Original Article

Basic fibroblast growth factor enhances proliferation and hepatocyte growth factor expression of feline mesenchymal stem cells

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A B S T R A C T

Introduction: The objective of this study is to evaluate the effect of basic fibroblast growth factor (bFGF) on the proliferation and secretion activity of feline adipose-derived mesenchymal stem cells (MSC).

Methods: Feline MSC isolated from the subcutaneous adipose tissue of cats were cultured with or without bFGF.

Results: The bFGF addition enhanced the proliferation of feline MSC to a significant great extent compared with that without bFGF, although the cell proliferation tended to increase with the bFGF concentration. In addition, adipogenic and osteogenic staining assay demonstrated that the bFGF addition allowed MSC to maintain the differentiation ability even after the proliferation. Moreover, no change in the surface markers of MSC was observed between the cultures with or without bFGF. A quantitative RT-PCR assay revealed that the HGF and TSG-6 expression significantly increased by the bFGF addition. The highest mRNA expression of MMP-2 was observed for cells cultured in 1000 ng/ml bFGF concentration.

Conclusions: The culture with bFGF is a promising way to enhance the proliferation, and HGF secretion ability of MSC as well as maintain their differentiation ability and immunophenotype nature.

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1. Introduction

Transplantation therapy of mesenchymal stem cells (MSC) is expected to be a new promising treatment for variety of refractory diseases not only in human, but also in animals. MSC have an interest ability to differentiate into multiple cell lineages including osteoblastic, adipogenic, chondrogenic, and neuronal lineages and to secrete a wide variety of cytokines which are beneficial for tissue repair/protection and immunomodulation via the paracrine action [10]. MSC of somatic and non-embryonic stem cells can easily be isolated from various tissues, such as bone marrow, adipose tissue, and umbilical cord, and expanded.

Numerous studies on the clinical trials of MSC transplantation therapy in human have been published [9,11,20,29,34], whereas little has been reported for their applications to companion animals especially cats. Although there are some research reports of MSC transplantation in cats to show the therapeutic improvements, most of trials fail to obtain the beneficial effects. For example, the MSC administration shows a remarkable improvement in the acute and chronic kidney disease/injury models of rats [7,31,38,40,42]. However, little good effects are not observed for the experimental acute kidney injury and naturally occurred chronic kidney disease (CKD) of cats [28,30]. An experimentally induced allergy asthma ameliorates by the MSC therapy in mice [4], but not in cats [37]. It might be possible to speculate that feline MSC used in the studies did not possess an ability effective enough to improve the diseases...
condition due to a reduced therapeutic potential in their preparation.

Comparing with MSC of other animals, there are some difficulties to prepare feline MSC for therapeutic use. Adipose tissue is the most common source to isolate feline MSC due to the easy accessibility. Cats have relatively small body sizes among companion animals, and it is not preferable to collect a large amount of adipose tissue from a cat in clinical practice. Despite absolute necessity to subculture MSC to obtain an enough number of cells, it may be therapeutically advantageous to use MSC at an early passage for therapy. It is well known that an increasing passage number of MSC results in decreasing their abilities to differentiate [19], to secrete cytokines [6] and to improve disease conditions [2]. Especially in case of feline MSC, even relatively small passage number often leads to show a flattened large shape with often caused by cell senescence or potentially infected feline foamy virus, and remarkable reduction in their proliferation and differentiation potentials [1, 18, 44]. These are main reasons practical difficulty to stably prepare an enough number and biological functions of feline MSC for therapeutic use. Therefore, it is of prime importance to develop reproducible culture conditions for production of feline MSC with a high therapeutic ability.

Basic fibroblast growth factor (bFGF) is a potent mitogen that stimulates the proliferation of various cells including MSC [27]. Moreover, it is reported that the addition of bFGF to the culture medium of MSC maintains their multipotent differentiation ability and enhances their secretion of growth factors throughout mitotic divisions in animals [8, 26, 32]. However, the effect of bFGF addition was not examined yet for feline MSC.

This study is undertaken to evaluate the effect of bFGF on the proliferation, morphology, and gene expression of feline MSC. We examine the differentiation ability of MSC after the proliferation culture in the presence or absence of bFGF.

