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A tissue-engineering model for the manufacture of auricular-shaped cartilage implants

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Abstract The established surgical methods of external ear reconstruction using autogenous tissue represent the current state of the art. Because of the limited possibilities for shaping conventional harvested autogenous rib cartilage, the cosmetic results of auricular reconstruction are frequently unsatisfactory. Tissue engineering could represent an alternative technique for obtaining a precisely shaped cartilage implant that avoids donor site morbidity and unsatisfactory cosmetic results. In this study, the reliability and quality of a tissue-engineering model for the manufacture of auricular-shaped human cartilage implants was investigated, focusing on the feasibility of the manufacturing process and the in vivo and in vitro maturation of an extracellular cartilage-like matrix. Implants were molded within an auricular-shaped silicone cylinder, and human nasal septal chondrocytes crosslinked by human fibrin within bioresorbable PGLA-PLLA polymer scaffolds were used. After an in vitro incubation of up to 6 weeks, defined fragments of the prefabricated auricular-shaped construct were implanted subcutaneously on the backs of nude mice for at least 6 to 12 weeks (n=7). Scaffolds without cell loading served as controls. Macroscopic and histochemical examination after 3 and 6 weeks in vito showed a solid compound of homogenously distributed chondrocytes within the polymer scaffold, leading only to a limited pericellular matrix formation. Analysis after 6 and 12 weeks of in vivo maturation demonstrated a solid tissue compound and neocartilage formation with the presence of cartilage-specific matrix components. Implants obtained shape and size during the entire period of implantation. The model of cartilage implant manufacturing presented here meets all biocompatible requirements for in vitro prefabrication and in vivo maturation of autogenous, individually shaped cartilage transplants.

Keywords Tissue engineering · Fibrin glue · Polymer fleece · Cartilage transplantation · Auricular reconstruction

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Introduction

The variety of implants used today in facial plastic and reconstructive surgery shows the necessity of the ongoing search for an ideal solution. Reconstructive efforts to reproduce a three-dimensional cartilaginous framework with a defined topography from the head and neck have challenged surgeons for decades [1, 2, 3, 4]. The ideal method of surgical reconstruction would be the replacement of tissue defects with autogenous vital tissue. In the case of cartilage defects, the limited quantity of autogenous cartilage, the fact that it cannot be shaped satisfactorily as well as undesirable donor site morbidity can make an acceptable reconstruction difficult to obtain in some patients.

The concept presented here, which is based on tissue engineering, involves the creation of an autogenous implant, starting with a minimal biopsy of the surplus human septal cartilage harvested during a nasal-septal operation. This is followed by the enzymatic isolation of the chondrocytes from the cell-covering extracellular cartilage matrix and by chondrocyte amplification. The implant manufacturing process is finished by a three-dimensional cell arrangement and transplant shaping.

Cartilage engineering in this study is based on human cartilage biopsies, and an attempt to follow the baselines for future clinical applications as closely as possible has been emphasized. In contrast to several other studies us-

ing fetal, non-amplified chondrocytes, this experiment deals with adult chondrocytes that are amplified and differentiated in vitro, which represents a totally different potential for amplification and re-differentiation. In the current experiment, the reliability and quality of a tissue engineering model for cartilage implant manufacturing is investigated. Focusing on technical and biological aspects, the artificial temporary PLLA-PGLA polymer and fibrin matrix has to guarantee a homogenous three-dimensional distribution of chondrocytes and a continuous nutrient supply that supports the physiological behavior of adult human chondrocytes in order to gain an optimal transplant configuration for in vitro and in vivo maturation. Additionally, the experiment has to deal with the manufacturing aspects of a bioresorbable scaffold of a defined anatomical shape; the need for immunological acceptance and a low risk of contamination also must be considered [5].

Materials and methods

The surplus of surgically removed human nasal septum cartilage was unwrapped from the bone and perichondrium, diced into pieces of approximately 1 mm and digested enzymatically using 2 mg/ml of type II collagenase (Seromed, Berlin) and 0.1 mg/ml hyaluronidase (Serva, Frankfurt) dissolved in RPMI 1640 medium (Seromed, Berlin) at 37°C for 12 to 18 h. The number of vital cells was determined by a hemocytometer counting using trypan blue.

Cell amplification was performed in culture flasks (Nunc, Naperville, USA) with modified RPMI 1640 medium (10% FCS, 2% Hepes, 1% penicillin/streptomycin) under standard conditions. The medium was renewed every second day. The multiplicative procedure was repeated in a maximum of three passages.

