Enhancement of osteoblastic differentiation of mesenchymal stem cells cultured by selective combination of bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor-2 (FGF-2)

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Introduction: It is well known that bone marrow contains mesenchymal stem cells (MSCs), which can show osteoblastic differentiation when cultured in osteogenic medium containing ascorbic acid, b-glycerophosphate, and dexamethasone. The differentiation resulted in appearance of osteoblasts together with bone matrix formation and thus, in vitro cultured bone (osteoblasts/bone matrix) could be fabricated by MSCs culture. The cultured bone has already been used in clinical cases having orthopedic problems. To improve the therapeutic effect of the cultured bone, we investigated the culture condition to show extensive osteoblastic differentiation.

Materials and Methods: 1. Preparation and culture of rat MSCs

Rat MSCs were isolated and primarily cultured as previously described methods. Bone marrow cells were obtained from the bone shaft of femer of 7-week-old rats. After confluence in the primary culture, MSCs were resuspended in basal medium containing 0.28 mM ascorbic acid-2-phosphate, 0.4 mM L-proline, 10 uM dexamethasone, and 10 mM b-glycerophosphate disodium salt and then seeded to 12-well tissue culture plates at a cell density of 2.0×104 cells/cm2 for culture. For fluorescence analyses of mineralization shown in below, 1ug/mL of calcein was added to the culture wells. The growth factors were added as follows: 10 ng/mL recombinant human fibroblast growth factor-2 (FGF-2) (Kaken, Tokyo, Japan), 100 ng/mL recombinant human bone morphogenetic protein-2 (BMP-2) (Wako, Osaka, Japan). The supplementation scheme of FGF-2 and BMP-2 is depicted in Table 1.

2. Measurements of ALP activities and DNA contents

The quantification of the DNA contents was performed by using Hoechst 33258. ALP activity was determined as described by Reddi and Sullivan with minor modifications. The ALP activity represented by amount of released p-nitro phenol (PNP) was normalized to DNA contents. Quantification of osteocalcin was performed using EIA plate immobilized with anti-rat osteocalcin monoclonal antibody to measure the concentration of osteocalcin with intact rat osteocalcin EIA kit (No. BT-490, Biomedical Technologies Inc., MA, USA).

3. Quantitative fluorescence analysis of calcein incorporated into bone matrices

In this study, we used this method for measuring mineralization levels of MSCs. The fluorescence of the incorporated calcein was visualized and quantified by using an image analyzer (Typhoon 8600, Molecular Dynamics Inc., CA, USA). After the assay, PBS (-) in culture wells was renewed to culture media containing 1 ug/mL calcein and the cells were preceded to culture.

Results: DNA contents of MSCs after 12 days of the culture are shown in Fig. 1a. Total cellular proliferation rate of the MSCs was not affected by the supplementation of BMP-2 or FGF-2. Osteocalcin contents of MSCs after 12 days of culture are shown in Fig.1. The content in group D was the highest and 7-folds higher than that in control group A. The real-time PCR results showed in fig.3. The osteocalcin mRNA levels in all groups at 6 days of the culture were negligible but noticeable at 12 days, especially the level in group D was the highest among all groups.

As seen in insert figure of Fig. 2a, group D culture at 12 days showed the highest intensity among the all groups. As shown in line graphs of Fig. 2b, obvious intensities were detected in both group C and D at 10 days of the culture and the intensities increased as time passed. At 12 days of the culture, considerable fluorescence were detected in all groups and the intensity of the group D was the highest and 4 times more than control group A. Therefore, the medium with initial 6 days FGF-2 followed by 6 days BMP-2 supplementation, enhanced the basal osteoblastic activity of the MSCs cultured in the presence of ascorbic acid, b-glycerophosphate and Dex.

Discussion: The supplementation of FGF-2 followed by BMP-2 resulted in prompt and more extensive osteoblastic activity together with abundant bone matrix formation. Based on the results, human MSCs' culture with these cytokine supplementations may increase the areas or volume of the cultured bone on the materials and show great therapeutic effects in the field of bone tissue engineering.

	1d-3d	4-6d	7-9d	10-12d
Group A	(-)	(-)	(-)	(-)
Group B	BMP	BMP	BMP	BMP
Group C	FGF	BMP	BMP	BMP
Group D	FGF	FGF	BMP	BMP
Group E	FGF	FGF	FGF	BMP
Group F	FGF	FGF	FGF	FGF

Table 1. Supplementation patterns of BMP-2 and FGF-2 during 12 days of rat MSCs culture.

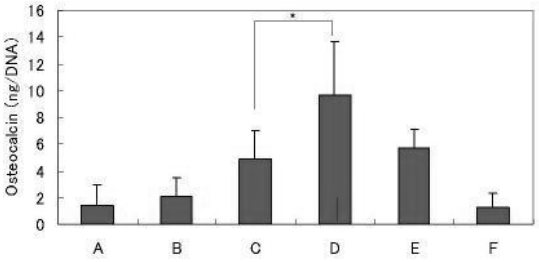

Fig.1 Osteocalcin contents after 12 days of rat MSCs culture.

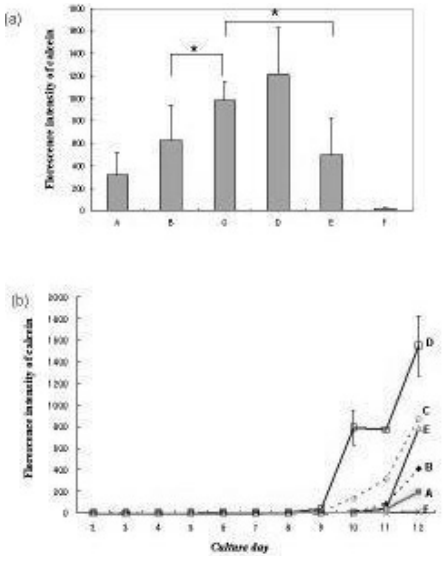

Fig.2 Time-course determination and 12 days of fluorescence intensity of calcein incorporated to bone matrices during rat MSCs culture.