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Simple evaluation method for osteoinductive capacity of cells or scaffolds using ceramic cubes

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ABSTRACT

Mesenchymal stem cells are good candidates for the clinical application of bone repair because of their osteogenic differentiation potential, but *in vivo* osteoinduction potential should be verified for culture expanded cells before clinical application. This study analyzed *in vivo* bone formation by MSCs quantitatively after implantation of MSCs planted porous biphasic ceramic cubes into athymic mice. MSCs were divided into osteogenic differentiation-induced and normal groups and also tested *in vitro* to evaluate the degree of differentiation into osteoblasts. The osteogenic induced group showed higher alkaline phosphatase and calcium level *in vitro* and corresponding higher level of bone formation *in vivo* compared to control group. Whereas there was no bone formation observed in fibroblast-implanted negative control group. In critical sized bone defect models, commonly used for evaluation of bone regeneration ability, it is difficult to distinguish between osteoinduction and osteoconduction, and quantitative analysis is not simple. However, this method for evaluating osteoinduction is both accurate and simple. In conclusion, the analysis of *in vivo* bone formation using porous ceramic cubes is a powerful and simple method for evaluating the osteoinduction ability of target cells and, furthermore, can be applied for evaluation of scaffolds for their osteoinductive properties.

1. Introduction

Mesenchymal stem cells (MSCs) in bone marrow support the micro-environment for hematopoiesis but also maintain diverse differentiation potential (Devine and Hoffman, 2000; Majumdar et al., 1998). Culture expanded MSCs differentiate into mesodermal lineages including osteoblasts, adipocytes, chondrocytes and myocytes *in vivo* after transplantation (Liechty et al., 2000; Mackenzie and Flake, 2001; Shake et al., 2002) and also differentiate into such lineages *in vitro* with proper supplements such as dexamethasone (Beresford et al., 1992; Gimble et al., 2008).

The diverse flexibility of MSC differentiation potential has driven extensive studies for the recovery of damaged tissues or organs in the field of regenerative medicine and produced many fruitful results, especially in skeletal system (Ankrum and Karp, 2010; Veronesi et al., 2012). Autograft is widely used for reconstruction of damaged bone tissue, but limitations in graft amounts make it difficult or impossible to repair large fracture. Culture expanded MSCs may be used to cover this limitation (Pourebrahim et al., 2013) but evaluation of cell characteristics and osteogenic potential are needed after long-term culture or large scale expansion.

Alkaline phosphatase activity and osteoblast marker genes, such as RUNX2 and osteopontin are widely used for evaluation of osteoblastic differentiation of MSCs *in vitro* (Chen et al., 1997; Komori, 2010) but genuine, histologically identifiable bone formation is impossible to produce *in vitro*, so the expression of such markers *in vitro* cannot guarantee bone formation *in vivo*.

Transplanted MSCs can be differentiated into osteoblasts and produce bone in isogenic tissue, but this process influenced by the complex *in vivo* micro-environment as well as by target cell characteristics. Qualitative analysis of ectopic bone formation is essential for meaningful evaluation of osteoinduction ability of transplanted MSCs. To more accurately evaluate the osteoinductive ability of culture expanded MSCs, with or without osteo-induction, we quantified new bone formation by MSCs after implantation of MSCs combined with biphasic ceramic cubes into athymic mice.

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2. Materials and methods

2.1. Isolation and culture of mesenchymal stem cells

Human bone marrow specimens were obtained from the iliac crest of patients undergoing non-emergency orthopedic surgery through an Internal Review Board approved protocol at Yeungnam University Hospital. An equal volume of Dulbecco's modified Eagle medium with low glucose (DMEM-LG; Sigma, St. Louis, MO, USA) was mixed with the specimen and centrifuged at $450 \times g$ for 10 min The pellets were suspended in DMEM-LG and fractionated on a density gradient centrifuge with density 1.08 Percoll (Sigma) solution at $480 \times g$ for 15 min. The low density, upper nucleated cell layer was collected and washed with 10% FBS (FBS; Gibco-BRL, Rockville, MD, USA) supplemented DMEM then plated at 1.8×10^5 cells per cubic centimeter. When cultures became confluent, cells were trypsinized and replated at 4×10^3 cells per cubic centimeter for continued passage. Second passage cells were used for experimentation.

Cells were cultivated in DMEM-LG with 10% FBS only for Fibroblast (Fib) and normal MSCs cultures (Con): 100 nm dexamethasone (Sigma), 50 μm ascorbic acid phosphate (Wako Chemical, Osaka, Japan) and 10 mm beta-glycerophosphate (Sigma) were supplemented for osteogenic induction culture (Osteo).

2.2. Flow cytometry

Cultured MSCs were analyzed for stem cell markers. The cells were trypsinized and incubated in primary antibodies at room temperature for 30 min Mouse anti-human CD105 (Abcam, Cambridge, UK), mouse anti-human CD90 (Abcam), mouse anti-human CD73 (Abcam), mouse anti-human CD45 (Abcam) were used for primary antibodies. After incubation with secondary FITC-labeled anti-mouse IgG (Sigma) in the dark for 30 min, cells were analyzed using FACScane fluorescence-activated cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with Cell Quest software (Becton Dickinson).