For three-dimensional cell arrangement, the cells were detached enzymatically and mixed with the fibrinogen component of the fibrin glue system [7] (Immuno, Heidelberg) in a 1:2 ratio. Bioresorbable PLLA-PGLA polymer scaffolds (Ethicon, Norderstedt, Germany) were soaked with the chondrocyte-fibrinogen suspension in a density of $10–15\times10^6$ cells/ml. Gel formation of the fibrin glue was induced by the addition of the thrombin solution. Cell culture was performed under standard conditions with a continuous supply of feed medium, using modified Hams F12 medium (Gibco, USA).

An auricular-shaped silicone cylinder was completely filled with the polymer fleece scaffold. The chondrocyte-fibrinogen suspension was soaked into the polymer construct in a concentration of 10×10^6 cells/ml. The thrombin solution completed the fibrin polymerization. The silicone cylinder was removed, and the auricular-shaped implant was cultured under standard conditions in a bioreactor (Minucell, Regensburg).

Because of ethical considerations, it was not appropriate to implant the entire auricular-shaped construct on the back of the nude mouse (CD1, nu/nu, Charles River, Sulzfeld, Germany). Instead, defined fragments (1.0×1.0×0.2 cm) of the prefabricated implant were implanted in subcutaneous pockets using aseptic surgical techniques. After 6 and 12 weeks, the mice were killed by an overdose of CO_2. The animal testing was performed at the appropriate department in accordance with the principles of the declaration of Helsinki (regional authorizing agency, Berlin 0151/98). The explants were investigated for macroscopic and microscopic characteristics.

Experimental groups I and II (n=7) were chondrocyte-fibrin-fleece constructs. Specimens of group I were harvested 6 weeks after implantation; specimens of group II were explanted 12 weeks after implantation. The specimens of group III (n=6), consisting of gel-polymer-fleece constructs without chondrocyte loading, were explanted after 6 (n=3) and 12 (n=3) weeks in vivo.

Results

In vitro experiment

The chondrocytes in a monolayer culture with a doubling time of 4–6 days showed a fibroblast-like morphology as a feature of dedifferentiation. After the transfer into the fibrin glue/polymer construct, the majority of cells regained their chrondrocytic round phenotype, passing through a hypertropic intermediate state, and remained phenotypically stable for up to 6 weeks.

After 3 and 6 weeks in vitro, the chondrocyte fibrin-polymer scaffolds showed a homogenous distribution of chondrocytes. The permanently degrading and decomposing fibrin glue component allowed the chondrocytes to start with the buildup of a small number of pericellular cartilage components. During the slowly progressing in vitro matrix formation, the polymer fleece, which holds longer, guaranteed the stability of the overall shape. Masson trichrome staining revealed that the newly synthesized extracellular matrix contained collagen. Pericellular accumulation of matrix compounds such as proteoglycan was demonstrated in PAS staining. In control samples of fibrin glue and polymer fleece without cells, no pericellular matrix staining could be observed.

A slow but continually increasing amount of collagen and proteoglycan could be observed by increasing the time of the in vitro cultivation (Fig. 1). After 6 weeks of cultivation, a significant but altogether only limited number of pericellular matrix components were achieved.

Implant manufacturing

After removing the implant-covering silicone cylinder immediately after the implant manufacturing process, the implant appeared hard but flexible and showed the well-

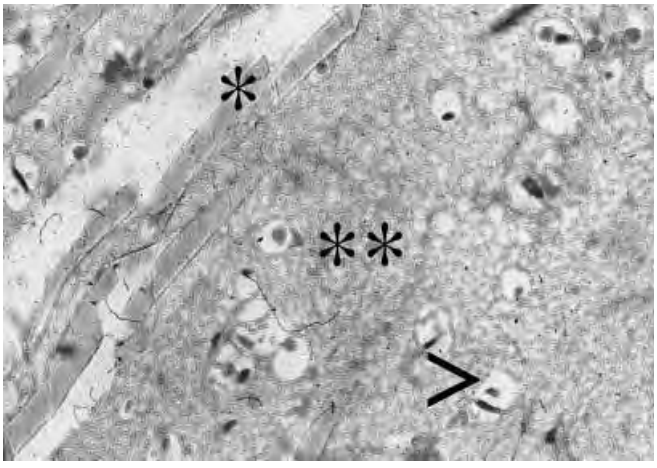

Fig. 1 Histologic section of the auricular-shaped implant after 3 weeks in vitro. Notice the large number of polymer fibers (*), the homogenous chondrocyte (>) distribution and the remnants of the fibrin matrix (**), HE, ×200