2. Materials and methods

2.1. Isolation and proliferation of feline adipose-derived MSC

Subcutaneous adipose tissues were isolated during an ovariohysterectomy procedure from five client-owned cats of 1–2 years old in clinically healthy conditions. All experiments and procedures were performed in accordance with the guideline for securing the safety of regenerative medicine and cell therapy in dogs and cats by JSVRM (The Japanese Society for Veterinary Regenerative Medicine). Before collecting, all owners signed an informed consent form according to the above guideline. Adipose tissues (approximately 1 g) were digested in collagenase solution (Collagenase D, Sigma-aldrich Co., USA) for 1 h at 37 °C. The digests were centrifuged for 5 min at 450 g on KOKUSAN Desktop centrifuge H-36 (KOKUSAN Co. Ltd., Japan), the supernatants including the fat layer was aspirated to remove. The remaining stromal vascular fraction (SVF) was resuspended in saline, filtered through a 70 μm Nylon filter (Falcon®, Corning Inc., USA), and centrifuged for 5 min at 450 g similarly. The SVF pellets were resuspend in alpha MEM (NACALAI TESQUE, INC., Kyoto, Japan) containing 10% vol% fetal bovine serum (FBS) (HyClone®, GE Healthcare UK Ltd., England) and penicillin-streptomycin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), seeded in a 10-cm tissue culture dish (IWAKI, AGC techno glass) and incubated at 37 °C in 5% CO2 - 95% air atmospheric condition. After 2 days, the medium was changed to remove unattached cells. When reached at 90% confluence, cells were detached with 0.05 wt% trypsin solution (NACALAI TESQUE, INC.), frozen with CELLBANKER 1plus (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) and stored in liquid nitrogen until to use.

2.2. Cell proliferation assay

To prepare the curve of cell proliferation, cryopreserved cells were thawed and seeded in a 24-well plate (IWAKI, AGC techno glass) at 2.5 × 103 cells/cm² in the culture medium without or with 100 ng/ml recombinant human bFGF (ReproCELL Inc., Yokohama, Japan). After 1, 3, 4 and 5 days, the cells were detached with 0.05 wt% trypsin and the cell number was counted. To investigate the concentration dependency of bFGF, cells were cultured without or with 1, 10, 100 or 1000 ng/ml bFGF. After 5 days, the cell number was counted. Assays were performed in triplicate unless otherwise mentioned.

2.3. Assay of adipogenic and osteogenic cell differentiation

Cryopreserved cells were thawed, seeded in a 6-well plate (IWAKI, AGC techno glass) at 2.5 × 103 cells/cm² and cultured without or with 10, 100 or 1000 ng/ml bFGF. After 5 days, cells were detached with 0.05 wt% trypsin, and reseeded in a 6-well plate at 2.5 × 103 cells/cm² followed by culturing in the cell culture medium for 2 days. After the normal cell culture, the cells were cultured in Canine Adipocyte Differentiation Medium (Cell Applications, Inc., USA) or Canine Osteoblast Differentiation Medium (Cell Applications) for 14 days. The culture mediums were changed every 4 days. Adipogenic differentiation was evaluated by staining with the Oil Red O staining solution (Sigma-aldrich Co.,). Osteogenic differentiation was confirmed by the Alizarin Red staining solution (PG research Co., Ltd., Tokyo, Japan).

2.4. Characterization of surface markers of feline MSC cultured with bFGF

To confirm the cell surface markers of feline MSC cultured in bFGF concentrations of 0, 10, 100, and 1000 ng/ml for 5 days, the cells were stained with the following FITC-conjugated: anti-CD14 (clone TUK4, GeneTex Inc., USA), anti-CD29 (clone TS2/16, Thermo Fisher Scientific K.K., Tokyo, Japan), anti-CD44 (clone IM7, BD Biosciences, USA), anti-CD90 (clone 5E10, BD Biosciences), anti-CD105 (clone SN6, Bio-Rad Laboratories, Inc., California, USA) antibodies, and then the cell surface markers were analyzed on a flow cytometer (BD FACSCant II, Becton, Dickinson and Company, USA).

