Enhancement of bone-related cell function by bone-like nanocomposite.

Masanori Kikuchi[1] and Atsushi Irie[2]

1. Biomaterials Center, National Institute for Materials Science, 1-1, Namiki, Tsukuba, Ibaraki 305-0044, Japan
2. Biomembrane Signaling Project, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113-8613, Japan

Introduction

Regenerative medicine is one of the most efficient treatments for repairing tissue defects. To realize the regenerative medicine, following four key technologies are considered as fundamental for tissue regeneration; cells, chemical stimuli (cytokines, hormones, vitamins etc.) [1, 2], mechanical stimuli (stretch, compress, hydrostatic pressure etc.) [3] and scaffold materials. The importance of the scaffold materials is generally considered as a supporting factor for cell proliferation and tissue construction; thus, importance in cell differentiation has not been disucussed very well. In fact, many researchers only reported "support" or "enhance" the effect of chemical stimuli for cell differentiation [4]. Recently, mesenchymal stem cell differentiation to osteoblasts by surface nanotopography was reported [5]. It can be useful to enhance direct bone bonding property of stem of artificial joint; however, applying it to the scaffold materials could be difficult because the scaffold materials usually requested its biodegradablility. Even though, the result suggested that bulk and biodegradable materials for scaffold with controlled nanostructure, *i.e.*, controlled nanotopological surface was continuously appeared even during material degradation, could enhance the cell functions.

On the other hands, the development of induced pluripotent stem (iPS) and embryonic stem cell technologies eagaly desire effective cell differentiation technique *in vitro* to realize regenerative medicine. This kind of differentiation is generally induced by cytokines and hormons; however, those chemicals are very expensive and have some difficulty to use practical medicine due to their biological safety for whole body. If cells can differentiate by using scaffold materials and conventional culture medium, safety and cost issues will be minimize.

A hydroxyapatite/collagen bone-like nanocomposite (HAp/Col) is the first artificial material incorporated into bone remodeling process [6]. Further, this material can easily be fabricated to appropriate forms for tissue engineering [6-9] and reported as osteogenic activity enhancer [8, 10].

In the present study, bone marrow cells and osteoblasts isolated from mice were cocultured on the HAp/Col disks with and without adding of osteoclastic differentiation inducers. Osteoclastic differentiation of bone marrow cells was evaluated by a tartrate-resistant acid phosphatase (TRAP) staining.

Experimental

Materials

The HAp/Col nanocomposite (HAp:Col=8:2 in mass ratio) was prepared according to the previous report [6]. Briefly, 200 cm^3 of 400 mM $Ca(OH)_2$ (decarbonated at 1050 °C and hydrated alkaline analysis grade $CaCO_3$ from Wako pure chemicals Inc., Japan) and 400 cm^3 of 120 mM H_3PO_4 (Reagent grade, Wako pure chemicals Inc., Japan) with 2 g of type-I atelocollagen (Biomaterials grade, Nitta Gelatin Inc., Japan) were simultaneously added to 200 cm^3 H_2O previously added in the reaction vessel for pH measurement. The pH of the reaction solution in the vessel was maintained to 9±0.05 with an auto-titration unit equipped with a pH controller, and the reaction temperature was controlled to 40 °C with a water bath. After filtration of the HAp/Col obtained, 10 g of the wet HAp/Col was packed in dehydration mold and press-dehydrated at 10 MPa for overnight. The plate obtained was punched out to 4 mm ϕ disk and crosslinked by a vacuum heating at 140 °C for 12 h or soaking in 0.5, 1.0 or 2.0%(w/v) glutaraldehyde (GA) solution for 90 min. The GA crosslinked disks were washed in dH_2O for 60 min. with shaking, soaked in 2 mass% glycine solution to remove free GA from the sample for 1 day and washed with dH_2O for 60 min. twice. The disks were dried, polished and sterilized with an ethylene oxide gas. The disks were soaked in the medium for 5 days before cell culture test to allow adsorption of Ca and Mg ions. Non-crosslinked disk was also prepared and served for the experiment without polishing and soaking in the MEM to prevent its surface degradation before the experiment. Further, the HAp/Col membranes [8] with and without 1% (w/v) GA crosslinkage were also examined.

Osteoclast Differentiation Assay

Osteoclast differentiation assay was performed as described previously [11]. Briefly, bone marrow cells and osteoblasts were isolated from C57BL/6 mice. They were cocultured on the HAp/Col disk for 6 days in 96-well plates in α-minimum essential medium containing 10% fetal bovine serum with or without osteoclast differentiation inducers, 10 nM 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and 1 μM prostaglandin E_2 (PGE_2). The cells on the HAp/Col disk were then fixed, and stained for TRAP, a marker enzyme of osteoclastic differentiation. Dentin slices with and without 1% (w/v) GA crosslinkage, sintered HAp disk and tissue culture polystylene (TCPS) were used as control.