Fig. 2 Transplant shaped by loading the polymer fleece with chondrocytes and fibrin glue in a silicone cylinder

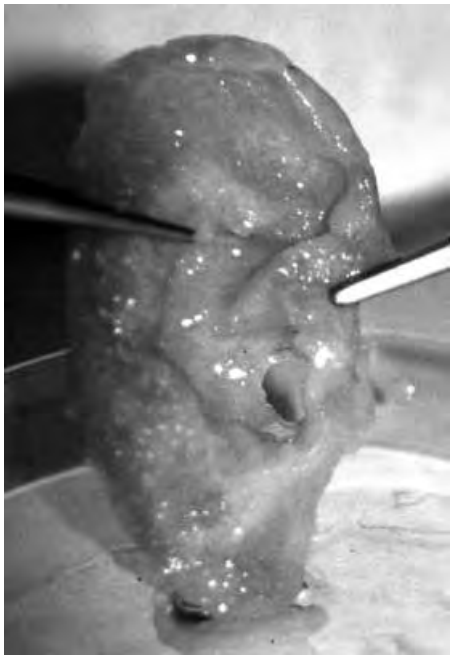

Fig. 3 The auricular-shaped implant after the removal of the silicone form

defined and stable contours of a human auricle (Fig. 2, Fig. 3). Although not further investigated, the mechanical properties were roughly estimated to be good; this was determined by the mechanical properties of the polymer fleece and the added fibrin glue. The surface of the implant appeared smooth with no protruding polymer fibers. Microscopic examination demonstrated homogenous chondrocyte distribution not only close to the polymer fibers, but also in the fibrin-filled space between the polymer fibers.

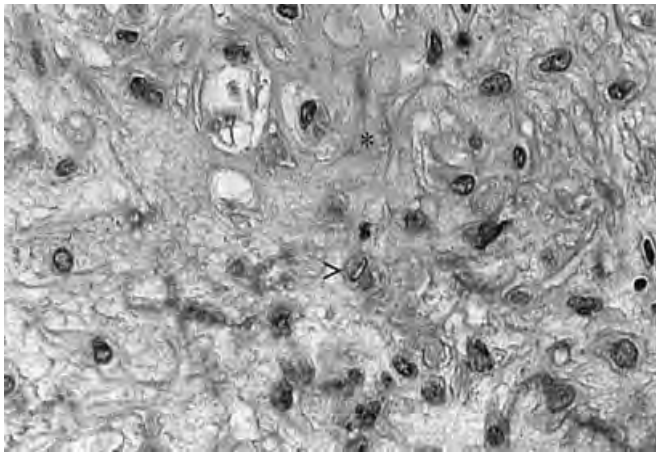

Fig. 4 Histologic section of an implant after 6 weeks in vivo. Homogenous neocartilage with synthesis of collagen in the matrix (*) and typically shaped chondrocytes (>), MG = Masson Goldner, (×200)

In vivo experiment

Macroscopic examination demonstrated that all of the explants showed stability in shape to a great extent, but with a varying slight decrease in size. None of the explants in group I or II showed a complete resorption. Every explant was surrounded by a thin, strongly adhering, capsule-like fibrous tissue layer. Specimens in group II (12 weeks in vivo) appeared harder and more compact than those in group I (6 weeks in vivo). After a clean cut through the explant, the surface at the cutting edge showed a compact, smooth, whitish, cartilage-like morphology. In the gross analysis, the surrounding fibrous tissue layer showed varying degrees of invasion of the fibrous tissue cords into the compact cartilage matrix. The microscopic aspect of the explants in groups I and II looked like native hyalin cartilage with homogenous neocartilage formation. A large degree of differentiation, showing round-shaped clusters

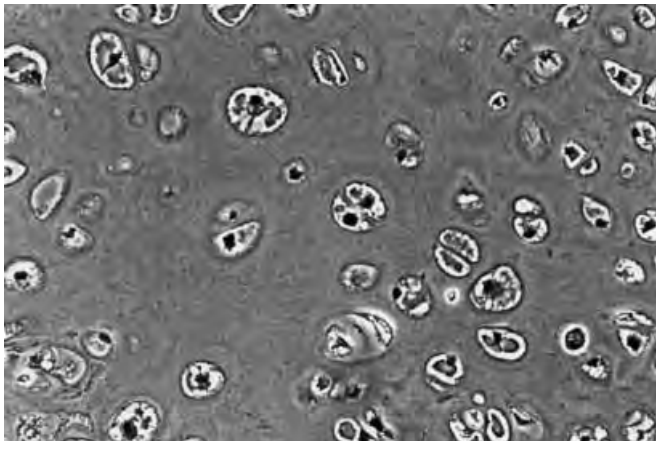

Fig. 5 Histologic section of a implant after 12 weeks in vivo. Lacunae with chondrocytes and a high amount of collagen, MG (×400)