2.5. Gene expression analysis

Cryopreserved cells were thawed, seeded in a 6-well plate and cultured without or with 10, 100 or 1000 ng/ml bFGF. After 2, 3 and 5 days, the culture medium was completely removed and cells were solved in 0.5 ml of TriPure Isolation Reagent (Roche Diagnostics K.K., Tokyo, Japan). The total RNA was extracted according to the instructions of manufacturer. The concentration and purity of RNA were checked by NanoDrop® 2000 Spectrophotometers (Thermo Fisher Scientific). The obtained total RNA was used for cDNA synthesis with Verso cDNA synthesis Kit (Thermo Fisher Scientific) according to the instructions of manufacturer.

Realtime PCR was performed by using THANDERBIRD™ SYBER® qPCR kit (TOYOBO Co., Ltd., Osaka, Japan) with StepOnePlus™ Real Time System (Thermo Fisher Scientific) in which feline specific oligonucleotide primers for hepatocyte growth factor (HGF), tumor necrosis factor-stimulated gene 6 (TSG-6), matrix metalloproteinase-2 (MMP-2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used (Table 1). The reaction condition was as follows: 95 °C for 3 min, followed by 35 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 20 s. Melting curve analysis was conducted after each cycle. Relative quantification of gene
expression was performed by the $2^{-\Delta\Delta Ct}$ method based on Ct values for both target and reference gene (GAPDH) [21].

2.6. Statistical analysis

Quantitative data were expressed as the mean ± standard deviation (SD). Statistical analysis among different groups was performed by unpaired Student’s t test, with $p < 0.05$ considered to be statistically significant.

3. Results

3.1. Effect of bFGF on the proliferation and morphology of feline MSC

Fig. 1 shows the effect of bFGF addition on the proliferation and cell morphology of feline MSC. In the bFGF-free culture, the number of feline MSC gradually increased with time. The cells proliferated for 3 days were of a fibroblast-like shape, but after 5 days culture, became more flattened and larger. On the contrary, in the bFGF concentration of 100 ng/ml, feline MSC proliferated to a significantly great extent with these cultured without bFGF. The shape of cells was either a quite small, bulging, elongated spindle/fibroblast-like or a round-up 3 days later, which is quite different from that observed in feline MSC bFGF-free cultured. The shape change continued up to approximately 4 days in culturing with bFGF, and then many cells tended to recover their original morphology although still kept be of non-flattened shape 5 days after culture.

Fig. 2 shows the effect of bFGF addition on the proliferation of feline MSC. The number of MSC proliferated increased with the concentration of bFGF added and became significantly higher at the concentrations of 100 and 1000 ng/ml than that of MSC cultured without bFGF. The high bFGF concentration seemed to be unstable in the proliferation effect. In the half of culture, the number of cells cultured for 5 days in 1000 ng/ml bFGF was smaller than that in 100 ng/ml. In 1000 ng/ml bFGF, the proliferation of feline MSC was obviously retarded at the initial culture period up to 3 days, but after that remarkably accelerated (data not shown). The negative effect on the initial proliferation of MSC was observed in the high bFGF concentration of 1000 ng/ml. A part of cells died to be in a shrunken floating condition and the cell proliferation was very slow (data not shown).

3.2. Adipogenic and osteogenic staining of feline MSC culture with or without bFGF

Fig. 3 shows Alizarin Red and Oil Red O staining images of feline MSC 14 days after culture in the presence or absence of 100 ng/ml bFGF following osteogenic and adipogenic induction cultures for 5 days. The extent of adipogenic and osteogenic staining was not influenced by the culture, irrespective of the bFGF addition. No difference in the differentiation ability was observed for cells before and after culturing for 5 days.

