Rice bran extract affects differentiation of mesenchymal stem cells potency into osteogenic cells

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Abstract As rice bran contains various nutrients and other proteins of which a part has biological effects on animal cells, we tested the effect of rice bran extract on rat mesenchymal stem cells (rMSCs) obtained from bone marrow. These rMSCs are pluripotent and can be readily induced to differentiate into a number of cell types, including bone and cartilage. rMSC was aggregated by culturing in serum-free condition with rice bran extract, but was not aggregated by culturing in serum-free condition or in serum-containing medium. Moreover, the longer aggregates of rMSCs

were cultured in serum-free condition with rice bran extract, the more the aggregates grew. After two passages in serum-free conditions, rMSCs lost their potency for differentiation into osteogenic cells; however, the addition of rice bran extract to serum-free medium successfully prevented the loss of this ability for differentiation. In addition, MSC makers *CD105* and *CD166* gene expression in serum-free condition with rice bran extract corresponded to these expressions in serum-containing medium. This result suggests that certain factors in rice bran could be bioactive and contribute toward retaining the ability of MSCs to differentiate into osteogenic cells after passaging.

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Introduction

Mesenchymal stem cells (MSCs), initially isolated from bone marrow-derived cells (Friedenstein et al. 1966, 1970), are multipotent stem cells that can be obtained from many adult and fetal tissues, including adipose tissue and umbilical cord blood (Lee et al. 2004a, b). MSCs can differentiate into multiple lineages, including osteoblasts, adipocytes, chondrocytes and neurocytes (De Bari et al. 2001; Barry et al. 2001; Deng et al. 2001). For this

reason, MSCs have been considered a useful source of stem cells for regenerative medicine and have been used in clinical applications, such as bone and chondral regeneration (Morishita et al. 2006; Wakitani et al. 2002). Before their use in cell-based therapies, the population of MSCs needs to be expanded sufficiently *in vitro*. The conventional culture medium for expanding MSCs is composed of a basal nutrient medium supplemented with fetal bovine serum (FBS) (Lennon et al. 1996). However, in clinical applications, as the addition of FBS to cultures causes concerns about the risk of zoonotic diseases, various serum-free media have been developed. These specialized media contain several growth factors, such as basic fibroblast growth factor-2 (bFGF-2), platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor- β (TGF- β) (Mimura et al. 2011; Chase et al. 2010). In our experience, we found that these media are not necessarily optimal for all MSC types, as the requirements of MSCs isolated from different tissues can be diverse.

Rice bran extracts (RBEs) or fermented RBEs contain a number of different components, including saccharides, proteins, polyphenols and oils. Recent studies have reported that these extracts exert a number of biological effects on animal cells, including the following: enhancement of human natural killer cell activity (Ghoneum 1998); anti-photoaging effects in human fibroblasts (Seo et al. 2010); and anti-inflammatory effects in mice (Akihisa et al. 2000). However, there is no report concerning the biological effects of RBE on MSCs. Thus, the purpose of this study was to investigate whether RBE has any bioactivity on rat MSCs as research model alternative to human MSCs, because they are easily isolated without informed consent in comparison with human MSCs.

Materials and methods

Rice bran extract

RBE, containing 55 % protein, was prepared as follows: RBE was extracted from rice bran, derived from Japonica rice, in an alkaline solution, precipitated with acid, and subsequently freeze-dried. RBE was used at a concentration of 100 $\mu\text{g/ml}$.

MSC preparation and culture

Bone marrow cells were isolated from the limbs of Wistar rats (3 weeks old) by flushing femurs with PBS, using a 21-gauge needle. The isolated bone marrow cells were mixed with α -MEM (Wako, Osaka, Japan), supplemented with 15 % fetal bovine serum (FBS) (Biowest, Nuaillé, France) and 1 % penicillin–streptomycin (Wako), and seeded onto 6-cm tissue culture dishes (Sumitomo Bakelite, Tokyo, Japan). After the cells were cultured for 3 days, the used medium was exchanged for fresh medium and the cells were cultured for a further 3 days. Following this culture period, the surviving cells were MSCs. When they reached 80–90 % confluence, they were subcultured in α -MEM with 15 % FBS at a density of 3.0×10^5 cells/dish.

