Novel Composite Scaffolds from Gelatin, Chitooligosaccharide, and Demineralized Bone for Bone Tissue Engineering

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Introduction: Biomaterial scaffolds play a major role in bone tissue engineering. A composite that utilizes the osteoinductivity of demineralized bone (D) and the attractive characteristics of chitosan may be potentially useful as a tissue-engineered bone substitute [1]. Demineralized bone is a well known osteoinductive biomaterial used for promoting new bone formation [2, 3]. Gelatin (G) is a natural polymer derived from collagen that has been used for biomedical applications [4]. Chitooligosaccharide (C) is one of the very low molecular weight water-soluble chitosan which provides similar structure to glycosaminoglycan of bone matrix [5]. Mesenchymal stem cells (MSCs) are multipotential cells which can differentiate into osteogenic, chondrogenic, and adipogenic lineages. MSCs have become the most used stem cells in the research field of tissue engineering [6]. The purpose of this study was to characterize physical and biological properties of novel scaffolds in combination of G, C, and D in vitro and in vivo bioassay.

Methods: Three dimensional scaffolds from the combination of G, C, and D were fabricated into 3 groups (G, GC, GCD) using chemical processes. The scaffolds were tested for cytotoxicity before culturing with hMSCs. The biological properties of scaffolds with MSCs were verified using alkaline phosphatase (ALP) activity assay, osteogenesis gene expression analysis. Calcium deposition was determined by o-cresolphthalein method. Elemental composition was determined by energy-dispersive X-ray spectroscopy at day 28. Cell morphology was investigated after cultured via scanning electron microscope (SEM). In the in vivo bioassay, GC and GCD acellular scaffolds were implanted in 8-week old male Wistar rats for 4 and 8 weeks, respectively. The explants were analyzed for new bone formation using hematoxylin and eosin (H&E) stain and Masson’s Trichrome stain. The study was approved by the MDCU-IRB animal care committee and was conducted in accordance with the national legislation on protection of animals.

Results: In vitro: All scaffolds did not exhibit cell toxicity. The MSCs could be attached and proliferated on all three groups of scaffolds (Fig.1A). Interestingly, the ALP activity of MSCs reached the maximum level at day 7 after cultured on the scaffolds (Fig.1B) whereas the calcium assay demonstrated the highest level of calcium containing in MSCs at day 28 (Fig.2A). Furthermore, weight percentage of calcium and phosphorus on MSCs surface after cultivation on the GCD scaffolds were increased among other scaffolds (Fig.2B). The hMSCs cultured with G, GC, and GCD scaffolds can express runx2, alp, and col-I (Fig.3). The SEM images showed that hMSCs can attach and proliferate on the scaffolds surface thorough cultivation time. Mineral crystal aggregation can be observed in GC and highly in GCD scaffolds (Fig.4).

In vivo: H&E staining demonstrated that GC and GCD scaffolds displayed osteoid at 8 weeks (Fig.5A, 5B). In addition, Masson’s Trichrome staining confirmed that there was new bone formation at 8 weeks in GC and GCD scaffolds (Fig.5C, 5D).

Discussion: Our findings demonstrate that G, GC, and GCD scaffolds provide abilities for cell attachment and cell proliferation after cultivation with hMSCs. They can exhibit the highest level of ALP at day 7. Especially, the hMSCs cultured in GCD scaffolds exhibited higher osteogenic differentiation than that cultured in G and GC scaffolds. Moreover, the GC and GCD scaffolds can promote osteogenesis in the Wistar rat model. The GCD scaffolds show the highest level of osteoinductivity than others and may be utilized for bone regeneration.

Significance: The present study demonstrates the feasibility of developing novel composite scaffolds from gelatin, chitooligosaccharide, and demineralized bone. These porous composite scaffolds are promising materials for bone tissue engineering.

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Elemental analysis of hMSCs after cultured on G, GC, GCD scaffolds

<table>
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<th>Type</th>
<th>O</th>
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<th>Mg</th>
<th>P</th>
<th>Ca</th>
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Fig. 1 hMSCs cultured on 3D G, GC, GCD scaffolds. A) Initial attachment and proliferation of hMSCs on G, GC, and GCD scaffolds. B) ALP activity of hMSCs cultured on G, GC, and GCD scaffolds. *represents the significant difference (p<0.05) relative to G. **represents the significant difference (p<0.001) relative to G.

Fig. 2 Mineralization of 3D scaffolds cultured hMSCs for 28 days. A) Ca deposition of hMSCs proliferated on G, GC, GCD scaffolds. *indicates the significant difference (p<0.05) relative to G; **represents the significant difference (p<0.001) relative to G. B) Elemental analysis of hMSCs after cultured on G, GC, GCD scaffolds.

Fig. 3 Osteogenic gene expression of hMSCs cultured with scaffolds. A) runX2B) alp C) ocl

Fig. 4 SEM image of hMSCs cultured on scaffolds (4,000X). A) G scaffold at day 7, 28 B) GC scaffold at day 7, 28 C) GCD scaffold at day 7, 28.

Fig. 5 Histology staining of explants at 8 weeks (20X). A) G scaffold explant (HE) B) GCD scaffold explant (MCA) C) GC scaffold explant (Masson's Trichrome) D) GCD scaffold explant (Masson's Trichrome). Black arrow indicates new bone formation.