A novel recirculating flow-perfusion bioreactor for periosteal chondrogenesis

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Abstract

Purpose Previous research indicated that engineered cartilage was soft and fragile due to less extracellular matrix than native articular cartilage. Consequently, the focus of this study was mostly confined to application in vitro function. In order to generate 3D engineered cartilage resembling native articular cartilage, we developed a recirculating flow-perfusion bioreactor to simulate the motion of a native diarthrodial joint by offering shear stress and hydrodynamic pressure simultaneously.

Materials The bioreactor we developed offers steady oscillating laminar flow (maximum shear stress of 250 dyne/cm²) and hydrodynamic pressure (increased from 0 to 15 psi) simultaneously. The periosteal explants were harvested from the proximal medial tibiae of rabbits and fixed onto PCL scaffold with four corner sutures and cambium layer facing upward, then these periosteal composites (periosteum/ PCL) were placed into the culture chamber of our bioreactor for six weeks in vitro culture.

Results The cartilage yield in our recirculating bioreactor was 75–85%. The outcome was better than the 65–75% in the spinner flask bioreactor (shear stress only) and 17% in static culture. In addition, there was a significant difference in the cell morphology and zonal organisation among the three methods of culture; the engineered cartilage in the

recirculating bioreactor presented many more characteristics of native articular cartilage.

Conclusions If the environment of culture provides the shear stress and hydrodynamic pressure simultaneously, the composition of the engineered cartilage resembles native articular cartilage, including their ECM composition, cell distribution, zonal organisation and mechanical properties.

Introduction

Articular cartilage is a specialised tissue, devoid of blood vessels, lymphatic channels, and innervation [1], and it has a limited capacity to heal when damaged [1–3]. It is also a specialised tissue that is precisely suited to withstand the highly force-loaded joint environment [4, 5]. Previously, research has focused on biochemical and biomedical application of tissues in vitro, but their overall function has been largely ignored. Most engineered cartilage becomes soft and fragile and has poor mechanical properties. However, the concept of “functional tissue engineering” [6, 7] has become a critical issue that involves the application of physical loading in order to promote the development of tissue constructions that can adapt the mechanical demands encountered in vivo, especially the bone, cartilage, and tendon tissues required in orthopaedics [8]. In our previous study [9], we found there was a high percentage of chondrocytes with a homogenous distribution in engineered cartilage grown in static culture. This engineered cartilage became soft and fragile because of the lower extracellular matrix (ECM) composition compared to native articular cartilage. Hence abundant ECM within the engineered cartilage is essential to improve this tissue’s mechanical properties.

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Periosteum, a natural tissue-engineering material, possesses three prerequisites for tissue engineered cartilage repair [10–12]. It contains undifferentiated mesenchymal stem cells with the potential to form cartilage in the presence of TGF- β 1 or shear stress [9, 13], it functions as a scaffold onto which these cells can adhere, and it produces bioactive factors that enhance chondrogenesis. According to previous experiments [14, 15], periosteum is sensitive to mechanical stress and, as such, it provides an opportunity to study the effects of mechanical stimuli on chondrogenesis and cartilage metabolism. Therefore, we designed an experimental model to investigate the relationship between a variety of mechanical stimuli and ECM production, heterogeneous cell morphology in order to improve the arrangement and zonal organisation of engineered cartilage.

Bioreactor culture systems provide the fundamental mechanisms of cell function in a 3D environment and improve the quality of engineered cartilage [16]. In our previous experience using a spinner flask bioreactor [9], only shear stress was encountered by the periosteal explants. The spinner flask bioreactor is too simple to offer a steady laminar flow and generated an irregular surface on the engineered cartilage due to turbulent flow. In addition to the shear stress, we believe dynamic compression force is also important for proper cartilage development. We therefore developed a recirculating flow-perfusion bioreactor that simultaneously offers shear stress and hydrodynamic pressure, and simulates the motion of native diarthrodial joint to generate 3D engineered cartilage with ECM composition, cell distribution and zonal organisation as close as possible to those of native articular cartilage.

