3차원 혼합배양을 통한 신생혈관형성을 촉진하는 뼈용 Scaffold

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THE SCAFFOLD FOR BONE FORMATION CONCOMITANT WITH IMPROVED ANGIOGENESIS THROUGH 3-DIMENSIONAL CO-CULTURE SYSTEM

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It is well known that endothelial cells play very important roles in differentiation of osteoblasts as well as angiogenesis. In this study, our aim is an understanding that cell-cell interaction and cell-matrix interaction depending on inoculation cell density ratio and implantation period. So, we manufactured scaffolds, which were based on collagen and tricalcium phosphate. These biocompatible and porous scaffolds are suitable to bone regeneration and angiogenesis induction. We obtained osteoblastic cells from HBMSCs (human bone marrow mesenchymal stem cells) and HUVECs (human umbilical vein endothelial cells) from umbilical cord.

We co-cultured osteoblastic cells and HUVECs in the manufactured scaffolds. We varied inoculation cell density ratio (osteoblastic cells : HUVECs) from 1:1 to 1:0.6 . After 14 days of culture *in vitro*, they were implanted subcutaneously as a pocket type on the dorsal region of nude mice. And then biopsy was performed from 2 to 4 weeks. Culture type was dynamic culture to endow shear stress. Capillary network formation and calcium deposition in the implanted scaffolds was observed using histological staining.

We found that inoculation cell density ratio was optimal at 1:1 and prolonged implantation period increased calcium deposition. As another result, we confirmed that there were more blood vessels under optimal condition than the other conditions. So we suggest that improved bone formation resulted from synergistic effects by cell-cell interaction (heterotypic gap junction). Also, we suggest that the increased ECM from intercommunication as well as endothelial cells promoted the capillary network formation (angiogenesis). Therefore these results demonstrate that the specific inoculation cell density ratio could improve the bone formation and moreover endothelial cells play very important role in bone formation.

1. Introduction

It is well known that VEGF plays an important role in bone formation or bone remodeling and induction of angiogenesis (1). VEGF was secreted by not only osteoblastic cells but also enthothelial cells. And it plays an important role in bone formation as well as angiogenesis by autocrine or paracrine fashion (2). So in this study we focused on synergistic effect through co-culture by depending on inoculation cell ratio in scaffolds (1, 2). Thus, we selected co-culture using two cell types and culture type is selected with dynamic culture to provide shear stress. Because it is known that shear stress increases secretion of ECM in cell culture (3). Scaffolds are composed of type Ⅰ collagen and tri-calcium phosphate. Scaffolds are manufactured using lyophilization and cross-linking process (4). Animal test is performed with pocket type implantation in subcutaneous using nude mice. So, we observed calcium deposition and angiogenesis as like in bone formation through histological staining. As a result we found optimal inoculation cell ratio improving calcium deposition and angiogenesis *in vivo.*

2. Materials and Methods

2.1 Preparation of porous biocompatible Scaffold

A porous sponge type scaffold is composed of type Ⅰ collagen and tricalcium phosphate. These materials are mixed in 0.05% acetic acid solution, frozen, lyophilized and cross-linked. We selected sterilization method with a soaking in ethanol before cell culture.

2.2 Isolation of endothelial cells and culture

We selected HUVEC (human umbilical vein endothelial cell) as an endothelial cell. Endothelial cells were isolated from fresh human umbilical cord vein using enzyme digestion method and cultured using EGM-2 media.

2.3 Differentiation of osteoblastic cells

Osteoblastic cells are derived from BM-MSCs(bone marrow mesenchymal stem cells). MCSs are cultured in low glucose DMEM (Dulbecco's modified Eagle's medium) containing 10% FBS and osteoblastic cells are differentiated from BM-MSCs using differentiation inducers such as dexamethasone, ß-glycerophosphate and L-ascorbic acid-2-phosphate for 2weeks *in vitro.*

2.4 Dynamic co-culture using scaffolds

We varied inoculation cell density ratio (osteoblastic cells : HUVECs) from 1:1 to 1:0.6. To provide shear stress to scaffold containing cells we used 3-D shaker. Culture was performed for 2 weeks.

2.5 Implantation

Cell-cultured scaffolds were implanted into subcutaneous region of nude mice as a pocket type. And then biopsy was performed from 2 to 4 weeks. Histological analysis was performed by microscopy.

