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ENGINEERING TUBLAR BONE CONSTRUCTS

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Abstract

Cell sheet techniques have proven effective in various soft tissue engineering applications. In this experiment, we investigated the feasibility of bone tissue engineering using a hybrid of mesenchymal stem cell (MSC) sheets and PLGA meshes. Porcine MSCs were cultured to a thin layer of cell sheets via osteogenic induction. Tube like long bones were constructed by wrapping the cell sheet on PLGA meshes resulting in constructs which could be cultured in spinner flasks，prior to implantation in nude rats. Our results showed that the sheets were composed of viable cells and dense matrix with a thickness of about 80-120μm, mineral deposition was also observed in the sheet. *In vitro* cultures demonstrated calcified cartilage-like tissue formation and most PLGA meshes were absorbed during the two-month culture period.. *In vivo* experiments revealed that dense mineralized tissue was formed in subcutaneous sites and the two-month implants shared similar microCT characteristics with native bone. The neo tissue demonstrated histological markers for both bone and cartilage, indicating the bone formation pathway in constructs was akin to endochondral ossification, with the residues of PLGA having an effect on the neo tissue organization and formation. These results indicate cell sheet approaches in combination with custom shaped scaffolds has great potential in producing bone tissue.

Introduction

A tissue engineering approach for addressing tissue reconstruction is to engineer new tissues by using selective cell transplantation on polymer scaffolds (1). In this cell-scaffold based tissue engineering concept, seeding of isolated cell suspensions into scaffolds often encounter problems in producing high cell density and delivery efficiency. To achieving superior cell delivery and preservation of cell-cell contact, several researchers have attempted to use cell sheet technique for tissue engineering. Okano's group invented the use of single sheet of cultured corneal epithelial cells and multilayered cardiomyocytes sheets for engineering transplantable corneal and myocardial tissues respectively (2,3). Cooper and Pouliot et al utilized dermal fibroblasts sheets for skin (4, 5) and L'heureux and colleagues reported human blood vessel engineering using endothelial and smooth muscle cell sheets (6).

Current cell sheet techniques provide sheets which are strong enough to allow careful manipulation in a laboratory to produce stacked or wrapped constructs, (7), however, they contract extensively upon detachment from culture surfaces and lack the mechanical strength demanded for bigger bone defects. Moreover, limited cells types are reported to form cell sheet and most are terminally differentiated cells with inadequate lifespan and differentiation potential. The drawbacks of cell sheet techniques restrict their application in engineering large-size tissues, especially bone tissue. To overcome these problems, cell sheet techniques can be combined with scaffolds used in current bone engineering strategies and this combination could achieve advanced cell delivery, which is not possible using the traditional cell

suspension or hydro gel systems, and can result in dense, mineralized tissue formation.

In bone tissue engineering fields, polymer scaffolds are considered promising bone substitutes for bone regeneration due to superior mechanical properties and biodegradability (8). Synthetic polymers are easy to process into predetermined shapes. Amongst them, PLGA has been well characterized and has shown good biocompatibility and fast degradation, and is possibly the most widely used synthetic polymer in biomedical area (9, 10). Studies demonstrated bone marrow mesenchymal stem cells (MSC) seeded on PLGA derived foam or scaffolds can lead to mineralized tissue formation. However, a study repairing rabbit defects revealed less optimal neo bone growth in PLGA derived scaffolds which may relate to the lower efficiency of cell loading (11).

In order to fully utilize the advantages of cell sheet techniques and overcome cell seeding problem in polymer scaffolds, this study exploited the feasibility of combining MSC sheets with PLGA scaffolds in bone tissue engineering. We firstly achieved the multipotent mesenchymal stem cell sheets through stimulation of matrix formation and then wrapped the cell sheets on PLGA meshes to form tube-like constructs. The constructs underwent dynamic culture and *in vivo* implantation and results demonstrated that it is possible to regenerate bone tissue by combining mesenchymal stem cell sheet techniques with scaffolds.