Materials and methods

Periosteal harvesting and culture

All work in this study was approved by the Institutional Animal Care and Use Committee of Kaohsiung Veteran General Hospital. All periosteal explants were harvested by

sharp subperiosteal dissection from the proximal medial tibiae of two-month-old New Zealand white rabbits (Fig. 1a). Immediately, the periosteal explants were secured onto poly- ϵ -caprolactone (PCL) scaffold with four corner sutures and cambium layer facing upward after harvesting (Fig. 1b). These periosteal composites (periosteum/ PCL) were placed into the culture chamber of our design of bioreactor (Fig. 1c). The bioreactor contained 700 ml of DMEM with penicillin/streptomycin (50 U/ml and 50 μ g/ml) and supplemented with ITS+(2.08 μ g/ml each of insulin, transferring, and selenious acid, plus 1.78 μ g/ml linoleic acid and 0.42 mg/ml BSA), 1 mM proline, and 50 μ g/ml ascorbic acid. The medium was replaced once every week, and the cultures were maintained at 37°C in 5% CO₂ and 95% air.

Recirculating flow-perfusion bioreactor

This bioreactor was a closed system (Fig. 2) and it could be divided into three parts: a culture environment, a control system, and a power source. First, there was one culture chamber and two reservoirs in the bioreactor. All intraluminal areas were maintained as sterile during medium recirculation. The periosteal composites were placed on the bottom of the culture chamber and received shear stress from oscillating flow due to transfer of the medium back and forth between the two reservoirs. Second, the control system was made up of a programmable controller (PLC) and electronic valves, which were used to control the hydrodynamic pressure and shear stress. Finally, we used an oil-free air compressor as the power source. The bioreactor requires the compressor to pump an air mixture, which contained 5% CO₂, into the medium and produce hydrodynamic pressure. Shear stress was generated from the back and forth motion of the medium between the two reservoirs. Hence, the bioreactor simultaneously offered shear stress and hydrodynamic pressure.

The bioreactor consisted of two reservoirs (Fig. 2), and there were four connectors on the top of reservoir A (left side). A mixture of 5% CO₂/air was pumped into reservoir A via the first connector (air in) before the air filter. The second connector (medium in) allowed the circulating

Fig. 1 **a** Periosteal explants were harvested from the proximal medial tibiae. **b** They were secured onto PCL scaffold with four corner sutures and cambium layer facing upward. **c** These periosteal composites were placed into the culture chamber

Fig. 2 Illustration of the experimental setup for culturing periosteal explants in recirculating bioreactors after suturing to PCL scaffolds. **a** The periosteal explants received hydrodynamic pressure (0–15 psi) when D1 and E1 were opened and E2 closed. **b** They received shear stress (maximum 250 dyne/cm²) when E2 was opened. The circulation was reversed from reservoir B to A when gates D2, E2 & E1 were open and gate D1 was closed

medium to pour into reservoir A from reservoir B (right side) via the culture chamber. The third connector (pressure sensor) monitored the pressure grading of the closed system. The last connector (air out) functioned as a one-way safety valve and served to drive out redundant air or overflow medium. The remaining connector (medium out) was located beneath the bottom of reservoir A.

First, a mixture of 5% CO₂/air was pumped into reservoir A. During this period, the pressure in the culture chamber is increased from 0 to 15 psi when D1 & E1 electronic valves were opened. After ten seconds, gate E2 was also opened by the PLC, allowing the medium to flow from Reservoir A, through the culture chamber, and into Reservoir B due to the different pressure grading between the two reservoirs. According to the equation presented in an earlier report, the medium flow could be controlled and provide a maximum shear stress of 250 dyne/cm². The redundant air was driven out when D1 & E1 electronic valves were closed after medium drain out in reservoir A. The circulation was reversed from reservoir B to A when gates E2 & D2 were open and gates E1 & D1 were closed. The shear stress was derived from oscillating flow driven by the different pressure grading between the two reservoirs.

Cartilage yield assay

A standardised cartilage yield assay and histological scoring were performed after six weeks in static culture, a

spinner flask bioreactor, or a recirculating bioreactor. Specimens were fixed in 10% neutral formalin buffer and embedded in paraffin. Sections were cut (3-μm thick) from the central portion of the parallel long axis of the engineered cartilage and stained with Safranin-O/fast green for histological analyses [17]. An automated histomorphometry method was used to determine the percentage of red staining in our samples (i.e., cartilage yield), as previously described and validated [18].

