x10^{6} cells each); Group 2, COS1NR+MSCs (5x10^{6} cells each, MSCs were neutralized with anti-IL-6 antibodies before injection); Group 3, COS1NR alone (5x10^{6} cells). Each group recieved isoprotelenol (3 mg/kg/day from week 4). MSCs, mesenchymal stem cells; IL, interleukin.

tivity was unchanged in MSCs under hypoxic conditions, whereas β2AR sensitivity demonstrated a significant decrease to 78±11.5% of the control in COS1NR cells (P<0.05). Consistent with this observation, HIF-1α expression in isoproterenol-treated MSCs was 115±8.3% of the expression in the untreated control cells under hypoxic conditions (P<0.05), whereas there was no significant change in isoproterenol-treated COS1NR cells (Fig. 4).

Co-culture of COS1NR cells and MSCs under hypoxic conditions. To examine whether MSCs may affect the *in vitro* response of COS1NR cells, a co-culture experiment was performed. Co-cultured cells were treated with 10 μM isoproterenol under hypoxic conditions for 48 h as described previously. In the presence of co-cultured MSCs, COS1NR cells demonstrated a significant increase in cellular proliferation following isoproterenol treatment (131±8.5%, P<0.05). Based on our gene profiling assay (Table I), IL-6 was then neutralized in co-culture medium. The neutralization of IL-6 significantly inhibited the effect of co-cultured MSCs (P<0.05, Fig. 5).

Effect of intravenous injection of MSCs on tumor growth in syngeneic rats. Subsequently, an *in vivo* tumor formation assay in syngeneic rats was performed. An hypoxic condition was confirmed in the rat model, with a tumoral oxygen pressure of 14±2.5 mmHg. No significant inter-group variation was revealed. Rats receiving isoprotelenol exhibited increased growth of tumors (Fig. 6A), however, the systemic administration of MSCs enhanced the growth of tumors in response to isoproterenol injection (Fig. 6B). Notably, this effect was inhibited by the IL-6 neutralization of MSCs prior to administration.

Discussion

Neurotransmitters are significant in cancer progression (22). Immune cells are involved in the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system, through their expression of receptors for glucocorticoids and catecholamines, respectively (22). In addition, the direct effects of stress-related neurotransmitters have been reported in various types of cancer through β2AR signaling (9-13). However, under hypoxic conditions *in vitro*, β2AR signaling does not appear to simply be a facilitator of cancer expansion. Baloğlu *et al* (14) reported that hypoxia caused the desensitization of the β2AR, owing to an increase in $G_{i/0}$ protein activity, or possibly, heterologous receptor desensitization by the activation of other receptors. The authors suggested that the effects are likely to depend on tissue type as well as the degree and duration of hypoxia (14).

The current study focused on the hypoxia-induced desensitization of β2AR on OS cells. It was observed that the sensitivity of COS1NR cells was significantly decreased under hypoxia, while that of MSCs was not. HIF-1α expression was unchanged in COS1NR cells while expression increased in MSCs. These observations may explain why the cellular proliferation of COS1NR cells was not increased by β2AR stimulation under hypoxia. In terms of cancer growth, this desensitization would be unprofitable for the neoplasms themselves, however, IL-6 stimulation by MSCs may compensate for this. In the present *in vivo* experiment, the systemic administration of MSCs resulted in increased tumor growth, while native MSCs were also recruited to tumors.

The correlation between OS- and bone marrow-derived MSCs remains uncertain. Mohseny *et al* (23) reported that OS originates from MSCs as a consequence of aneuploidization and the genomic loss of cyclin-dependent kinase inhibitor 2, which suggests an obstacle to the clinical use of MSCs. Shimizu *et al* (24) reported that the overexpression of *c-myc* with a loss of Ink4a/Arf transforms bone marrow-derived cells into OS. These two groups focused on early genetic events in the pathogenesis of OS, where MSCs are candidates for tumor origin. Conversely, several groups have emphasized a supportive role of MSCs, a heterogeneous stromal cellular population, as a member of the tumor microenvironment. Johann *et al* (25) reported that tumor stromal cells derived from 11 cases of pediatric malignancies, including two cases of OS, exhibited MSC-like properties and impaired NK cell cytotoxicity. Brune *et al* (26) examined six cases of OS and reported that non-malignant MSCs were isolated from human primary OS samples, stressing the hypothesis that bone-marrow MSCs may be related to tumor stromal MSCs. Previously, Bian *et al* (4) reported that human MSCs promoted OS growth through a positive feedback loop involving IL-6. Although a number of previous researchers have studied the secretion of cytokines, including IL-6, IL-10, CCL5 and vascular endothelial growth factor (2) by MSCs, the importance of IL-6 in the enhancement of MSC proliferation by OS cells has been stressed and vice versa. The gene expression profile analysis of the current study confirmed the expression of IL-6 and IL-6 receptors on MSCs and COS1NR cells and successfully demonstrated that the IL-6 antibody inhibited the effect of MSCs on COS1NR cells in a co-culture experiment and tumor-forming assays in rats.

MSCs may compensate for the desensitization of β2AR signaling on OS cells. The inhibition of the β2AR pathway in MSCs recruited to the tumor may be a therapeutic target in the field of musculoskeletal oncology.

Acknowledgements

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