Materials and methods

PLGA mesh scaffold

PLGA fibers were obtained by de-braiding of Vicryl sutures (Johnson and Johnson, USA). The fibers were formed into thin non-woven meshes in the size of $2x4cm^2$.

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Cell isolation and culture

Three month old Duroc/Yorkshire cross pigs were obtained from the animal holding unit of National University of Singapore. The study has been reviewed and approved by the animal holding unit of National University of Singapore. Porcine bone marrow MSC were isolated and cultured as reported previously (Chen et al). Briefly, Porcine MSCs were aspirated from marrow and after gradient centrifugation; cells were cultured in low glucose Dulbecco's Modified Eagle's medium (DMEM) (GIBCO, Invitrogen, CA, USA) containing 2% fungizone (Sigma, MO, USA) and 1% antibiotics (100μg/ml penicillium and 100μg/ml streptomycin) at 37°C and 5% CO_2 humidified environment. Cells of the first passages were used for all experiments. Cells were seeded and cultured in $150cm^2$ flasks until confluent.

Construction of hybrid of cell sheet and PLGA mesh

Confluent cultures were differentiated along the osteogenic lineage using ascorbic acid 2-phosphate (50 μg/ml), β-Glycerophosphate(10mM) and Dexamethasone (100nM) in DMEN high glucose. Culture medium was changed every two days. After approximately 2-3 week's culture, a thick sheet of cells was obtained. The cell sheets were lifted carefully with cell scrapers as shown in Fig 1.

The sterilized PLGA meshes were placed in a culture dish. Tubes from 1ml syringes with 3 cm length were used as mold for construction of the hybrid constructs. One cell sheet was wrapped around the PLGA meshes, and the hybrid was then rolled around the syringe. After the rolling of the first sheet, the next sheet was wrapped over the previous tube. The tube was transferred into a 6-well plate for three days before being transferred to a dynamic spinner flask. During the dynamic culture, the syringe support was removed. The rotating speed of tube in the flask was 12 rounds per minute and the culture period was 4 or 8 weeks. The composites of 8 weeks were split into half and one half was used for *in vivo* implantation.

Microscopy observation

For SEM, samples were fixed with 5% gluteraldehyde (Sigma) for 2 hours followed by serial dehydration with ethanol. Samples were then sputtered with gold and examined by XL30SEM (FEI Inc, OR, USA) at 10Kv.

For TEM, the sheets were fixed with 5% glutaraldehyde for 2 hours and post fixed in 1% osmium tetroxide. The samples were embedded in resin after serial ethanol dehydration. Ultra-thin sections of about 50-60 nm were prepared and stained with 2% uranyl acetate and Reynold's lead. The sections were observed under JEM1220 (JEOM Ltd, Tokyo, Japan).

Implantation

Immuno-deficient nude rats, aged 3-4 months, were used as subcutaneous transplant recipients. Operations were carried out under anesthesia in accordance with protocols approved by the IACUC. After aseptic preparation, four subcutaneous pockets were produced by small scissors on the back of each rat, wherein the constructs were implanted. Two months after implantation, the animals were sacrificed and specimens were harvested. The specimens were fixed with 10% phosphate-buffered formalin for further analysis.

MicroCT scan

Skyscan *in vivo* microtomograph, 1076 (Philiphs, UK) was used at a scanning resolution was 35 µm, with an averaging of 5. A 1mm thick aluminum filter was used, with a rotation angle of 180° and a step size of 0.8°. Approximately 500 scan slices were taken and files were reconstructed at a step size of 4 using a modified Feldkamp algorithm as provided by Skyscan. The output was a series of 120 serial 1968 x 1968 bitmap images. 3D visualization was achieved using Mimics. A threshold was carried out and the range used to visualize bone mineralization was -314 to 2265 HU. In order to ascertain the degree of mineralization, the threshold range was subdivided into 3 regions. They were -314 to 546 HU, 547 to 1407 HU and 1408 to 2265 HU. The highest range indicated a high degree of bone mineralization.

