R-2. Evaluation on the biocompatibility, bone cell activity and bone regenerative capacity of chitosan-PLLA bilayer porous membrane

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Chitosan is a biodegradable natural biopolymer that has been shown to improve wound healing, and to enhance bone formation. It is non-immunogenic and regulates the release of bioactive agent. It can be produced in various forms including film, sponge, and fiber. The chitosan functions as potential vehicle for the growth factors to promote osteogenesis and as high biocompatible surface of membrane. The poly(L-lactic acid)(PLLA) is intended to both support the chitosan fibrous mesh and separate the soft tissue. The purpose of this study was to investigate the application of chitosan-PLLA membranes as barrier membrane and tissue engineering scaffold for bone formation in osteoblasts culture.

Chitosan fibers were prepared by extruding 4% chitosan solution in 4% acetic acid into pH 13 aqueous solution. Chitosan fibrous meshes were fabricated by pressing chitosan fibers. PLLA-methylene chloride-ethylacetate solution cast on between the two chitosan fibrous meshes using a doctoring blade, and solvents were evaporated in air at 25°C for 24h and further dried under vacuum for 24h to remove residual solvents. Surface and cross-section structures of the membranes and the configuration of their inner pores were observed by scanning electron microscopy(SEM). Prewetted matrices with complete media were placed in 24-well plates. Aliquots of 100l of osteoblast-like cell line(MC3TC3E1) were seeded onto the top of the sponges resulting in a seeding density of 104 cells/matrix. Medium was changed every 2-3 days and cultures were maintained in a humidified atmosphere consisting of 95% air and 5% CO2 at 37℃. Osteoblast proliferation was determined at 1, 7 and 14 days. The adherent cells were released by incubation in 300l of 0.25% trypsin in 4 mM EDTA for 10 minutes. After digestion, the matrices were washed with 300l of HBSS for collecting retained cells. Cells in trypsin/HBSS solution were counted by the hemacytometer . Cultured cell-membrane constructs were incubated at low temperature(in ice) in a fixative of 2.5% of glutaraldehyde for 20 minutes and then washed in PBS for 10 minutes twice. The complexes were then incubated for 20 minutes in a postfixative of 1 % aqueous OsO4 and subsequently washed with PBS for 10 minutes. Samples were then sequentially washed with in ethanol of increasing(70, 80, 90, 95, and twice in 100%) concentration for 10 minute-wash. This step was performed to dehydrate the cells. Cell-matrix samples were allowed to air dry overnight. They were coated with 20nm of gold-palladium and examined by SEM. RNA was isolated from trypsinized cells and isolated RNA was used to create a total cDNA library with the Kit for RT-PCR Kit. The resulting reverse transcriptase product were expanded using the primers specific to the sequences of interest

(collagen type , osteopontin, and osteocalcin). The determination was done by gel electrophoresis on agarose gel. Sprague-Dawley rats were used in this study. Rats were anesthetized by intraperiotoneal injection of ketamine(30mg/kg body weight). The surgical site was made in the sagittal plane across the cranium. A full-thickness flap including periostem was reflected, exposing the parietal bone. An 8 mm diameter full-thickness calvarial defect was made using saline cooled trephine drill. Extreme care was taken to avoid injury to the brain. After removal of the trephined calvarial bone, chitosan coated PLGA matrices were implanted into the defects. No carriers or other regenerative materials were placed in the control group defects. The periosteum and skin were closed using 5-0 chromic gut and 4-0 silk sutures, respectively. Bone regenerative efficacy of the

PLLA membrane having 2-10㎛ pore size was observed between the two chitosan fibrous meshes. The pores were generated in the sublayer of the chitosan-PLLA membrane by using an in-air drying phase inversion technique. This pore size may be proper to prevent connective tissue (~30㎛) ingrowth and permeate the nutrients. In cell attachment level, the membrane was similar to polystyrene culture plate which was known for its good cell attachment an proliferation. The osteoblasts continued to proliferate throughout the period. The SEM of the attachment of osteoblasts to the membrane revealed well attached state showing cytoplasmic extension. The number of cells were evidently increased and some of the pores of the membrane were partially filled with the proliferated cells at the14th day. The expression of collagen type , osteopontin and osteocalcin mRNA level at 1, 4, 7, and 14 days. Seeded osteoblasts retained their biochemical phenotype-specific characteristics for bone formation throughout the entire culture period as evidenced by the production of collage type , osteopontin and osteocalcin. No evidence of inflammatory tissue reaction was revealed at any specimen and all wounds showed a good healing response. At 4 weeks postsurgery, non-membrane-treated defects were invaded by thin, loosely organized connective tissues. In the membrane-treated defect, bone regeneration had advanced from the periphery and from the dural surfaces of the defect beneath the membrane.

Chitosan-PLLA membrane showed high cell attachment level attributed by tissue compatibility of chitosan. The chitosan-PLLA barrier membrane might be a valuable modality in periodontal regenerative therapy.

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