Results and Discussion

It is well known that the role of endothelial cells on the osteoblastic cells is crucial in bone regeneration as well as angiogenesis (1, 2). So, in this study we tried to know optimal inoculation cell ratio in co-culture using scaffolds. We varied inoculation cell density ratio (osteoblastic cells : HUVECs) from 1:1 to 1:0.6 (1×10^6 cells : 1×10^6 cells/scaffold, 1×10^6 cells : 8×10^5 cells/scaffold and 1×10^6 cells : 6×10^5 cells/scaffold). After 14 days of culture *in vitro*, they were implanted subcutaneously as a pocket type on the dorsal region of nude mice. And then biopsy was performed from 2 to 4 weeks.

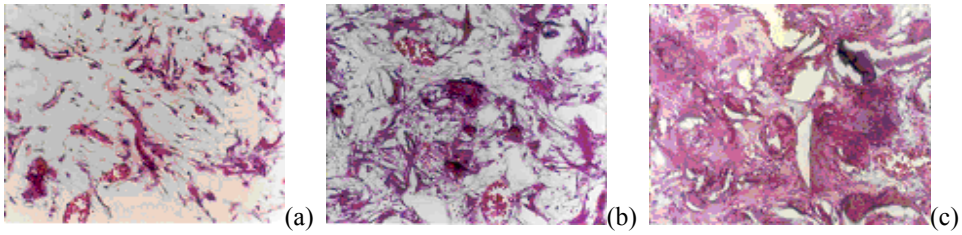
Fig. 1. Mouse before biopsy (left) and biopsy after 4weeks of implantation (right)

Culture type was dynamic culture to endow shear stress (3). Capillary network formation and calcium deposition in the implanted scaffolds was observed using histological staining. Cell characterization was confirmed by histological and histoimmunohistological staining following to Table.1.

	Control	**Endothelial cell**	**Osteoblastic cells**	**Co-culture**
H&E staining	-	+	+	+
M.T staining	-	-	+	+
vWF staining	-	+	-	+
VEGF staining	-	+	-	+
Von Kossa staining	-	-	+	-
osteocalcin staining	-	-	+	+
osteopontin staining	-	-	+	+

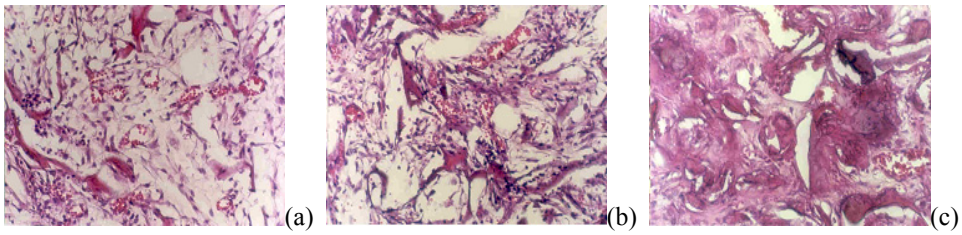
Table.1. Hisological and immunohisotological staining characterizing single or co-cultured cells after 2 weeks of implantation

In the microscopy it is means that dark purple color is a better calcium deposition (Fig.2, 3). So we found that inoculation cell density ratio was optimal at 1:1 and prolonged implantation period increased calcium deposition. As another result, we confirmed that there was more blood vessel induction under optimal condition than the other conditions.



(a) (b) (c)

Fig.2. H&E staining of scaffolds depending on inoculation cell ratios from 1:0.6 to 1:1 at 4 weeks post-implantation (a; 1×10^6 cells : 6×10^5 cells/scaffold, b; 1×10^6 cells : 8×10^5 cells/scaffold, c; 1×10^6 cells : 1×10^6 cells/scaffold)



(a) (b) (c)

Fig.3. H&E staining of scaffolds depending on implantation periods
(a; 2 weeks post-implantation, b; 3 weeks post-implantation, c; 4weeks post-implantation)

So we suggest that the improved bone formation resulted from synergistic effects with endothelial cells. Also, we suggest that the increased ECM by endothelial cells promoted the capillary network formation (angiogenesis). Therefore these results demonstrate that the specific inoculation cell density ratio could improve the bone formation and moreover endothelial cells play very important role in bone formation as well as angiogenesis promotion.

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