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論　文　の　要　旨

Growth factors are powerful therapeutics that control cell function by modulating cellular activities such as cell proliferation, migration and gene expression. In tissue engineering, it is important to keep the bioactivity of these labile molecules for an optimal duration in 3D microenvironment. Porous scaffolds can provide structural support as well as a functional platform for release of these drugs in a controlled and regulated manner. Controlled and local release of these cell inductive molecules from porous scaffolds can provide an efficient strategy to control the complex and dynamic regulation of cellular processes in 3D microenvironment and can improve the regeneration potential of engineered scaffolds.

Technology involving spatial localization of these drugs via carrier based system such as drug delivery devices (DDS) made from natural and synthetic polymers is considered as one of the effective tools to develop appropriate porous scaffolds with controlled release function. Growth factor loaded biodegradable microbeads prepared from synthetic and natural polymers can be promising in the area of tissue engineering. The use of biodegradable microbeads can be expected to protect the biological activity of these labile molecules and thus can be able to avoid the rapid clearance. The technology can also be useful to release the molecule exactly at the local microenvironment in a controlled and regulated manner in a therapeutic limit.

The objective of this research is to prepare a few kinds of such bioactive porous scaffolds with controlled release function for application in cartilage, skin and bone tissue engineering. Porous scaffolds were prepared by freeze drying method and pore structure was introduced and controlled using ice particulates or ice-collagen particulates of controlled diameters. Controlled release function was introduced via biodegradable microbeads of synthetic polymer, poly lactic-co-glycolic acid (PLGA), or natural polymer, collagen. In 1st and 2nd parts of the work, insulin loaded PLGA microbead functionalized collagen porous

scaffolds with controlled pore structures were prepared and investigated for their application in cartilage and skin tissue engineering. In 3rd part of the work, dexamethasone loaded collagen microbead functionalized poly(L-lactide) (PLLA)-Collagen hybrid scaffolds with controlled pore structures were prepared as an osteoinductive platform for bone tissue engineering.

1. Preparation of collagen scaffolds with controlled insulin release for cartilage tissue regeneration

Scaffolds for cartilage tissue engineering require well controlled pore structures and controlled release of growth factors. Appropriate pore structures are important for spatial homogeneous cell seeding and distribution while controlled release of growth factors allows spatiotemporal provision of growth factors to control cell proliferation and differentiation. For example, during long term *in vitro* chondrocyte culture, internal necrosis is quite a common problem. To solve the problem, incorporation of insulin in porous scaffolds with a controlled release manner has been proposed because insulin is one of the growth factors applicable for survival of chondrocytes. And collagen has been frequently used to prepared porous scaffolds for cartilage tissue engineering. Therefore, in this part, collagen scaffolds with a controlled pore structure and controlled insulin releasing function were prepared and used for cartilage tissue regeneration. Insulin loaded PLGA microbeads were incorporated in collagen porous scaffolds to construct collagen-microbead hybrid scaffold by a freeze-drying technique. The pore structures of the hybrid scaffolds were controlled by using pre-prepared ice particulates having a diameter range of 150-250 μm as a porogen material. At first, insulin incorporated PLGA microbeads were prepared by using an emulsion solvent evaporation technique and ice particulates with a diameter 150 μm - 250 μm were prepared by spraying pure water in liquid nitrogen and sieving. Subsequently, the microbeads and ice particulates were homogeneously mixed with 2% (w/v) collagen solution. Finally, the mixture was freeze-dried and cross-linked to form collagen-microbead hybrid porous scaffolds. *In vitro* insulin release from hybrid scaffolds was studied by incubation in phosphate-buffered solution at 37 ºC under shaking condition. The hybrid scaffolds were used to culture bovine articular chondrocytes. After 1 week culture, cell viability and proliferation were evaluated.

The results indicated that collagen-microbead hybrid scaffolds had a controlled pore structure and interconnected pores. The microbeads showed an even spatial distribution throughout the pore walls of hybrid scaffolds. *In vitro* insulin release profile from the hybrid scaffolds suggested a zero order release kinetics up to period of 4 weeks without the appearance of a significant initial burst. The controlled insulin release from hybrid scaffolds was useful for survival of cells at inner pores of porous scaffold. Furthermore the hybrid scaffolds were useful to maintain higher cell proliferation potential than control groups. The collagen-microbead hybrid porous scaffolds with controlled pore structure and controlled insulin release should be useful as a candidate scaffolding material for cartilage regeneration.