Results

Bioreactor

Our bioreactor can offer oscillating shear stress and hydrodynamic pressure simultaneously for engineered cartilage culture in vitro. A finite element method analysis of the flow field was done using ANSYS software (ANSYS, Canonsburg, PA, USA) to show that the fluid flow in the bioreactor was laminar flow not turbulent. There were smooth surfaces of all engineered cartilage cultured in the bioreactor. It also provided oscillating fluid flow to evenly distribute shear stress on the periosteal explants (maximum shear stress of 250 dyne/cm²) and simulate the motion of the diarthrodial joint completely. The hydrodynamic pressure was generated from the mixture of 5% CO₂/air pumped into the reservoir, which also served to drive out

the redundant air after the medium had flowed out of the reservoir. The bioreactor also provides hydrodynamic pressure from 0 to 25 psi. This is important because ECM synthesis increases relative to the growth of the engineered cartilage, resulting in decreased diffusion of nutrients and oxygen over time. As such, the hydrodynamic pressure should also be increased over the duration of the experiment.

Cartilage yield in the engineered tissue

Although there was no mechanical stimulation in the periosteal composites, the cartilage yield [18] (based on percentage of cartilage) of 17% in the composites was suspended/immobilised in the spinner flask without flow. The periosteal explants were fully expanded, and the cambium layer of the periosteum was also kept completely exposed and facing upward. It's easy to diffuse nutrition, oxygen and waste and lower cell proliferation and slightly increase ECM synthesis (Fig. 3a). The use of fluid flow shear stress in the spinner flask bioreactor groups resulted in approximately a four- to fivefold increase in cartilage yield compared with static culture (Fig. 3b). Although the extracellular space was full of matrix under shear stress, cartilage yield of 65–75% resulted because the cell ratio was too high. The cartilage yield of 75–85% developed in our recirculating bioreactor because of lower density of cell (Fig. 3c). On the other hand, hydrodynamic pressure

can stimulate more ECM product in our bioreactor (Fig. 4a).

Cellular organisation

There was a significant difference in the cell morphology and zonal organisation between the tissue samples grown in the two bioreactors and those in the static culture group. Homogenous cell morphology and uneven distribution over the engineered cartilage was observed in static culture group (Fig. 3a), which produced histological scores [19] of zero or one. Increased heterogeneity and organisation of the cells resembling articular cartilage was apparent in the tissues exposed to shear stress. Two different zones based on variations in cell morphology and matrix distribution were clearly observed in tissues grown under shear stress in the spinner flask bioreactor (Fig. 3b). We found small, high density, round cells with a high ratio of ECM in the superficial layer because of the larger shear stress that occurred during culture and large round cells with lower density ECM in the deep layer. Although a cartilage yield of 65–75% and two different layers were identified, the cell morphology and zonal organisation in the shear stress group was unlike that of native articular cartilage, and this group received histological scores of one to two. Three different zones were apparent in the engineered cartilage grown in our recirculating bioreactor (Fig. 3c). Many flat cells were found on the surface of the engineered cartilage

Fig. 3 **a** Homogenous cell morphology and distribution and low density ECM were found over the engineered cartilage in this static culture. **b** The shear stress enhanced different cell morphologies, increased the ECM ratio, and promoted the formation of two different zones (yellow arrow head area) in a spinner flask bioreactor. **c** The significantly different three layers of engineered cartilage were grown in a recirculating bioreactor, including many flat cells in the superficial zone, a large amount of ECM with large round chondrocytes in the lacuna within the middle zone and high density small round cells concentrated in the deep zone. The cell morphology, arrangement and zonal organisation of this group were similar to native articular cartilage (by Safranin-O stain)

Fig. 4 **a** This increase was significant ($p < 0.0001$), with a cartilage yield of 17% in the static culture compared with 65–75% in the spinner flask (shear stress only). The cartilage yield of 75–85% in our recirculating bioreactor was a significant increase ($p < 0.05$) to the spinner flask bioreactor because of a lower cell density. **b** These scores

reflect cell morphology, amount of matrix, and zonal organisation, with a score of 3 indicating a resemblance to native articular cartilage. There were significant differences in zonal organisation and heterogeneity scores among the different culture groups ($p < 0.05$)

due to the shear stress stimuli, and a low density of large round cells within lacuna and abundant ECM were found in the middle layer because of the hydrodynamic pressure stimuli. Most uniform-sized small round cells with a tight arrangement were observed in the deep zone. We believe these cells obtained sufficient nutrition and oxygen due to the increased diffusion promoted by hydrodynamic pressure. Furthermore, the cell morphology and zonal organisation of these tissues was similar to native articular cartilage. A cartilage yield of 75–85% was observed in this recirculating bioreactor group, which received histological scores of two to three (Fig. 4b).

