Engineering of human tracheal tissue with collagen-enforced poly-lactic-glycolic acid non-woven mesh: A preliminary study in nude mice

Wei Wu a, Xue Feng b, Tianqiu Mao a, Xinghua Feng a, Hong-Wei Ouyang c, *, Guifang Zhao d, Fulin Chen a, d, *

a Department of Oral and Maxillofacial Surgery, Qindu Hospital, The Fourth Military Medical University, Xi’an, PR China
b Department of Orthodontics, Qindu Hospital, The Fourth Military Medical University, Xi’an, PR China
c Tissue Engineering Center, School of Medicine, Zhejiang University, Hang Zhou, PR China
d Xi’dai-Rege Lab of Tissue Engineering and Regenerative Medicine, Northwest University, Xi’an, PR China

Accepted 14 September 2006

Abstract

The purpose of the current study is to fabricate tissue engineered trachea with poly-lactic-glycolic acid (PLGA) non-woven mesh enforced by collagen type I. PLGA fibres coated with collagen solution were put together and fabricated into the shape of a human trachea, after drying and cross-linking treatment, a non-woven mesh with “C” shape formed. Chondrocytes from sheep nasal septum cartilage were expanded in vitro and seeded into PLGA/collagen non-woven mesh in the density of $5.0 \times 10^7$ mL$^{-1}$. After 5 days of in vitro incubation, six Cell-PLGA/collagen composites were implanted subcutaneously into the back of 6 nude mice to prefabricate a tissue engineering trachea. Eight weeks later, the cartilage formation was observed by gross inspection and histological examination. Cartilage like tissue in the shape of the initial PLGA/collagen scaffold had been regenerated successfully without obvious inflammatory response. The tissue engineered trachea cartilage consisted of evenly spaced lacunae embedded in matrix stained red with safranin-O staining. The amount of GAGs in tissue engineered trachea cartilage reached 71.42% of normal value in native cartilage. This study demonstrated that collagen-enforced PLGA non-woven mesh facilitated the adhesion and proliferation of chondrocytes, it also owned adequate mechanical strength to serve as an ideal scaffold for trachea tissue engineering without internal support.

Keywords: Tissue engineering; Cartilage; Collagen; PLGA; Trachea

Introduction

Cartilage repair remains a clinical obstacle because of the poor regenerative capacity of cartilaginous tissue and the limited number of donor sites. Tissue engineering provides a promising solution for the repair of cartilaginous defects in the head and neck. Cartilage tissue engineering, which constructs cartilage in scaffolds with a predetermined shape such as nose, outer ear, temporomandibular joint, and trachea, has attracted much attention in recent years. To obtain a good repair, scaffolds should not only possess properties that support cell adhesion, growth, and differentiated function, but also create a stable, three-dimensional microenvironment with enough porosity for chondrocytic ingrowth. During the past decade, natural biomaterials and scaffolds including collagen, chitosan, hyaluronan derivatives, silk protein, and synthetic degradable polymers have been investigated extensively for their ability to support the growth of chondrocytes isolated from various animal species. However, these scaffolds have their respective
problems concerning mechanical strength and efficiency of cell seeding. For engineering tracheal tissue, the scaffold should have specific physical properties that can be moulded into a “C” shape. Non-woven mesh made of polyglycolic acid (PGA), polyactic acid (PLA), and their co-polymers (PLGA), were used because of their ideal three-dimensional structure, good mechanical properties, and adjustable degradation speed compared with natural matrices.\(^5,7\)

In tracheal tissue engineering, one method of fabricating the scaffold in the shape of a ring is to apply internal supports made of biomaterials that are not biodegradable but are bio-inert. Ruszynah et al. mixed human nasal septum chondrocytes into poloxamer 407 (Pluronic F127), and then the chondrocyte-hydrogel admixture was painted on to the high-density polyethylene as an internal support in the predetermined shape of a trachea. The composite was then implanted subcutaneously in athymic mice, and 8 weeks after implantation tissue-engineered tracheal cartilage had formed.\(^8\) Kojima et al. placed cell-PGA complex into the grooves of a 20 mm diameter, 50 mm long, helical template made from a Silastic E RTV silicone rubber mould-making kit, and implanted the complex subcutaneously in the neck of sheep. Eight weeks after implantation, tissue-engineered trachea was harvested and anastomosed into a 5 cm defect in a sheep trachea.\(^9\) In all these cases, internal supports were necessary to maintain the shape during the chondrogenesis of the cell-scaffold composite in vivo. However, this process would be complicated, and the risk of rejection increased by the use of non-degradable materials for long-term implantation.

