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Original

Origin of Osteoblasts Involved in the Mechanism of Ectopic Bone Formation Induced by KUSA/A1 Cells with Honeycomb Carrier

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Abstract: The basic principle of bone tissue engineering is to use seeded stem cells in porous scaffold. Stem cells can proliferate and differentiate into various types of mature cells. A kind of stem cell called KUSA/A1 is a marrow stromal cell, capable of differentiating into three mesenchymal phenotypes: osteocyte, adipocyte, and myocyte by treating with 5-azacytidine in cell culture. Moreover, it has been reported that the mechanism of bone induction by KUSA/A1 cells is similar to intramembranous ossification.

In order to clarify the origin of osteoblasts implicated in new bone formation, KUSA/A1 cells alone and combined with Honeycomb carrier were implanted in Transgenic Green Fluorescent Protein mice (GFP) mice. The presence of GFP positive host cells with osteoblastic morphology as well as GFP negative cells, clearly of KUSA/A1 cells in origin were observed around the bony trabeculae. These results indicated that the new bone was not only produced by KUSA/A1 cells but also by host cells from the surrounding connective tissues.

To our knowledge, this is the first study to describe that host cells play an important role in ectopic bone induced by implanted marrow stromal cells, which would need special attention in bone tissue engineering.

Key Words: Ectopic bone formation, Host cells, KUSA/A1 cell, Honeycomb carrier

Introduction

Recently, implantation of cells onto scaffold has taken a great importance in tissue engineering and is expected to provide new applications for creation of new organs and tissues. KUSA/A1 cell is a marrow stromal cell line from primary bone marrow culture of female C3H/He mouse[1]. KUSA/A1 cells are capable to differentiate into osteoblasts and induce mineralized bone matrix[2]. We have reported that a large amount of new bone can produce using KUSA/A1 cells with honeycomb scaffold[3]. Moreover, we have also published that their mechanism of bone induction is similar to intramembranous ossification[4,5]. Until now, the origin of the ectopic bone has not been reported yet.

In this study, we evaluated the origin of osteoblasts involved in the mechanism of ectopic bone formation induced by KUSA/A1 cells combined with honeycomb carrier.

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Materials and Methods

Atelocollagen honeycomb carrier

The atelocollagen honeycomb scaffold (KOKEN, Japan), 3x3x2 mm in size, composed by multiple collagen membranes (1mm in thickness) with honeycomb-shape were used. The scaffold presents parallel pores extended from surface to surface.

Cell culture

KUSA/A1 cells were courtesy of Dr. A. Umezawa from Keio University, Tokyo, Japan. The cells were cultured in minimum essential medium alpha medium (a-MEM, GIBCO BRL, Inc., USA) supplemented with 10% fetal bovine serum (SIGMA, USA) and 1% antibiotic-antimycotic (GIBCO, USA). They were seeded in 10 cm petridishes (Falcon, Inc., USA) and incubated at 37ºC in humid air with 5% CO_2.

Three-dimensional culture

When the cells became nearly confluent, they were harvested with trypsin-EDTA and placed at a concentration of $2x10^5$ cells/

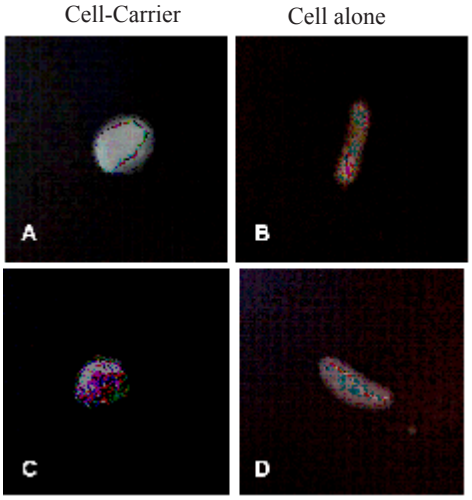

Fig.1. Soft X-ray photograph of Cells-Carrier and Cells alone after implantation. Cells-Carrier at 2 weeks (A) and 4 weeks (C) showed larger radiopacity compared to cells alone alone at 2 weeks (B) and 4 weeks (D).

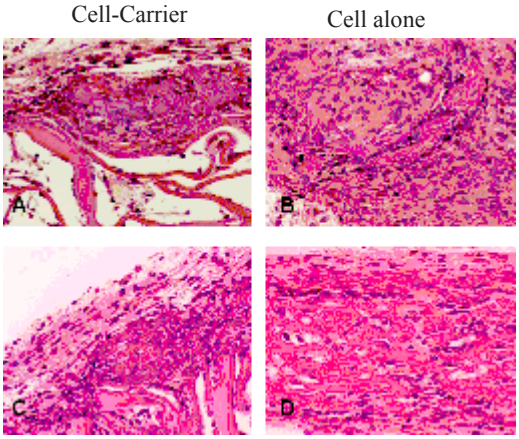

Fig.2. Histological examination of Cells-Carrier and Cells alone after implantation. Immature bone formation in Cells-Carrier at 2 weeks (A) and 4 weeks (C) and cells alone at 2 weeks (B) and 4 weeks (D). Areas of new bone (arrow). H-E staining X40

