論　文　内　容　要　旨

Differentiation of Human Bone Marrow-derived Mesenchymal Stem Cells into Early Chondrocytes in Three-dimensional Culture

（ヒト骨髄由来間葉系幹細胞の三次元培養下における軟骨細胞への分化）

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Introduction

Human bone marrow-derived mesenchymal stem cells (hBMSCs) are considered to be promising as cell sources for cartilage regeneration. However, differentiation of hBMSCs into chondrocytes can ultimately lead to hypertrophic chondrocytes which later become osteoblasts and contribute to the full osteogenic lineage. Therefore, suppressing hypertrophic chondrocyte differentiation must be carefully taken into consideration for cartilage regeneration. This study aimed at studying the effect of conditions, such as differentiation stages and nutrient concentrations, in three-dimensional (3D) pellet culture of hBMSCs on the promotion of chondrogenic differentiation and also the prevention of hypertrophic chondrocyte conversion or, in other words, the stabilization of early chondrocytes differentiated in the pellets.

Materials and Methods

hBMSCs (UE6E7T-3, JCRB Cell Bank) were cultured in growth or chondrogenic medium. After cells reached confluence, 2.5×10^6 cells were centrifuged at 500 *g* for 5 min in a 15 ml conical tube to obtain a pellet. Then the pellet was cultured under growth medium or chondrogenic medium, and the medium was changed every 2–3 days. Pellets were sliced with a thickness of 5 μm and stained with hematoxylin–eosin and toluidine blue O (TBO). Moreover, the sections were immunologically stained to detect HIF-1α (a marker for hypoxia), type X collagen (a marker for hypertrophic chondrocytes), and necrotic and apoptotic cells were analyzed by propidium iodide (PI) staining and TUNEL assay, respectively. Quantitative PCR was performed to analyze the expression of chondrogenic marker genes (Sox9 and collagen type II) and the activity of mitochondria. ImageJ software was used to quantify the stained cells. Statistical analysis was performed by ANOVA.

Results and Discussion

First, the difference in medium conditions was considered: hBMSCs were cultured under growth or chondrogenic medium in monolayer. It was seen that cells under the chondrogenic medium exhibited a lower level of mitochondrial activity than those under the growth medium. This result suggests that chondrocytes differentiated under the chondrogenic medium consume less oxygen than growing hBMSCs. In 3D culture, pellets in chondrogenic medium contained a smaller number of necrotic cells than those in growth medium. This may be due to the fact that, as mentioned above, differentiated chondrocytes require less oxygen and therefore that these cells could survive even in the core regions of a pellet where microenvironment might be under

hypoxia conditions.

To gain deeper insights into the effect of hypoxia conditions on the differentiation of chondrocytes, pellets were prepared separately from undifferentiated and pre-differentiated cells, and then cultured under chondrogenic medium for 10 - 28 days. It appears that necrotic cells distribute unevenly within a pellet prepared from undifferentiated cells and are more abundant in the core regions of a pellet. This result can be simply explained from the theoretical consideration for the oxygen gradient established within a pellet. However, when pellet culture was initiated with more differentiated chondrocytes, the density of necrotic cells are relatively low in the core regions, probably because of reduced requirement of oxygen by differentiated chondrocytes. This result suggests that hypoxia condition, found especially in the core regions of a pellet, has rather a positive effect on the enrichment of chondrocytes.

As one of the most important molecules that promote chondrocyte differentiation and also suppress mitochondrial activity, a special attention was paid here to hypoxia inducible factor-1α (HIF-1α). This protein is known as a transcription factor that binds to Sox9 promotor to induce chondrogenesis, while the expression of several proteins forming mitochondrial redox carriers is impaired by HIF-1α. The later effect gives rise to a decrease in intracellularly-generated reactive oxygen species, a by-product of ATP synthesis, which may cause cell necrosis. It was observed in the present study that pellets prepared with pre-chondrogenic differentiation produced cartilage-like extracellular matrices, as shown by TBO staining, more abundantly than those with undifferentiated cells. In addition, the level of HIF-1α gene expression was higher in pellets prepared with pre-differentiated cells than those with undifferentiated cells. The both results can be well explained from the diverse functions of HIF-1α as described above.

During chondrogenesis, progenitor cells ultimately differentiate into early and subsequently late chondrocytes, a so-called hypertrophic chondrocytes, and finally contribute to full osteogenic lineage. Therefore, hypertrophic chondrocyte conversion should be suppressed as much as possible for cartilage regeneration. In this study, it was found that culturing pre-differentiated pellets in a medium at low glucose concentration facilitated to significantly decrease hypertrophic chondrocyte conversion in the pellets.

Conclusion

Chondrocyte consume less oxygen than that of hBMSCs. Since chondrocyte and hBMSCs consume oxygen at different rate, oxygen gradient and oxygen consumption influence cell necrosis and chondrogenic differentiation in the pellets. HIF-1α seems to play a key role in these effects. Hypertrophic chondrocyte conversion can be suppressed in a medium at low glucose concentration. Accordingly, pre-chondrogenic differentiation with low glucose concentration may provide optimal conditions for the formation of early chondrocytes and thus for cartilage

regeneration.