Another way of fabricating a PLGA scaffold in a special shape involves coating fibrous PGA mesh with solutions of PLA in methylene chloride, and then evaporating the solvent to deposit PLA on the mesh. Despite the increased mechanical stability, the chemically and biologically inert properties of PLA are unlikely to induce adhesion of cells and formation of tissue.\(^10\) Other approaches have also been adopted to produce porous polymer scaffolds with appropriate pore sizes, porosity, and mechanical properties.\(^11\) They typically involved high temperature or organic solvents,\(^12,13\) which would be expected to denature proteins that presented during the process.

Naturally occurring polymers extracted from the native extracellular matrix, like collagen, have been widely used to modify the synthetic material to improve the cells’ adhesion.\(^14\) In this study, we have made a trachea-like scaffold by combining cross-linked type I collagen and PLGA fibres to produce tissue-engineered cartilage in the shape of human trachea. We hypothesised that this complex may have better biocompatibility and appropriate mechanical properties that can support formation of cartilage in vivo in specialised shapes. We examined formation of ectopic cartilage in the shape of human trachea by using a chondrocyte/PLGA-collagen composite in nude mice.

Material and methods

Making the scaffold

Polymer fibres of glycolic acid and lactic acid co-polymer were obtained by disassembling polyglyactin 910 (Vicryl) sutures (Fig. 1a). The fibres were immersed into the solution containing 0.5% (w/v) digested bovine pepsin acid-soluble type I collagen (Sigma, USA) for 30 min. It was then poured into a calcium alginate impression mould 3 mm high with an inner diameter of 12 mm and an outer diameter of 14 mm (Fig. 1a). The mould filled with the polymer fibres was centrifuged to remove the redundant liquid reagent from the scaffold. The scaffold was then left to dry overnight. The dried porous scaffold was thoroughly washed in distilled water for 30 min, and then left to dry again. To crosslink the collagen coated on to the surface of PLGA fibres, the dried scaffold was exposed to ultraviolet irradiation in a laminar hood for 3 h on each side. After they had been sterilised with ethylene oxide gas, the scaffolds were sealed and stored at \(-20^\circ\text{C}\) until use. The pore structure and the fibre diameter of the material were measured by scanning electronic microscopy (EM).

Isolation and expansion of chondrocytes from sheep nasal septa

Cartilage from the nasal septa of 2-month-old sheep was dissected and minced into pieces of about 1 mm\(^3\); after it had been rinsed three times with phosphate buffered saline (PBS) supplemented with penicillin (200 U/mL) and streptomycin (100 \(\mu\)g/mL), samples of the cartilage were digested with 0.2% collagenase type II (Gibico) in Dulbecco’s modified Eagle’s medium (DMEM) at 37 \(^\circ\text{C}\) for 12 h. The digested cell suspension was filtered through a 250 \(\mu\)m nylon mesh filter to remove the debris of the matrix and then centrifuged at 1000 rpm for 10 min; the resulting cell pellet was washed twice with PBS and resuspended with DMEM containing 10% fetal bovine serum (Hyclone), L-glutamine (272 \(\mu\)g/mL), and ascorbic acid (50 \(\mu\)g/mL). Cells were counted with a haemocytometer, and their viability was assessed using a trypan blue exclusion test. The isolated chondrocytes were cultured at a density of 2.0 \(\times\) 10\(^4\) cm\(^{-2}\), and the medium was changed every 3 days. After they had been subcultured twice, chondrocytes were collected by trypsin digestion and suspended in culture medium at a density of 5 \(\times\) 10\(^7\) cells/mL for seeding.

Seeding and in vitro incubation of cell–scaffold complexes

A suspension of cells in a volume of 0.6 mL was carefully seeded into the scaffold. The cell–scaffold complexes were put into dishes, transferred into an incubator, and incubated in 5% carbon dioxide with 100% humidity at 37 \(^\circ\text{C}\) for 4 h to make sure that most cells adhered to the scaffolds. Then 2 mL of medium was carefully added around the complexes.

Twelve hours later, an additional 10 mL of medium was added. The complexes were incubated in vitro for 5 days before implantation, and were observed under a photomicroscope. One small piece of the complex was cut with shape scissors and processed for scanning EM before implantation.