Discussion

In most studies, chondrocytes are seeded onto 3D scaffolds and grown in static cultures. However, researchers must worry about central necrosis of the engineered cartilage as it is growing because it is usually difficult to diffuse nutrients, oxygen, and waste within the scaffolds. The native articular cartilage is a thin layer (approximately 1–5 mm thick) of hydrated soft tissue covering the ends of all synovial joints [2, 3]. We think that periosteal chondrogenesis is a good experimental material for generating replacement articular cartilage, as it may be useful for creating an appropriately sized (larger) slice of cartilage. We designed an experimental model for these periosteal explants by securing them to PCL scaffolds with four corner sutures and the cambium layer facing upward. There are many advantages to this model. First, the periosteal explants were fully expanded and immobilised on the PCL

scaffolds, avoiding periosteal contracture and keeping the cambium layer of the periosteum completely exposed and facing upward. Second, there was an increased diffusion of nutrients, oxygen, and waste because the periosteum was very thin (<1 mm) under full expansion. As such, the cells obtained adequate nutrients and oxygen (avoiding central necrosis) and were also stimulated by growth factors present in the culture medium. Third, the undifferentiated stem cells of the cambium layer were easily stimulated by shear stress and hydrodynamic pressure. In this study, the use of a porous PCL scaffold as a solid support for the explanted periosteum enabled us to determine the effects of directional fluid flow shear stress and hydrodynamic pressure on this tissue using our recirculating bioreactor. Furthermore, we hope the thinner, wider, and smoother surface of the engineered cartilage manufactured by periosteal chondrogenesis will significantly aid in the repair of larger cartilage defects.

As we know, native articular cartilage consists of a low density of chondrocytes (<10%) and a high percentage of ECM [2]. The ECM provides mechanical strength to the hyaline cartilage. In the past, we found a high percentage of homogeneously distributed chondrocytes and low amounts of ECM in the engineered cartilage grown in static medium cultures. These engineered cartilages were soft and fragile and demonstrated poor mechanical strength. However, in our recirculating bioreactor, the hydrodynamic pressure can control chondrocyte over-growth and stimulate increased ECM secretion from chondrocytes. These chondrocytes produce more ECM to resist the increased pressure, protect themselves, and to increase the mechanical properties of the engineered cartilage. Therefore, the ECM/chondrocyte ratio

was highest in the engineered cartilage grown in our recirculating bioreactor. On the other hand, we also found many small flat cells on the surface of the engineered cartilage (due to shear stress stimuli) and a large amount of ECM with large round chondrocytes in the lacuna within the middle layer (Fig. 3c). This increased amount of ECM likely resulted in superior mechanical properties of the engineered cartilage.

The native articular cartilage is an anisotropic material that can resist a variety of mechanical forces. It is divided into three zones based on differences in cell morphology, arrangement, and matrix biochemistry. Similarly, we believe various mechanical forces also influence cell morphology, arrangement, collagen orientation, and matrix content and distribution. In our studies, the histological appearance of the engineered cartilage grown in the different culture environments showed significant differences in zonal organisation and heterogeneity.

In view of these results, we propose that engineered cartilage must be cultured in a mechanically stimulated environment. We have designed a recirculating flow-perfusion bioreactor that simultaneously offers oscillating shear stress and hydrodynamic pressure, simulating the motion of a diarthrodial joint, for growing engineered cartilage in vitro. Shear stress enhanced cell proliferation and differentiation and a lower amount of matrix synthesis, and hydrodynamic pressure stimulated increased ECM production, heterogeneous cell morphology, and improved the arrangement and zonal organization of the tissue. The recirculating flow-perfusion bioreactor engineered cartilage with good mechanical properties, similar to native articular cartilage.

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