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学位授与の要件	医歯薬学総合研究科機能再生・再建科学専攻 （学位規則第４条第１項該当）
学位論文の題目	Cellular Fragments as Biomaterial for Rapid In Vitro Bone-Like Tissue Synthesis （細胞断片は，in vitro 環境下において骨様組織を急速に誘導した）
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学位論文内容の要旨

Tissue engineering is an interdisciplinary field to fabricate tissues or organs *in vitro* and *in vivo* to replace or support the function of defective or injured body parts. Many researchers and scientists have attempted to regenerate tissues by combining cells, signaling molecules (e.g., growth factors and small molecules) and biomaterials (e.g., ceramics, natural or synthetic polymers).

In regenerative dentistry, bone tissue formation utilizing artificial substances remains as big challenges. Recently, regeneration of bone tissues using stem cells has attracted worldwide attention. Despite of having huge advantages, differentiation of stem cells into mature osteoblasts or chondrocytes usually require 2 to 3 weeks to promote mineral formation.. Hence, researchers seek to achieve rapid bone tissue formation *in vitro.*

For this purpose, it is very important to understand the physiological mechanisms of bone formation *in vivo* from an engineering point of view, and then develop bioinspired techniques and materials based on the obtained knowledge. As a cell-free approach, utilization of isolated matrix vesicles (MVs) from osteoblasts has also been attempted for rapid and robust bone-like mineral formation. However, previous studies were unable to persuade faster mineralization using MVs. Recently, our research group found that the cellular nanofragments dispersed in the extracellular region caused by cell rupture would be the nucleation site for initial mineralization. Therefore, in this study, we developed methods to fabricate cell fragments from fully confluent intact cells. The methods were ultrasonication (30 sec and 3 min), non-ionic detergent (triton 0.1% and 1%) or freeze-dry-mash methods. Mineralization assay followed by alizarin red S and alkaline phosphatase (ALP) staining were performed by incubating the freshly prepared cell fragments in normal culture medium supplemented with beta glycerophosphate (β-GP) *in vitro*.

The ALP enzyme activity assays were performed to compare its activity between intact live cells and cell nanofragments. The morphology of the cell nanofragments as well as those of the obtained minerals were analyzed by scanning electron microscopy (SEM), energy dispersive X-ray spectrometer (EDS) and transmission electron microscopy (TEM). Mineral characterization was carried out by using X-ray diffraction (XRD) analysis. Finally, cell nanofragments were mixed with collagen gel for reproducing bone-like mineralization *in vitro.*

Our results showed that mechanical fragmentation by ultrasonication for 3 min allowed the fabrication of smaller (< 150 nm) and more homogeneous cell nanofragments among all methods. Moreover, the cell nanofragments fabricated by ultrasonication 3 min induced markedly rapid mineralization *in vitro* within just 1 day. Cell disruption by ultrasonication 3 min induced an immediate increase in ALP activity. Moreover, a loss-of-function experiment with a phosphatase inhibitor cocktail completely suppressed the mineralization of cell nanofragments. These results indicate that the mineralization of cell nanofragments is not just a spontaneous precipitation of minerals onto the organic matter, but in fact, that ALPs play crucial roles in cell nanofragment mineralization by cleaving phosphate-containing molecules (e.g., pyrophosphate, phosphoproteins, β-GP) and promoting the release of free phosphate ions that subsequently bind with calcium to form calcium phosphate minerals.

Additionally, a concentration of 10 mM and 100 mM of β-GP induced faster mineralization of cell fragments. On the other hand, fetal bovine serum inhibited the cell nanofragment mineralization in a concentration-dependent manner. Characterization of initial minerals formed by the cell nanofragments revealed them to be amorphous calcium phosphate (ACP) until 5 days of incubation. After 7 days of incubation, ACP was shown to crystallize into hydroxyapatite as demonstrated by XRD analysis. Interestingly, the minerals formed from the cell nanofragments showed no significant difference in size and shape (sphericity) compared to those formed from live cells (osteoblasts and chondrocytes) cultured in osteogenic media. Along with these results, SEM observations of the cell nanofragment-mineral microstructure inside the collagen gel confirmed the bone-like mineral deposition onto the collagen fibers after 3 days of incubation.

Together, these results indicate that cell nanofragments would be a promising substrate for realizing rapid bone-like mineralization *in vitro*.

論文審査結果の要旨

In tissue engineering, materials scientists focused to synthesize biomimetic and bio responsive biomaterials that fulfil the main requirements for tissue formation *in vitro* or *in vivo*. Especially, bone tissue engineering *in vitro* is one of most attracted area of application. For rapid bone tissue formation *in vitro*, several trials using stem cells and growth factors have been done recently. Nevertheless, stem cell-based bone formation *in vitro* takes usually 2 to 3 weeks to promote mineral formation. Therefore, it is in high demand to develop advance methods and materials to achieve more rapid bone tissue formation *in vitro*.

Previously ruptured chondrogenic cell membrane nanofragments were observed as an enucleation site during initial mineral formation but the optimization of methods for cell nanofragments fabrication was not done until then. Therefore, in this study, objectives set to optimize the method to fabricate cell nanofragments and utilize them as bioinspired materials for rapid 3D bone-like tissue synthesis *in vitro*.

Applied five individual methods were ultrasonication (30 sec and 3 min), non-ionic detergent (triton 0.1% and 1%) or freeze-dry-mash methods. Ultrasonication for 3 min could produce smaller (<150 nm) and homogeneous cell nanofragments, which induced markedly rapid mineralization. Additionally, a concentration of 10 mM and 100 mM of β-GP induced faster mineralization while FBS inhibited the cell nanofragments mineralization in a concentration-dependent manner. Minerals formed by cell nanofragments were crystallized into hydroxyapatite after 7 days of incubation. Minerals formed by the sonicated cell nanofragments showed no significant difference in size and shape compared with those by live ATDC5 or MC3T3-E1 cells. Along with these, a cell nanofragment-containing collagen gel induced the mineral deposition onto collagen fibers after 3 days of incubation.

In conclusion, 3 min ultrasonication was the optimal method to prepare cell nanofragments which can be used as bioinspired substrate for synthesizing rapid bone-like tissue *in vitro*.

This paper has already been published in the International Journal of Molecular Science and it has been evaluated internationally.

The thesis defense committee hereby accept this article as a doctoral dissertation in dentistry.