Histology

Specimens were demineralized with formic acid, dehydrated and embedded within paraffin. The sections were stained with hematoxylin and eosin (H&E) staining; safranin-O/fast green (S/F) staining, and alizarin red staining. The protocol for H&E, S/F and alizarin red staining has been reported previously. Immunostaining was carried out with monoclonal antibody against osteocalcin (OCN) (Sigma) with the DAKO Animal Research kits (Dako, USA).

Results

Fabrication of cell-sheet/PLGA

The MSC proliferated fast and formed cell sheets within 3 weeks after confluence in the presence of osteogenic cocktail, the cell sheet formed could be lifted easily with a cell scraper (Fig. 1). Mineralized nodules could be observed on the cell sheet, both microscopically and macroscopically. Transmission electron microscopy (TEM) analysis showed that there was abundant rough endoplasmic reticulum in the plasma with fine crystals of minerals in the matrix (Fig.2). SEM revealed that the cells were trapped in the dense matrix and cell frame could not be recognized (Fig.2). The thickness of sheet formed in culture flasks was about 80-120μm and extensive collagen type I expression was observed (Fig.2F).

When the sheet was assembled onto the PLGA meshes, the cell sheets adhered to the mesh after l hr as demonstrated by a lack of detached cells-sheet in the medium. After three days of static culture, the composite became a whole unit and was ready to transfer into spinner flasks.

***In vitro* culture**

During the culture in the spinner flask, the composites of sheet and PLGA mesh maintained the initial shape of the tube. The tissue in constructs comprised two distinct parts; one the dense sheet tissue and the other was PLGA fibers with fibrous tissue located in the middle after one month culture (Fig 3A,B). In two month culture samples, two layers of cartilage-like tissue were observed throughout the composites with typical lacunae within cells and positive safranin –O staining. (Fig.3 C-F). The cartilage like tissue was located on both sides of PLGA mesh. Very few PLGA fibers were detected in the constructs, instead, fibrous tissue was present. Calcium depositions were detected in the middle of constructs in both 4 week and 8 week samples as indicated by positive alizarin red stain. The cartilage-like tissue in constructs showed no calcium deposition (Fig. 3G,H).

Implantation

Gross inspection showed that the newly formed bone graft maintained the initial shape of the cylindrical tube (Fig.4A). Micro CT scanning results showed that the density of mineralized tissue was either similar to that of cancellous or cortical bone (Fig 4B-D).

The tissue engineered bone was more unevenly distributed in the inner part (Fig.5 A,B). Typical osteocyte-like cells were embedded in dense matrix surrounded by marrow cavities. Cartilage-like tissues were also present displaying hypertrophic

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chondrocyte morphology and positive staining for safranin-O (Fig.5 C-F). The cartilage tissue was randomly and discontinuously located in the constructs. Consistent with the fibrous tissue formed *in vitro*, the fibrous tissue was detected in the center of constructs. Specifically, the bone matrix protein OCN was detected in limited neo bone tissue areas and very weakly detected, if at all, in the cartilage like tissue (Fig.5G,H).

Discussion

The report demonstrated that the MSC can form dense cell sheets *in vitro* and the MSC sheets can be shaped into tubular-like form with the support of a PLGA scaffolds. The hybrid of PLGA meshes and MSC sheets formed highly mineralized tissue. The neo bone tissue and the cartilage-like tissue were observed in constructs after two months implantation, and showed a similar composition to cortical bone as shown by microCT scans.