### Table 1

| Name      | Sequence (5'-3') |
|-----------|------------------|
| HGF       | Forward ATTCCATGGGATTATTGTCCTATTT |
|           | Reverse TTCAACTAAACCATCCATCTACAT |
| TSG-6     | Forward ATATGAAGGTGGCCGTCTCG |
|           | Reverse TTCCAAATCAGCTGGGCCC |
| MMP-2     | Forward TGGCAGCAAGCAGATGGATA |
|           | Reverse GTACTTACGGCGACACTTT |
| GAPDH     | Forward ACGATGACATCAAGAAGGTG |
|           | Reverse CATACAGAAATCAGCTTT |

![Fig. 1. Effect of bFGF addition on the proliferation and cell morphology of feline MSC; A, Feline MSC proliferation in the presence or absence of 100 ng/ml bFGF. *p < 0.05; significant difference against the number of cells cultured without bFGF at the corresponding time. B, Light microscopic images of feline MSC 3 and 5 days after culture with or without bFGF.](image1)

![Fig. 2. Effect of bFGF concentration on the proliferation of feline MSC 5 days after culture in bFGF concentrations of 0, 10, 100, and 1000 ng/ml *p < 0.05; significant difference between the two groups.](image2)

![Fig. 3. Shows Alizarin Red and Oil Red O staining images of feline MSC 14 days after culture in the presence or absence of 100 ng/ml bFGF following osteogenic and adipogenic induction cultures for 5 days. The extent of adipogenic and osteogenic staining was not influenced by the culture, irrespective of the bFGF addition. No difference in the differentiation ability was observed for cells before and after culturing for 5 days.](image3)
**Fig. 3.** Alizarin Red and Oil Red O staining images of feline MSC 14 days after culture in the presence or absence of 100 ng/ml bFGF following osteogenic and adipogenic induction cultures for 5 days.

**Fig. 4.** The result of flow cytometry assay of feline MSC cultured in bFGF concentration of 0, 10, 100, and 1000 ng/ml for 5 days stained with FITC-conjugated: anti-CD14, anti-CD29, anti-CD44, anti-CD90, and anti-CD105 antibodies. The light gray areas represent unstained cells. The percentage indicates gated positive area.
3.3. Determination of the immunophenotype of feline MSC cultured with bFGF

Fig. 4 shows the result of flow cytometry assay of feline MSC cultured in bFGF concentration of 0, 10, 100, and 1000 ng/ml for 5 days. The cells were all positive for CD29, CD44, CD90 and CD105 and negative for CD14 antibodies, which are the typical phenotype of feline adipose-derived MSC. No significant difference in the phenotypes was observed between cells cultured with or without bFGF (Table 2).

3.4. Gene expression of HGF, TSG-6, and MMP-2 for feline MSC cultured with bFGF

Table 2

| bFGF concentration (ng/ml) | Positive markers (% gated [SD]) | Negative marker (% gated [SD]) |
|---------------------------|--------------------------------|--------------------------------|
|                           | CD29                          | CD44                          | CD90                          | CD105                          | CD14                          |
| 0                         | 95.35 ±2.33                   | 100.00 [±0.00]                | 99.50 ±0.24                   | 96.20 ±1.22                    | 1.27 [±0.12]                  |
| 10                        | 98.60 ±0.05                   | 99.83 ±0.05                   | 98.90 ±0.45                   | 95.63 ±1.20                    | 0.73 [±0.33]                  |
| 100                       | 99.50 ±0.22                   | 100.00 [±0.00]                | 99.13 ±0.41                   | 98.17 ±0.61                    | 0.80 [±0.37]                  |
| 1000                      | 99.93 ±0.05                   | 100.00 [±0.00]                | 99.83 ±0.09                   | 98.53 ±0.29                    | 1.63 [±0.39]                  |