Results and Discussion

Figure 1 shows that TRAP stained mouse bone marrow cells cocultured with osteoblasts with and without adding of osteoclastic inducers. When cultured with the osteoclastic inducers, strong TRAP staining, *i.e.*, presence of active osteoclasts, was observed for cells cultured on all specimens including the HAp/Col, dentine and HAp wells as well as a positive control, TCPS wells. This suggested that the HAp/Col disk did not inhibit osteoclastic differentiation *in vitro*. On the contrary, no TRAP positive cells were observed on the HAp disk and TCPS without adding of the osteoclastic inducers; however, TRAP positive osteoclasts were observed on all the HAp/Col disks. In the case of dentin, weak or no TRAP positive cells were observed. (It meant the dentin had unstable effect of osteoclastic differentiation.) This result demonstrated that the HAp/Col induced osteoclastic differentiation of mouse bone marrow cells cocultured with osteoblasts.

In general, the dentine is usually used as a bone simulating material for osteoclast researches due to its nanostructural and chemical similarity to bone; however, it has no or very weak osteoclastic differentiation ability in comparison to the HAp/Col bone-like nanocomposites. The dentine has the same primary structure, *i.e.*, bone-like nanostructure in which *c*-axes of HAp nanocrystals are oriented along collagen fibers [13] as well as the HAp/Col nanocomposite [6]. Their important differences could be in their surfaces. Generally, the dentin slice was prepared by cutting the dentin cylinder of which circle surface is perpendicular to long axis of ivory; therefore, major plain revealed in the slice is perpendicular to long axis of the HAp/collagen bundle, *i. e.*, *c*-plain of the HAp nanocrystals and functional group of collagen terminal could mainly interact with cell attached. On the other hand, the HAp/Col nanocomposite fibres lay on the HAp/Col disk surface, *i. e.*, *a*-plain of the HAp nanocrystals and all functional groups on the collagen molecules could interact with cells attached. This interaction manner is very similar to regular attachment manner of bone related cells, because dominant surface of bone is the side of HAp/collagen nanocomposite bundles. This bundle structure is native for the HAp/Col bone-like nanocomposite and can be used for every shaped materials fabricated by it.

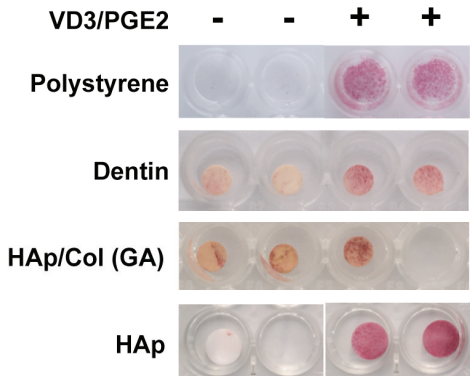

Figure 1. Tartrate resistance acid phosphatase staining of bone marrow cells cocultured with osteoblasts after 7 days culture on tissue culture polystyrene, dentine, hydroxyapatite/collagen bone-like nanocomposite disk (HAp/Col) and sintered HAp with (+) and without (-) adding osteoclastic inducers.

Conclusion

The HAp/Col disk demonstrated that induction of osteoclastic differentiation of mouse bone marrow cell cocultured with mouse osteoblasts. Nano- and micro-topographies as well as chemical and mechanical properties of materials have a possibility to enhance cell differentiation *in vitro* and expected to be one guideline for future design of tissue engineering scaffolds.

References

1. Noda, M. & Rodan, G.A., *J. Cell Physiol.* **133**, 426-437 (1987).
2. Maegawa, N., Kawamura, K., Hirose, M., Yajima, H., Takakura, Y., Ohgushi, H., *J. Tissue Eng. Regen. Med.* **1,** 306-313 (2007).
3. Mizuno, S., Tateishi, T., Ushida, T, & Glowacki, J., *J. Cell Physiol.* **193,** 319-327 (2002).
4. Liu, G., Zhao, L., Cui, L., Liu, W. & Cao, Y. *Biomed. Mater*. **2**, 78-86 (2007).
5. Dalby, M.J., Gadegaard, N., Tare, R., Andar, A., Riehle, M.O., Herzyk, P., Wilkinson, C.D. & Oreffo, R.O. *Nat. Mater.* **6,** 997-1003 (2007).
6. Kikuchi, M., Itoh, S., Ichinose, S., Shinomiya, K. and Tanaka, J., *Biomaterials* **22,** 1705-1711 (2001).
7. Kikuchi, M., Ikoma, T., Syoji, D., Matsumoto, H.N., Koyama, Y., Itoh, S., Takakuda, K., Shinomiya, K. and Tanaka, J., *Key Eng. Mat.* **254-256**, 561-564 (2004).
8. Kikuchi, M., *Key Eng. Mat.* **330-332**, 313-316 (2007).
9. Kikuchi, M., *Key Eng. Mat*. **361-363**, 431-434 (2008).
10. Yoshida, T., Mizuno, I., Kikuchi, M., Koyama, Y. and Takakuda, K. *Key Eng. Mat*. **361-363**, 1079-1082 (2008).
11. Irie, A., Takami, M., Kubo, H., Sekino-Suzuki, N., Kasahara, K. & Sanai, Y. *Bone* **41,** 165-174 (2007).
12. Kikuchi, M., Matsumoto, H.N., Yamada, T., Koyama, Y., Takakuda, K. and Tanaka, J., *Biomaterials* **25**, 63-69 (2004).
13. Su, X.W. & Cui, F.Z. *Mater. Sci. Eng. C* **7,** 19-29 (1999).

Acknowledgement

The study is supported in part by Research Promotion Bureau, Ministry of Education, Culture, Sports, Science and Technology, Japan under the contract No.19-207.