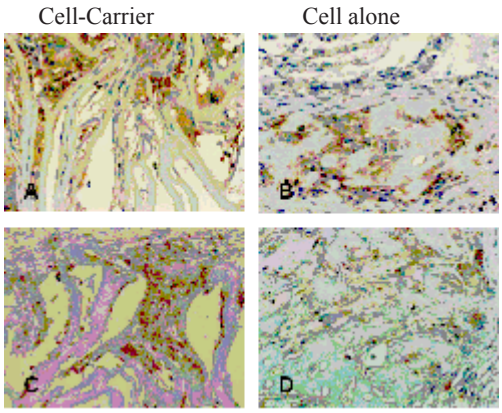

Fig.3. Immunohistochemical examination of Cells-Carrier and Cells alone after implantation. Note evidence of invasion of host cells in the carrier at 2 weeks (A) and 4 weeks (C). The presence of GFP positive host cells with osteoblastic morphology as well as GFP negative cells, clearly of KUSA/A1 cells in origin were observed around the bony trabeculae in Cells alone at 2 weeks (B) and 4 weeks (D). Positive cell expression (arrow). GFP Immunostaining, X40

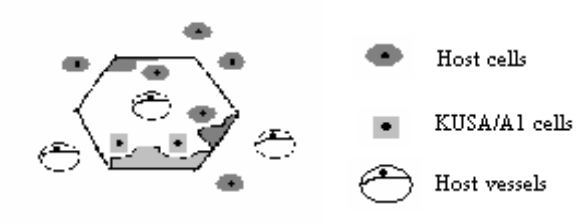

Fig.4. Scheme of ectopic bone formation induced by KUSA/A1 and Host cells

ml onto atelocollagen honeycomb carrier in suspension culture treated dishes. The cells were grown for 10 days at 37ºC in humid air with 5% CO_2 and the medium was changed every 3 days.

Bone induction assay in mice

In order to elucidate the origin of the new bone, KUSA/A1 cells with or without honeycomb carrier, were also transplanted into the subcutaneous tissue of GFP mice. The specimen were then extracted at 2 and 4 weeks and examined radiographically (soft X-ray, SOFTEX), histologically and immunohistochemically with GFP (Santa Cruz, US). (Fig.1)

Results

1.Origin of the newly bone

Cells-Carrier groups at 2 weeks, revealed a weak but large, diffuse radiopacity in a soft X-ray photograph (Fig.1A).While Cells alone groups at 2 weekspresented a weak and small radiopacity with indistinct border (Fig.1B). At 4 weeks, in **c**ells-carrier groups, the carriers were filled with areas of different radioopacities representing various degrees of calcification (Fig.1C). In Cells alone groups at 4 weeks, small and irregular but dense and homogeneous islands of radiopacities with well defined border were detected (Fig.1D).

Histologically, Cells-Carrier groups demonstrated areas with cell proliferating and invading into the carrier and also with apparent vessel invasion (Fig.2A-C). Areas with newly formed bone and areas with degenerative hard tissue could be also observed. Inflammatory reaction surrounding the collagen membrane of the carrier was observed. In Cells alone groups, bone nest formation (Fig.2B-D) with trabeculae surrounded by active osteoblast-like cells were observed. Degenerated areas with inflammatory reaction coulb be also detected.

Interestingly, by GPF immunohistochemestry (Fig.3), the ectopic bone formed by both groups, demonstrated the presence of host cells immunopositive to GFP as well as KUSA/A1 cells completely negative to GPF marker around and inside the bony trabeculae. In Cells-Carrier, GFP positive host cells were mainly located at the periphery of the carrier. These host cells seems to

invade inside the carrier to form active osteoblasts surrounding the trabeculae bone and osteocytes inside the bone matrix, which were also immunopositive for GFP.

Discussion

Tissue regeneration is a biological process to renew the damaged tissue. In order to stimulate regeneration different kinds of biomaterials such as L-lactide/epsilon-caprolactone copolymer foams, calcium alginate, CO_3apatite-collagen sponge can be used[6-8].

Tissue engineering is the biomedical technology or methodology to create a favorable environment in order to generate tissue, by using cells; scaffold, growth factor, or appropriate combinations of these. The bone marrow stromal cells are shown to have potential to differentiate into a variety of mesenchymal cells, such as adipocytes, chondrocytes and myocytes. The properties of marrow stromal cells as multipotent progenitor cells make them an attractive target for use in therapeutic and bioengineering applications, and the regulation of their commitment to specific cell types is a field of primary interest [9,10].

Several stromal cells were established from murine bone marrow cultures and one of the KUSA subclones, KUSA/A1 cells are capable of regenerating mature bone in vivo [11,12]. Importantly, the osteogenesis by these cells was reproducible and irreversible, and the transplanted KUSA/A1 cells never transformed into malignant cells or induced any inflammatory reactions. It is noteworthy that the oxygen concentration could play a crucial role for chondro-osteogenic cell differentiation [13]. Osteoblastic differentiation in the presence of excessive vessel formation as in this case, is a feature of membranous ossification [4,5].

KUSA/A1 cells alone and in combination with honeycomb carrier implanted subcutaneously into GFP mice clarified the origin of the cells responsible for ectopic bone formation. All of the tissues of these GFP transgenic mice, with exception of erythrocytes and hair, were green under excitation light and the fluorescent nature of the cells from these mice would facilitate their use in many kinds of cell transplantation experiments [14]. Presence of GFP positive host cells with ostoeblastic morphology around the trabeculae bone was observed in both groups (Fig.3D), indicating that the new bone was not only produced by KUSA/A1 cells but also by host stem cells from the surrounding connective tissues (Fig.4). It has been reported that he role of capillary invasion is not only to increase oxygen delivery but also to supply osteoprogenitor cells derived from perivascular mesenchymal cells [15] We also would like to suggest that these GFP positive host cells can be pericytes migrated from the surroundings tissue along the invaded blood vessels. The next step will be to identify the character of these host cells involve in the bone formation induced by KUSA/A1 cells and to evaluate the origin of the engineered bone induced by autologous marrow stromal cells.

To our knowledge, this is the first study to describe that host cells play an important role in ectopic bone induced by implanted marrow stromal cells, which would need special attention in bone tissue engineering.

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