The similar effects of bFGF on the morphology, proliferation, differentiation, and cytokine expression of feline MSC to those of other MSC were reported [14,19,35]. This suggests that bFGF can act on feline MSC in the same manner as MSC of other animals. However, there was a slight difference in the dose-dependent response of bFGF. Several ng/ml concentration of bFGF in the culture medium is enough to enhance the cellular functions of human MSC [33], whereas the maximum proliferation of feline MSC was observed in 1000 ng/ml among the bFGF concentrations investigated in this study. There might be two reasons to account for the different bFGF sensitivity between feline and human MSCs. One possibility is that recombinant human bFGF was used to feline cells in this study. Although exact amino acid sequence of feline bFGF protein has not been published yet, the amino acid sequence of feline bFGF predicted from the genomic sequence (377 amino acids, GenBank ID: XP_0210109163.1 XP_023109163.1) has two mismatched amino acid residues by comparison with the human bFGF product (146 amino acids, the bioactive region of bFGF) used in this study. Perhaps, human bFGF might have a lower affinity for the feline bFGF receptors. It is likely that a higher concentration is required to effectively act on feline bFGF receptors in feline MSC. Another possibility is that the instability or consumption of bFGF added to the culture medium. Indeed, at the beginning of culture, 10 ng/ml of bFGF did induce the morphological changes of feline MSC equivalent to that in 100 and 1000 ng/ml of bFGF, suggesting that feline MSC are able to respond to such a low concentration of bFGF. However, the morphological change by the low concentration of bFGF was quite transient and reversed within one or two days (data not shown). On the other hands, the morphological changes were kept for 3–5 days in 100 or 1000 ng/ml of bFGF. However, the initial prolongation was delayed at the bFGF concentration of 1000 ng/ml. The inhibitory effect of high bFGF concentrations on the MSC proliferation are also reported in other animals [14,41]. After the delay, MSC could well proliferate throughout culturing period similarly to those cultured in 100 ng/ml of bFGF. The similar delayed, and prolonged strong response in 1000 ng/ml bFGF were also observed in the expression of HGF mRNA. Probably, several ng/ml bFGF is enough to stimulate cell proliferation and the HGF expression of feline MSC, but the effect would not be maintained due to instability of bFGF protein and/or its consumption by cells during cell culturing at 37 °C. In addition, a high bFGF concentration of 1000 ng/ml seemed to initially act in an inhibitory or toxic manner to feline MSC by unknown mechanisms, and then this inhibitory effect may be canceled only when concentration of “active” bFGF is decreased by the instability and/or consumption. A favorable effect

4. Discussion

To obtain good outcomes in MSC therapy, it is critical to prepare MSC with a high therapeutic potential for transplantation. A methodology to accelerate the proliferation rate of MSC is absolutely required to produce an enough number of MSC for cell transplantation. However, the cells expansion by the in vitro culturing often causes a gradual loss of original MSC properties as observed by a changed cell morphology into senescent cells, reduced cell proliferation, impaired differentiation capacity, and altered gene expression [39]. It is well known that feline MSC are susceptible to prominent deterioration in cell expansion compared with the MSC of other animals. For example, human MSC can be expanded by 10 passages or more [5,43], and the MSC expanded for several passages are mostly used for human clinical trials [13]. However, feline MSC easily lose their original properties even after only a few passages [18,44]. For this reason, feline MSC of passage 1 (P1) generation are mainly used in MSC therapy for cats. In this connection, we investigated the effect of bFGF addition on the proliferation of feline MSC and the change in the biological and morphology with culture.

Table 2 Percentage of surface markers of feline MSC cultured with bFGF expressions.
of cells cultured in 1000 ng/ml bFGF in all four preparations of feline MSCs from clients-owned healthy young cats. However, bFGF is a costy protein. Although an optimization of bFGF concentration to be added should be investigated, 100 ng/ml bFGF is likely to be the most advantageous in term of the proliferation and biological abilities maintenance.

It is known that cultured MSC show a spindle or a fibroblast-like morphology [16]. Similarly to this, feline adipose tissue-derived MSC primarily cultured in the conventional medium containing 10 wt% FBS exhibited a small, bulging, elongated spindle or fibroblast cell-like morphology. However, when subcultured, the cells often altered their morphology to be a flattened polygonal shape with an expanded cytoplasm, and their proliferation slowed down. After further subcultured, the cells heavily extended their cytoplasm to be of a flattened giant cell. While they hardly proliferated after 2 to 4 passages on our culture system although it depended on the donor type of adipose tissues. It is demonstrated that the flattened morphology of MSC is caused by cell senescence [39]. On the contrary, when subcultured in the culture medium containing bFGF, feline MSC continued to show a bulging, spindle, triangle or star shape and much more compact in size. This will concomitantly associated with their active proliferation. In addition, it is also evident that most cells showed spherically raised shapes in the process of cell division. Feline MSC cultured with 10 ng/ml of bFGF actively proliferated only when their morphology was changed to be of small bulging shape. Thus, it is possible that morphological change of feline MSC in response to bFGF is closely related to their