In vivo implantation and harvesting of specimens

Ten of 7-week-old nude mice were obtained, and acclimatised for a week before use. The implantation procedure and the care of the mice were regulated by the Experimental Animal Centre of the Fourth Military Medical University. After it had been prepared aseptically, the skin on the back was incised, and a subcutaneous pocket was made. One complex loading of $3 \times 10^7$ cells was implanted into each of 6 mice, which made up the experimental group. Four scaffolds without cells were also implanted, and they acted as controls. Eight weeks after implantation, the animals were killed and specimens were carefully harvested and examined macroscopically. Part of each specimen was randomly cut and fixed in 10% phosphate-buffered formalin for histological analysis, and part of the composites were digested for measurement of glycosaminoglycan.

Histological examinations

The fixed specimens were dehydrated by immersion in a series of ethanol solutions, then embedded in paraffin, and sectioned. Sections were stained with haematoxylin and eosin, and safranin-O.

Quantification of glycosaminoglycan

Six samples of engineered trachea and native tracheal cartilage from sheep were analysed for quantification of glycosaminoglycan. Samples were digested by lysis buffer containing papain 125 $\mu$g/mL (Sigma), sodium phosphate 100 mmol/L, sodium EDTA 10 mmol/L, cysteine hydrochloride 10 mmol/L (Sigma), and EDTA 5 mmol/L, at 60°C for 24 h. Glycosaminoglycan in the tissue engineered cartilages was measured quantitatively by reaction with dimethylmethylene blue dye (Aldrich, USA) as described by Farn-dale et al. The intensity of the change in colour was quantified immediately in a spectrophotometer by measuring absorbance at 520 nm. According to the standard curve obtained from shark chondroitin sulphate (Sigma), the amount of glycosaminoglycan was extrapolated. Values were compared with those from the specimens of sheep tracheal cartilage.

Statistical studies

With the help of the Statistical Package for the Social Sciences (SPSS) Version 10, Student’s $t$-test was used to evaluate the differences in the glycosaminoglycan content in the
Fig. 2. (a) Phase contrast photomicrograph (original magnification ×100) of sheep nasal septal chondrocytes in monolayer culture. (b) Phase contrast photomicrographs of chondrocytes on PLGA and collagen non-woven mesh for 5 days (bar scale 100 μm), and (c) proliferation and adherence on to the fibres (arrow). Bar scale 100 μm. (d) Scanning electron photomicrograph of chondrocytes cultured in PLGA and collagen non-woven mesh, which showed cells adherent to, and spread on to, the PLGA fibres (bar scale 20 μm).

Macroscopic appearance

All 10 animals survived the experiment and there were no signs of inflammation, infection, or extrusion postoperatively. Before they were harvested, the grafts could be seen clearly subcutaneously on the animals' backs and they kept the initial shape of the PLGA/collagen scaffold in the experimental group. The specimens could be separated easily from the surrounding tissue, and each specimen showed a pearly opalescence in its gross morphology and was resistant to compression. The newly formed cartilage kept the shape of the trachea without obvious deformation (Fig. 3). The control group had been totally absorbed with no formation of cartilage.

Histological analysis

Histologically, cartilage formed in all six experimental specimens. Haematoxylin and eosin staining on the cross-section showed formation of mature and homogeneous cartilage, and the PLGA/collagen scaffold had been replaced completely by tissue that consisted of evenly spaced lacunae cells embedded in matrix (Fig. 4a). The lacunae cells varied in shape from round to oval with slight polymorphism, which showed...
that the tissue resembled mature hyaline cartilage. In some regions, cells were arranged in lines in accordance with the original PLGA fibres (Fig. 4b). Safranin-O staining on these sections showed homogeneous red staining, which showed that the engineered tissue was rich in proteoglycans (Fig. 5a). Single chondrocytes were embedded in round or angular lacunae (Fig. 5b).

**Quantification of glycosaminoglycan in tissue-engineered cartilage**

The mean (S.D.) content of glycosaminoglycan in the tissue-engineered cartilage and native tracheal cartilage were 69.4 (3.8) and 97.1 (3.1) μg/mg, respectively (Fig. 6); the difference between the two groups (p = 0.03) is significant.

**Discussion**

Autologous implants of chondrocytes has been used successfully in patients with defects of the articular cartilage. However, unlike engineered articular cartilage, engineered tracheal tissue needs a scaffold that can maintain its specific shape and size in vivo. In addition, the scaffold, acting as a space-filler, will facilitate the uniform distribu-

---

**Fig. 3.** Tissue engineered tracheal constructs harvested from nude mice after 8 weeks’ implantation showing macro-appearance of hyaline cartilage in the shape of a tracheal ring. There was a pearly opalescence on gross morphology, but no obvious deformation.