2. Preparation of collagen porous scaffolds with sustained release of insulin for skin tissue regeneration

Based on the results from Part 1, we further hypothesized that introduction of an appropriate size microbead to a collagen porous scaffold could generate long term insulin releasing collagen-microbead

hybrid scaffolds. The development of bioactive collagen-microbead hybrid porous scaffolds with a long term insulin releasing ability could be useful for regeneration of ulcer skin in insulin dependent diabetes patients. To demonstrate the hypothesis, insulin loaded PLGA microbeads of two distinct sizes, 4.4 ± 0.9 μm and 19.4 ± 1.6 μm, were prepared using the emulsion solvent evaporation technique. Microbead size was controlled by controlling the homogenization speed of double emulsion. Microbeads were tested for their release feature from collagen porous scaffold of controlled pore structure prepared from 2% (w/v) collagen using similar method as described in Part 1. The hybrid scaffolds were used for culture of human dermal fibroblasts for 2 weeks. The prepared hybrid scaffolds had controlled pore structures and interconnected pores. The hybrid scaffolds demonstrated a controlled and sustained release of insulin. DNA quantification results showed that the released insulin from the hybrid scaffolds promoted proliferation of fibroblasts. The hybrid scaffolds prepared with 19.4±1.6 μm microbeads showed a more stable and higher sustained release of insulin and had higher promotion effect on cell proliferation than did the others. Incorporation of the microbeads of a size of 19.4 ± 1.6 μm in collagen porous scaffolds was found to be ideal for development of long term insulin releasing collagen scaffolds. The prepared scaffold should be useful for regeneration of ulcer skin of diabetes patients.

3. Preparation of a dexamethasone loaded collagen microbead functionalized PLLA-collagen hybrid scaffold for osteogenic differentiation of mesenchymal stem cells.

Direct differentiation of stem cell after proliferation in porous scaffolds capable of releasing bioactive instructive cues is important for development of osteoinductive porous scaffolds for functional bone regeneration. In this part, we prepared long term dexamethasone (Dex) releasing PLLA-collagen hybrid scaffolds of controlled pore structures as an osteoinductive platform for human bone marrow-derived mesenchymal stem cells (MSCs).

PLLA-collagen-Dex hybrid scaffolds were prepared by incorporating Dex loaded collagen microbeads in PLLA-collagen porous scaffolds by combining emulsion freeze drying method and porogen leaching method. Pre-prepared ice-collagen particulates (425 μm-500 μm) were used as a porogen material to control the pore structures and deposit collagen thin layers on the pore surface for easy cell seeding and adhesion. The amount of Dex in the hybrid scaffolds was controlled as 0.0, 2.5 and 5.0 weight % to the total dry weight of PLLA. The results indicated that hybrid scaffolds had controlled pore structures and interconnected pores deposited with collagen thin layers. The deposition of collagen thin layers in scaffold micropores facilitated homogeneous cell seeding. The spatial localization of collagen microbeads in the hybrid scaffolds provided spatiotemporal sustained release of Dex. The released Dex concentration from the hybrid scaffolds prepared with 2.5% of Dex was found to be the most effective formulation condition for induction of osteogenic differentiation in MSCs, which was confirmed from the elevated expression level of specific osteogenic marker genes after 1 and 3 week cell culture. Incorporation of 2.5% of Dex should be ideal for preparation of long term Dex releasing osteoinductive PLLA-collagen hybrid scaffold of controlled pore structure for application in bone tissue engineering.

In summary, the present research has introduced the design and preparation of three bioactive porous

scaffolds with controlled pore structures and controlled release function for tissue engineering. The scaffolds were prepared by freeze drying which is a simple and cost effective method. The pore structure was created and controlled using pre-prepared ice or ice-collagen particulates of selective diameters as a porogen material. Controlled release function was introduced using precise integration of drug delivery devices carrying specific bioactive molecules applicable for tissue regeneration. The bioactive molecule carrying drug delivery devices were spatially distributed in all the three dimensions of the porous scaffolds and facilitated a sustained and stable release of the cell inductive molecules in a bioactive form for quite a longer duration. The released bioactive molecules were useful for promotion of cell function such as viability, proliferation and differentiation. These hybrid scaffolds should be useful for tissue engineering of various tissues such as dermal tissue, cartilage and bone.