Rat MSCs (rMSCs) from first to third passage were used for all experiments; they were removed from the culture dish with 0.1 % trypsin–EDTA, suspended in STK-1 serum-free medium (DS Pharma Biomedical Co., Ltd, Osaka, Japan) or α -MEM with 15 % FBS as a positive control, with or without RBE, and subsequently seeded in a 96 well tissue culture plate (Sumitomo Bakelite) at 4.0×10^3 cells/well or 6-cm tissue culture dishes at 3.0×10^5 cells/dish and cultured for 3 days.

Induction of osteogenic differentiation

After culture for 3 days in α -MEM or STK1 with or without RBE, the medium was replaced with osteogenic medium; α -MEM supplemented with 10 % FBS, 10 mM β -glycerol phosphate (Merck KGaA, Darmstadt, Germany), 0.05 mM L-ascorbic acid 2-phosphate (Sigma, Gillingham, United Kingdom), 10 nM dexamethasone (Sigma), 1 % penicillin–streptomycin solution (Wako), and cultured for 4 weeks. Osteogenic differentiation was detected with Arizalin red S (Sigma) (Gregory et al. 2004) and the threshold area (%) containing osteogenic cells was analyzed with Image J software and the level was estimated. All error bars represent standard deviation (SD; $n = 8$).

Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR)

Total RNA was isolated and quantified by Pure Link^{TM} RNA Mini Kit (Life technologies, Carlsbad, CA, USA) and then, from 2 μg of each RNA sample, cDNA was

synthesized with superscript^{TM} (Life technologies). Real time PCR was performed using the following primers and probes: CD44 Rn 00681157_m1, CD105 Rn0143378772_m1, CD166 Rn 00582112_m1, GAPDH Rn 99999916_sl (Applied Biosystems, Calrsbad, CA, USA) (for more details, see supplementary table 1). These cDNAs were analyzed by LightCycler R480 (Roche, Basel, Switzerland) and the results were normalized with respect to GAPDH. Relative mRNA quantify was calculated using the comparative $\Delta\Delta CT$ method (Livak and Schmittgen 2001).

Results

Treatment of MSCs with rice bran extract:
inducing aggregation of MSCs

In our recent study, 100 $\mu\text{g}/\text{ml}$ of rice bran extract (RBE) stimulated the proliferation of several cell lines, including hybridoma, hepatoma, CHO and HeLa cells. Thus, in this study, rMSCs were seeded into a 96-well plate at 4.0×10^3 cells/well using serum-free medium STK1 or α -MEM supplemented with 15 % FBS (serum-containing medium) as a positive control. The cells were then cultured in the absence or presence of 100 $\mu\text{g}/\text{ml}$ RBE for 3 days. Cells cultured in STK1 with RBE did not adhere to the bottom of the well but aggregated, while cells in STK1 without RBE adhered to the plate, along with the cells in serum-containing medium (Fig. 1). Furthermore, cells did not aggregate at lower concentration than 100 $\mu\text{g}/\text{ml}$ RBE. The longer rMSCs were cultured in STK1 with RBE, the more the cell aggregates grew. In addition, the aggregates adhered and migrated from the cell mass after replacing in serum-containing medium. Since various studies imply that aggregation contributes to cellular activity, we focused on aggregation of rMSCs and decided using 100 $\mu\text{g}/\text{ml}$ RBE for this study.

Bone differentiation potential of MSCs maintained in medium supplemented with rice bran extract

Several studies have reported that three-dimensional spheroids of MSCs have enhanced biological functions, including the ability for osteogenic or adipogenic differentiation, or anti-inflammatory properties (Wang et al. 2009; Miyagawa et al. 2011; Bartosh et al. 2010). Therefore, we hypothesized that the aggregation of

Fig. 1 Effect of RBE on rMSC morphology. rMSCs (passage = 3) were cultured for 3 days in α -MEM supplemented with 15 % FBS or STK1, with or without RBE. The scale bar represents 90 μm

rMSCs in STK1 with RBE would increase their potential for osteogenic differentiation. rMSCs, which were expanded in serum-containing medium for one, two or three passages, were seeded in 96-well plates using STK1 or serum-containing medium, with or without RBE, and cultured for 3 days. Following this, the media were replaced with osteogenic medium and cultured for 4 weeks. The cells were stained by Arizalin red S and the threshold area (%) containing osteogenic cells was analyzed with Image J software. rMSCs expanded for only one passage successfully differentiated into osteogenic cells, while rMSCs expanded for two or three passages partially lost their potency (Fig. 2a). After expanding for two passages, cells cultured in STK1 without RBE did not differentiate, but in the presence of RBE, they maintained their potency and differentiated into osteogenic cells.