**Fig. 4.** (a) Histological picture showing engineered tissues consisting of evenly spaced lacunae cells embedded in a basophilic matrix (bar scale 500 μm). (b) The lacunae cells varied in shape from round to oval with slight polymorphism, in some regions. Chondrocytes lined up along the track of PLGA fibres (arrow). Bar scale 200 μm. Haematoxylin and eosin stain.

**Fig. 5.** (a) Histological evaluation of the tissue-engineered trachea showing strong orange staining (bar scale 500 μm). (b) Orange red staining correlating with abundant production of proteoglycans indicating synthesis of mature hyaline cartilage matrix resembling native human hyaline cartilage (bar scale 200 μm, safranin-O stain).
Fig. 6. Glycosaminoglycan (GAG) content of native trachea and tissue-engineered trachea (TET) (n = 6).

tion and better trapping of cells in a comparatively large defect.

In this study, we used collagen solution instead of PLA solution as a biocompatible adhesive for the construction of the tracheal ring. The results showed successful fabrication of tissue-engineered trachea by the use of chondrocytes and PLGA non-woven mesh enforced by collagen without internal support. The viscosity of the collagen solution can bind PLGA fibres efficiently and cross-linking treatments can also increase the physical properties of the collagen. In this study, ultraviolet irradiation was chosen because it is toxicologically acceptable, and different from chemical cross-linking methods. After cross-linking treatment, the collagen coating on the surface of the fibres improved the mechanical properties of the scaffold without changing its structure, which enabled the scaffold to resist deformation after implantation, and avoided the insertion and removal of an internal support. This method can be used to fabricate a scaffold into individual shapes according to clinical need.

Another advantage of using collagen as the adhesive is that it is more biocompatible than polymers. The drying process avoided leaving a residue of organic solvent when PLA was used to link PGA mesh mechanically. Scanning EM showed good adhesion and spread of cells on to the fibres of the scaffold. Five days after in vitro incubation, seeded cells had proliferated significantly on the fibres, as shown on phase contrast photomicrography.

Histological analysis also showed that the structure of the cartilaginous tissues was similar to that of the native cartilage. As can be seen in Fig. 4b, in some regions chondrocytes lined up along the original PLGA fibres, which showed that chondrocytes adherent to the scaffold survived well and secreted extracellular matrix during tissue formation. In addition, we also noted that the cartilaginous matrices were homogeneously distributed, which indicated that the seeding of cells into the scaffold was uniform, which may be the result of the increased hydrophilicity after being coated with collagen.

Concentrations of glycosaminoglycan in tissue-engineered cartilage at 8 weeks reached 71% of the amount in native tracheal cartilage, which indicates that tissue-engineered trachea contained less glycosaminoglycan than native trachea; this is consistent with the results published by Kojima et al. We ascribed it chiefly to the fact that tissue-engineered cartilage grew for only 8 weeks in vivo. In addition, the subcutaneous microenvironment may also influence the secretion. Longer incubation time in vivo and implantation in situ may allow the content of glycosaminoglycan to approximate native tracheal cartilage more closely.

One of the main advantages in tissue engineering is that it needs only a small biopsy specimen from the donor site, avoiding traumatic harvesting operations. However, in cartilaginous tissue-engineering, the source of cells remains a problem that impedes its clinical application. Recently, some investigators have reported that chondrocytes from the nasal septum could be used successfully to fabricate hyaline cartilage, and can be regarded as a promising source of chondrocytes for tracheal cartilage tissue-engineering. Based on these considerations, we also chose nasal septal chondrocytes as seeded cells. In vitro culture showed that chondrocytes from the nasal septum can be isolated conveniently and the chondrocytes proliferated well. In vivo experiments also confirmed the feasibility of using chondrocytes from the nasal septum as seeded cells. However, because of dedifferentiation phenomena that exist in chondrocytes during subculturing and proliferation in vitro,21,22 we still cannot obtain enough chondrocytes with monolayer cultures. In future research, we plan to reconstruct cartilage by the co-seeding of mesenchymal stem cells and chondrocytes on to the scaffold. Results from the current study have also indicated that the nasal chondrocytes attached to the PLGA fibres coated with collagen continue to proliferate rapidly. The microscopic results showed that the proliferated chondrocytes adhere not only between cell-material, but also between cell and cell.

Acknowledgement

This study was supported by the National Natural Science Foundation of PR China, numbers 30270373 and 30370374.

References


