Kartogenin Enhance Chondrogenic Differentiation of MSCs in 3-D Tri-copolymer Scaffold and Functional-Closed Perfusion System

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Introduction:
The rapidly emerging field of tissue engineering holds great promise for the generation of functional cartilage tissue substitutes. Current treatments for cartilage repair are still less than satisfactory, and rarely restore full function or return the tissue to its native normal state (Tuli R, et al. 2003). Adult bone marrow derived mesenchymal stem cells offer the potential to open a new frontier in medicine (Ryan JM, et al., 2005). Adult mesenchymal stem cells (MSCs) can be isolated from bone marrow or marrow aspirates and because they are culture-dish adherent, they can be expanded in culture while maintaining their multipotency. These tissue-engineered materials show considerable promise for use in rebuilding damaged or diseased mesenchymal tissues. A small molecule, kartogenin (KGN), can promote chondrocytes differentiation both in vitro, and in two osteoarthritis animal models. Kartogenin induces chondrogenesis by regulating the CBFβ-RUNX1 transcriptional program. It can stimulate MSCs to differentiate as matrix-producing chondrocytes, while up-regulate type II collagen, SOX9, Aggrecan and TIMPs; meanwhile, KGN also down regulate RUNX2 related downstream genes, make this drug feasible to prevent chondrocytes hypertrophy (Johnson K, et al., 2012). The purpose of this research is aimed to optimize the chondrogenesis process in MSCs based chondrogenic construct. However, the possible application about combining KGN with biomaterial is still not reported. To fulfill the goal, we cultured rat MSCs on 3-D tri-copolymer porous scaffolds with KGN in a dynamic functional-closed perfusion system; in this study, MSCs were demonstrated to differentiate into chondrocytes phenotype without hypertrophic change. This result provide a potential for KGN usage in MSCs based cartilage tissue engineering.

Methods:
The procedures were performed in accordance with the guidelines for animal experimentation by the Institutional Animal Care Committee, National Taiwan University College of Medicine. The mesenchymal stem cells (MSCs) of male wistar rats were isolated using their plastic adherence. The fabrication of hyaline-collagen-chondroitin sulfate- tri-copolymer was according to Chang et al. (Chang CH, et al, 2003). The scaffold and chondrogenic cell morphology inside tri-copolymer scaffolds was observed by field emission gun scanning electron microscopy (TM 3000, Toshiba, Japan). Briefly, cells in scaffolds were fixed with 4% para-formaldehyde (PFA) for 2 h and 2% osmium tetroxide (OsO₄) solution for 1 h. All the samples were dehydrated in a graded series of ethanol, before critical-point drying (CPD) method, and were sputter-coated with gold to a thickness film before observation. Fourier transform infrared (FTIR) spectra of pure gelatin and EDC crosslinked tri-copolymer scaffold were measured to confirm the formation of amide bond, ester bond, and the presence of OSO₃, functional group of chondroitin sulfate. For condensation examination in 2D, alcian blue staining were used. Bioreactor design and operation
were described previously (Lin YJ, et al., 2009) and be carried out using functionally-closed process bioreactor system. Quantitative real-time PCR, immunofluorescence and histology examination were performed.

Results:

Part I: Static culture

Condensation process of chondrogenesis was examined in static 3D culture by live/death staining, dead cells were also found in nodule by PI staining. As shown, MSCs sparsely scattered within the scaffold in the control group, 1.0 μM KGN promote condensation of MSCs within the scaffold, while without significant effect on the cellular viability. When compared with 0.1 μM, 1.0 μM KGN significantly up-regulated Aggrecan gene expression (p< 0.0001). However, Col2a1 expression is down-regulated when compared with control (monolayer culture by DMEM with 10% FBS). When 1.0 μM KGN was added to MSCs culture for 14 days, Aggrecan gene expression was significantly up-regulated (p< 0.0001); SoX-9 gene expression was up-regulated during the first 7 days’ culture, while down-regulated in the second week’s period. However, SOX-9 expression is still more obvious when compared with that of 0.1µM KGN. After 7 days static culture in 3D scaffold, chondrogenesis related genes were examined and compared with 2D culture. MSCs cultured in 3D scaffold up-regulated aggrecan, Col2a1 and Sox9 genes expression. The result indicates 3D tri-copolymer scaffold culture can enhanced the chondrogenesis effect together with KGN.

Part II: Culture in bioreactor system

In the presence of 1.0 μM KGN is presents, phenomenon of condensation can be observed. The SEM images showed that the individual cells were attached at the surface of scaffolds on day 0; scattered cells can condense and exist as cluster on day 7. At the day 14 or 21, the secreted extracellular matrix gradually accumulated, but the size won’t growth much as culture days expand to 21 days. With hematoxylin and eosin staining, seeding MSCs sparsely scattered on the scaffold; at 7 days’ culture, condensed cell cluster around 100 μm can be found in section; after 21 days culture, lacunae like structures with singular-like cell around 10-20 μm can be found embedding in scaffold. With Safrain O staining and confocal image study, GAGs secretion within the cell cluster was clearly demonstrated after 21 days culture. Direct measurement of sulfated glycosaminoglycans (GAGs) content in the medium by the cationic dye 1,9-dimethylmethylene blue (DMMB assay), we found that the GAGs contents were significantly increased with the longer culture period. As noted above, MSCs cultured in 3D tri-copolymer scaffold had significantly higher chondrogenic genes (including aggrecan, Col2a1 and Sox9) expression than monolayer control. Further study of MSCs behavior on 3D cultures showed that the level of collagen type II expression at day 21 was found to be significantly higher than its expression at day 7 culture in bioreactor system. This result shows that the MSCs could retain the chondrogenic phenotype up to 21 days’ culture. TIMP-1, a metallopeptidase inhibitor, was also up-regulated. During 7 - 21 days’ culture, the expression of Col1a1 and RUNX II were retained stationary. For Col10a1, however, its gene expression was down-regulated at day 21.

Discussion:

To validate the chondrogenesis process in functional closed bioreactor system, we examined the cell morphology and genes expression in the scaffold. We found out that with the addition of KGN, the pellet cell mass were formed. Although the dimension of the cell mass retained, the cells mass shows sulfated glycosaminoglycans deposition. Besides the condensed cell mass, we also found out the lacunae
like structure in histological study, which was seldom formed in artificial constructs. By the histology section, we can propose that the differentiated cells, instead of wholly synthesized its own ECM, formed the lacunae-like structure in scaffold material.

**Significance:**
Based on the current results, the combination of tri-copolymer/MSCs with KGN successfully induced the chondrogenesis process in both genetic and morphologic aspect. We expect that this research provide an example for small molecule regulated practice and thus provide a new combination for cartilage tissue engineering.

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