Fig. 5. Effect of bFGF addition on the mRNA expression of HGF, TSG-6, and MMP-2 for feline MSC 2 ( ), 3 ( ), and 5 days ( ) after incubation in bFGF concentrations of 0, 10, 100, and 1000 ng/ml: (A) HGF, (B) TSG-6, and (C) MMP-2. GAPDH was used as a reference gene and various conditions was compared with 0 ng/ml bFGF at day 2. *p < 0.05; significant difference from the group of bFGF concentration 0 ng/ml at the same day.

Fig. 6. The media of mRNA expression level of HGF for feline MSC prepared from 4 different cats ( ), 3 ( ), and 5 days ( ) after incubation in bFGF concentrations of 0, 10, 100, and 1000 ng/ml. GAPDH was used as a reference gene and various conditions was compared with 0 ng/ml bFGF at day 2. *p < 0.05; significant difference between the group of bFGF concentration 0 ng/ml at the same day.
enhanced proliferation. Upon reaching to a confluence, the number of cells proliferated in the bFGF-supplemented culture by comparison was at least twice as long as that of the conventional culture condition. This may be explained in terms of their non-flattened compact cell shape. We can say with certainly that the compact cell shape is one of the possible reasons to achieve a larger production of feline MSC.

HGF is one of the key cytokines for MSC that contribute to recovery from various diseases via its mitogenic and anti-inflammatory effects. HGF exerts various physiological functions and contributes to recovery from various disease conditions [15,22]. Since HGF is a growth factor that promotes the proliferation of various types of cells [24], it is highly conceivable that the administration of the HGF expression-upregulated feline MSC generally contribute to the repair of injured tissues more efficiently than the conventional feline MSC. HGF also exerts an anti-inflammatory effect [25]. It is apparent from Figs. 5 and 6 that feline MSC enhanced the HGF expression and provide better therapeutic effects in MSC therapy to cats than the conventional feline MSC do.

Cats sometimes develop various refractory inflammatory diseases, such as stomatitis, allergic asthma, CKD, cystitis, chronic pancreatitis, inflammatory bowel disease, and dermatitis, etc. However, the administration of feline MSC prepared with the conventional method failed to obtain clinically observable therapeutic outcomes in allergic asthma [37] and CKD [28,30] in cats, although rat or murine MSC could show remarkable therapeutic improvement in rat or murine models of these diseases [4,7,31,38,40,42]. Especially, the CKD is a disease which is often encountered is not clinically treated yet. In the rat model of CKD, MSC exert anti-inflammatory and anti-fibrotic effects through HGF secreted and TSG-6, respectively [42]. Our results showed that bFGF addition enhanced the expression of TSG-6 for feline MSC similarly to human.

Moreover, MMP-2 is one of the MMP families that are known as the main mediator of extracellular matrix (ECM) remodeling, and it is repeated to suppress renal fibrosis [12,17]. Thus, it is expected that bFGF induced the up-regulated expression of HGF, TSG-6, and MMP-2 for feline MSC would more efficiently improve refractory inflammatory diseases including CKD in cats than the conventional feline MSC.

After cultures with bFGF, feline MSC still retained their ability to differentiate into osteoblastic and adipogenic linages. In addition, the cells presented a typical feature of MSC immunophenotype indicated by the CD14, CD29, CD44, CD90, and CD105. These findings suggest that feline MSC cultured with bFGF surely retain the interest properties of original MSC when after their proliferation to expand the large number of cells.

For a safety reason after transplantation to cats, any effects of residual human bFGF in the feline MSC preparation should be investigated. The bFGF product of a cell culture grade, used to iPS cell long-term passage culture, and cryopreservation on tonsil-derived mesenchymal stem cells. Cell Physiol Biochem 2015;36:85–99.

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