The percentage area of osteogenic differentiation (threshold area) was calculated with Image J software. Figure 2b shows that the osteogenic differentiation of passage two rMSCs cultured in STK1 with RBE was significantly increased compared to rMSCs cultured in STK1 without RBE. Passage three rMSCs cultured in STK1 with RBE were also superior to those in STK1 without RBE. In the serum-containing cultures, treatment with RBE did not improve the potency of the cells for osteogenic differentiation potency at any passage.

These results suggest that rice bran may contain certain bioactive factors that can contribute to retaining the ability of MSCs to differentiate into osteogenic cells.

Fig. 2 Effect of RBE on rMSC differentiation **a** after rMSCs (passage = 1–3) were cultured for 3 days in serum-free STK1 medium or α -MEM supplemented with 15 % FBS with or without RBE. Cells were subsequently cultured in osteogenic medium for 4 weeks. Cells were then stained with *Alizarin Red S*. **b** Threshold area (%) of cells stained with *Alizarin Red S* was analyzed with Image J software. All error bars represent SD (N = 8). Asterisk indicates a statistically significant difference ($P < 0.05$). (Color figure online)

Surface markers of MSC

CD44, CD105 and CD166 are among the well-defined markers for MSCs (Barzilay et al. 2009; Harting et al. 2008; Bruder et al. 1998). Therefore, we investigated

whether the gene expression was affected during the treatment with RBE. rMSCs were seeded into 6-cm tissue culture dishes at 3.0×10^5 cells/dish using STK1 medium with or without RBE or serum-containing medium as positive control, and cultured

Fig. 3 Effect of RBE on expression of MSC markers after rMSCs were cultured for 3 days. Expression levels of MSC markers were determined by real-time RT-PCR and normalized to the abundance of *GAPDH*. The culture conditions were: α -MEM containing 15 % FBS as positive control, STK1 serum-free medium, and STK1 supplemented with 100 μ g/ml RBE

for 3 days. Gene expression in these cells was analyzed by real-time PCR. Figure 3 shows that expression levels of *CD105* and *CD166* in the cells treated with RBE were quite similar to those in serum-containing medium. Among the three markers, expression of the two markers were similar, implying that RBE might contain certain factors supporting serum-free culture of rMSCs.

Discussion

rMSCs cultured in serum-free STK1 medium with RBE for 3 days aggregated and formed spheroid structures, whose ability for osteogenic differentiation was superior to cells cultured in STK1 without RBE. Recent studies reported that MSCs readily aggregate and formed spheroids by culturing in particular substrates, such as micro-patterned substrates (Wang et al. 2009), hydrophobic nano-pillars (Brammer et al. 2011) and chitosan membranes (Hsu et al. 2012), or by specialized methods, such as the hanging drop method

(Bartosh et al. 2010). Additionally, a recent study reported that N-cadherin is involved in the aggregation of MSCs (Hsu et al. 2012), implying that RBE might induce aggregation of MSCs through expression of N-cadherin. The expression level of N-cadherin of the cells treated with RBE will be examined to check this possibility.

Aggregation formed through such adhesion molecules might contribute to maintaining differentiation ability via some mechanisms. Aggregation enhanced by cell-cell interaction would form a niche, and this might maintain the ability of MSCs as well as of other stem cells for differentiation. Hypoxia occurred by aggregation might also contribute; in the center of aggregates, cells would be under low oxygen condition and it is reported that the potentiality of MSCs was maintained under hypoxia culture (Jin et al. 2010).

MSCs express several characteristic surface markers including CD44, CD105, and CD166. We investigated the gene expression of these markers. Among them, the expression of CD105 and CD166 in the cells treated with RBE corresponded to that in serum-

containing medium, thus through the expression of these MSC markers, RBE could contribute in maintaining the differentiation potential of MSCs in serum-free culture. In addition, some studies reported that CD105 and CD166 contribute to differentiation but not CD44 (Duff et al. 2003; Arai et al. 2002), suggesting that the relevant expression is related to the maintaining of differentiation potential of MSCs while higher expression of CD105 and CD166 indicate differentiation.

In this study, we found that the differentiation ability of aggregated rMSCs treated with RBE is increased compared with adherent rMSCs. Furthermore, the aggregation of rMSCs with RBE might prevent a loss in their differentiation potential. Thus, RBE could be used as a novel supplement for maintaining the differentiation ability of MSCs in serum-free culture conditions.

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