In the present study, MSC formed dense cell sheets within 2 weeks in the osteogenic induction condition, which comprised ascorbic acid (AA), glycerophosphate (GP) and Dex. AA is thought to be involved in collagen synthesis and deposition, GP stimulates mineral deposition and Dex stimulates cell proliferation and promotes cell ECM synthesis (12, 13). Therefore, MSC grew as multilayered cells intertwined within a mineralized collagen matrix. After wrapping on the PLGA mesh, the multilayered sheets formed condensed layers with high cell density. This demonstrated a clear sheet -technique advantage in cell delivery. The sheets could preserve the cell-cell interaction as cells were connected by their own matrixes and then seeded into scaffolds with high efficiency. At the same time, when cell sheets were wrapped into multilayered dense sheets, the cells in constructs should experience the condensation process due to the cell self-contraction (14). This condensation could promote the dense sheets further integrating as whole tissues and resulted in a well organized tissue pattern as observed *in vitro* constructs shown in Fig 3.

The hypothesis of this current study was that it is feasible to engineer a bone substitute construct without alien materials before implantation. This could be achieved after the degradation of the PLGA scaffold during *in vitro* culture. Before implantation, the construct was composed of living autogenous cells and matrix secreted by the cells themselves. In the sheet-scaffolds construction, PLGA meshes worked as a support to sustain the construct's morphology and were strong enough to resist the physical loading of cell sheets and hydro pressure.. After a months culture, the PLGA degraded by hydrolysis or cell actions and cell matrix was formed and replaced the function of PLGA mesh scaffolds. The PLGA voids were mainly replaced with fibrous tissue after *in vitro* culture and implantation and the fibrous tissue showed less clear nuclear stain than other tissues formed in constructs (Fig 3). This indicated that acidic milieu arising from PLGA degradation might interfere with cellular interaction and the matrix formation, and might even be toxic to cells. Meanwhile, the byproducts from PLGA may also attribute to the discontinuous and limited neo bone formation in implants. This observation was consistent with reports from other groups, who demonstrated that PLGA byproducts cause inflammatory response and interfere with tissue formation (11, 15, 16). However, some groups state that PLGA is a promising candidate-biomaterial for bone formation in both *in vitro* and *in vivo* (17, 18). The discrepancy may be related to the use of different PLGA sources since PLGA has various molecular weights and different compositional ratios derived from manufacturers. Another possibility is that the long observation time in our study (over 4 months) should make it possible to see the inflammatory cell

response to PLGA by-products whereas some other studies incorporated only a one to two month observation period. This period is too short to study the breakdown of the PLGA.

Cartilage-like tissue with distinct glycosaminoglycan (GAG) staining was observed in the *in vitro* constructs which were cultured in osteogenic medium. The formation of cartilage-like tissue should be the possible consequence of the specific spinner culture system. Constructs in spinner flasks were deeply immersed in the medium and experienced much higher water pressure, shear force and lower oxygen conditions than conventional culture. These hypoxia and higher biomechanical forces are reported as possible stimulation factors for the chondrogenesis process (19, 20). Moreover, the intrinsic, cell generated contraction forces might play a critical role in the specific type of tissue development (21).

Histologically, the neo bone and cartilage-like tissues were scattered and unevenly distributed in the constructs even though the constructs demonstrated similar mineral density as native bone. The possible reasons may be attributed to the PLGA by-product effects or the long *in vitro* culture time, which has previously been reported to lower the MSC differentiation capability (22). Moreover, the *in vivo* remodeling process may also affect the pattern of neo tissue distribution. The implants have a size over 10x10x10mm^3 and were encapsulated by nude rat skin. This closed contact with rat skin could exert extensive compressive force on the implants and resulted in the disorganized neo bone distribution (23). Further experiments aiming at better neo tissue organization could utilize polymers with slow degradation

properties and could also shorten the *in vitro* culture time points or create an optimal space for neo tissue growth.

Conclusion

This study reports the possibility of manufacturing MSC sheets and the techniques of engineering a tubular bone construct. The results suggest that the concept of combining MSC cell-sheets with scaffolds offers great promise to provide a template for engineering tube like bone which can be used for bone reconstruction or tissue engineering research. However, further studies still need to underscore the optimal culture conditions and choice of scaffold material.

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