2.3. Scanning electron microscopy

Samples were fixed in 2.5% glutaraldehyde (Polysciences, Niles, IL, USA), followed by further fixation in 1% osmium tetroxide (Polysciences) in 0.1 M PBS and dehydration in graded series of ethanol. After dehydration, samples were critical-point dried, mounted on aluminum stubs with conductive silver paint, and then sputtered with Pt–Pd in ion sputter (Hitachi, Naka, Japan). The samples were observed using a scanning electron microscope (Hitachi) at 25 keV.

2.4. In vivo analysis of bone formation

Biphasic porous tricalcium phosphate-hydroxyapitite (60:40) ceramic with mean pore size of 200 μm donated from Zimmer (Warsaw, IN, USA) were cut into $3 \times 3 \times 3$ mm and sonicated in distilled water to remove dust particles. Autoclaved sterile ceramic cubes were immersed in 100 μg/ml human fibronectin (Sigma) and dried overnight in a laminar flow hood. Human dermal fibroblasts and MSCs were trypsinized, rinsed, counted and suspended in DMEM-LG without serum at 5×10^6 cells/ml. Fibronectin-coated ceramic cubes were added to a tube containing cell suspensions and incubated at 37 °C for 2 h with mixing every 20 min, and then implanted into mice for standard MSC bone assay. For osteogenic inducing studies, cell-loaded cubes were incubated for 3 weeks in osteogenic induction or normal culture to examine the bone formation ability of different cell groups. Cubes were transferred to a 24-well culture dish and switched to each media. After 3 week's culture, cubes ($n = 8$ in each groups) were implanted subcutaneously on dorsum of the mice. After 6 weeks the ceramic cubes were harvested (preliminary test showed increase of bone formation reached plateau after 6 weeks), fixed for 24 h with 10% neutral buffered formalin phosphate, washed overnight in

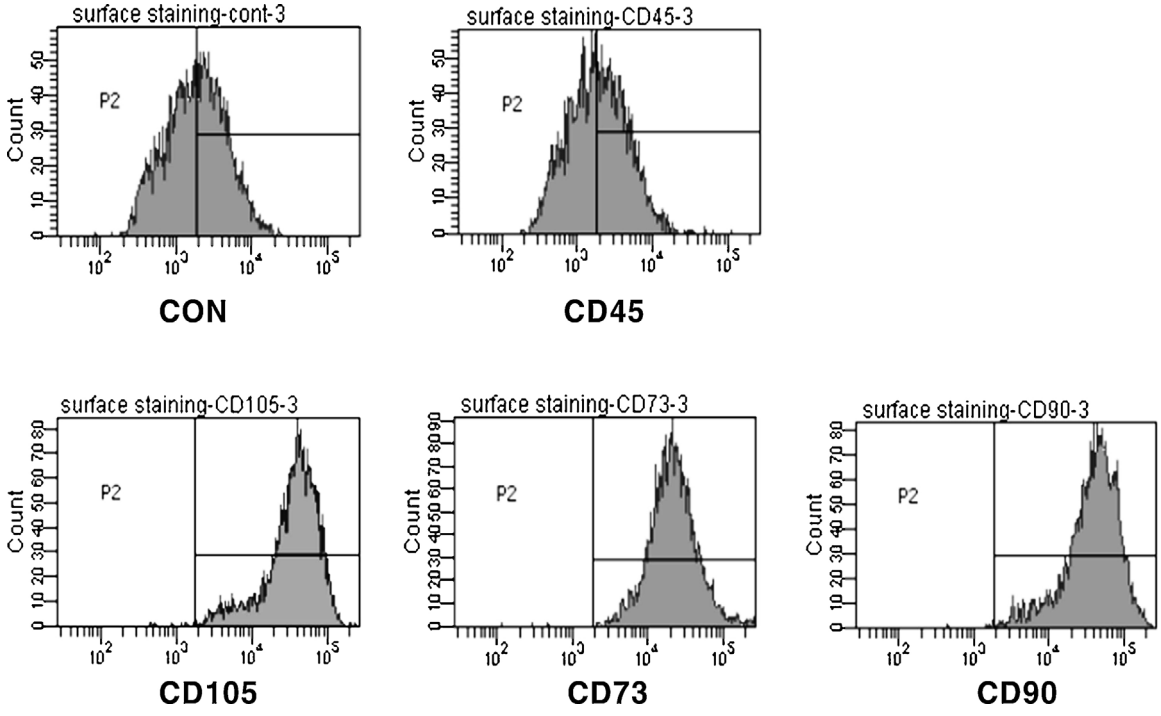

Fig. 1. Flowcytometric analysis of surface CD markers expression on cultured MSCs. Mesenchymal stem cell markers, CD90, CD73, and CD105, were highly positive while the hematopoietic cell marker, CD 45, was negative.