審　　査　　の　　要　　旨

〔批評〕

本論文では、軟骨や皮膚、骨などの生体組織を再生するために、薬物徐放性を有する多孔質足場材料の開発を目指して研究を行った。まず、ダブルエマルション法を用いて、インスリンを内包する乳酸/グリコール酸共重合体(PLGA)のマイクロビーズを作製した。作製した PLGA マイクロビーズとコラーゲン、ポローゲンとして直径 150～250 μm の氷微粒子を混合し、凍結乾燥法によりコラーゲン-PLGA マイクロビーズ複合多孔質材料を作製した。複合多孔質材料は氷微粒子と同じ大きさの細孔を持ち、インスリンを持続的に放出した。ウシ関節軟骨細胞を培養した結果、複合多孔質材料は軟骨細胞の増殖を促進した。このことから、本複合多孔質材料は軟骨組織の再生に有用であると考えられる。また、皮膚組織再生のための PLGA マイクロビーズ複合多孔質材料を作製し、PLGA マイクロビーズのサイズによる組織再生への影響を調べた。直径が 4.4 ± 0.9 μm と 19.4 ± 1.6 μm の2種類の PLGA マイクロビーズを作製し、コラーゲンと複合化した。作製した複合多孔質材料を用いてヒト皮膚の線維芽細胞を培養した。19.4 ± 1.6 μm のマイクロビーズと複合化した複合多孔質材料は、ほかの複合多孔質材料と比べて、より持続的なインスリンの放出挙動を示し、線維芽細胞の増殖をより促進した。これらの結果より、19.4 ± 1.6 μm のマイクロビーズの複合多孔質材料は皮膚組織の再生に有用であることが示唆された。さらに、骨組織を再生するために、デキサメタゾンを徐放するポリ乳酸(PLLA)−コラーゲン複合多孔質材料を作製した。デキサメタゾンを徐放するキャリアとしてコラーゲンマイクロビーズを用いた。PLLA 多孔質材料の細孔表面を親水化するために、コラーゲン水溶液を凍結した微粒子（直径 425～500 μm）をポローゲンとして用いた。デキサメタゾンを含有するコラーゲンマイクロビーズと PLLA 溶液及びコラーゲン水溶液の氷微粒子を混合し、凍結乾燥することにより、デキサメタゾンを徐放する PLLA-コラーゲン複合多孔質材料を得た。この複合多孔質材料を用いてヒト骨髄由来の間葉系幹細胞を培養し、間葉系幹細胞の骨分化を調べた。複合多孔質材料は間葉系幹細胞の接着を支持し、細胞が均一に分布していた。複合多孔質材料より放出されたデキサメタゾンは間葉系幹細胞の骨分化を促進した。特に、2.5%のデキサメタゾンを導入した複合多孔質材料は高い促進効果を示した。以上の結果により、インシュリンやデキサメタゾンを含有する PLGA 及びコラーゲンのマイクロビーズとコラーゲンや PLLA と複合化し、これらの生理活性物質の時間

的・空間的な徐放を制御できる複合多孔質材料を開発した。細胞培養の結果から、これらの複合多孔質材料は軟骨や皮膚、骨などの組織の再生に有用であることが示された。本研究で得られた結果は、組織工学のための多孔質足場材料の設計と創出に重要な学術的知見を含むものと考えられる。よって、本論文は博士（工学）の学位論文として十分な学術的な価値をもつものと認める。

〔最終試験結果〕

平成26年6月23日、数理物質科学研究科学位論文審査委員会において審査委員の全員出席のもと、著者に論文について説明を求め、関連事項につき質疑応答を行った。その結果、審査委員全員によって、合格と判定された。

〔結論〕

上記の論文審査ならびに最終試験の結果に基づき、著者は博士（工学）の学位を受けるに十分な資格を有するものと認める。