of homogenously distributed chondrocytes within a homogenous pericellular matrix, was observed after 6 and 12 weeks in vivo (Fig. 4 and Fig. 5). Masson trichrome staining demonstrated higher amounts and an obviously more homogenous distribution of collagen in group II than in group I. PAS staining showed a lower level of proteoglycan in group I than in group II. None of the implants of group III could be identified for explantation and macroscopic or microscopic examination because of resorption after 6 and 12 weeks in vivo.

Discussion

The multitude of implants used today in facial plastic and reconstructive surgery shows the necessity of continuing to search for an ideal solution. Faced with the projected increase in head and neck malignancies and the baseline incidence of microtia representing cosmetic and functional disorders in the field of head and neck surgery, the challenge to find a viable and durable implant continues. Reconstructive efforts to reproduce a three-dimensional cartilaginous framework with a subtle topography from the cranium have challenged surgeons for decades. Satisfactory results demand the manipulation of less than ideal components to create a pleasing result. The ideal method in surgical reconstruction would be the replacement of tissue defects with autogenous vital tissue, performed and developed by Brent, Tanzer and Weerda many years ago [1, 2, 3, 4]. In the case of cartilage defects, a limited quantity of usable autogenous tissue as well as undesirable donor site morbidity can make it impossible to obtain this ideal reconstruction in some patients.

The new biotechnology of tissue engineering represents an alternative technique that maintains the principles of autologous transplant surgery. The tissue-engineering-based concept of autologous transplant creation presented here involves the surgical replacement of a minimal human septal cartilage biopsy, followed by cell isolation from the cell-covering extracellular cartilage matrix, cell amplification and the final three-dimensional cell arrangement and transplant shaping. Earlier investigations in cartilage engineering, based on juvenile bovine, articular-cartilage biopsies and using carriers such as agarose gel, showed respectable results. Unfortunately, these models could not be transferred to a clinical application for several reasons [1]. Juvenile chondrocytes, with their immense potential for amplification and redifferentiation, are not available within the adult human body [2]. A heterogenous transplantation of bovine cartilage chondrocytes is excluded because of immunological and infectious reasons [3]. Agarose gel carriers lack biocompatibility and shapable stability during in-vitro transplant formation.

Cartilage engineering based on human cartilage biopsies, which simulates the baselines for following clinical application as closely as possible, deals with differentiated and adult chondrocytes. These cells have a totally different potential for amplification and re-differentiation. The objective of the present work was to provide human chondrocytes in vitro with a microenvironment generating conditions to support their physiological behavior in order to gain the optimal transplant configuration. The artificial temporary cell matrix has to guarantee a homogenous three-dimensional distribution of the chondrocytes and a constant supply of nutrients. Additionally, we have to deal with the practical aspects of a bioresorbable scaffold of a defined anatomical shape, also taking into account the need for immunological acceptance and anti-infectious security. Excluding all of the disadvantageous properties of the above-described carriers, in this study we investigated chondrocyte behavior and cartilage matrix formation in fibrin gel and fibrin gel/polymer carriers. These substances have long histories of clinical use and well-known biocompatibility.

Histochemical analysis of the in vitro experiment showed the limited expression of collagen and proteoglycan in accordance with the results of recent studies performing cartilage engineering within cell perfusion chambers [8, 9].

During the proliferation period, the chondrocytes shift their collagen type II synthesis to that of collagen types I and III and show a fibroblast-cell-like phenotype. This dedifferentiation induced by the monolayer culture is stopped and reversed when the cells are placed in a three-dimensional scaffold [10, 11, 12]. Several investigators have shown that dedifferentiated chondrocytes placed in a three-dimensional agarose gel are able to redifferenciate [13, 14]. A major advantage of gel carriers such as agarose is the homogeneous three-dimensional cell distribution. So far, the lack of stability, the poorly investigated biodegradability and the lack of infectious security of agarose gel restrict its prospective clinical applications. In a different approach, Vacanti and co-workers focused on biodegradable polymer scaffolds and showed that chondrocytes grown on rigid synthetic, three-dimensional, bioresorbable polymer fiber meshes are fully differentiated and capable of producing type II collagen in vitro and in vivo [15, 16]. Homopolymers of lactid acid and glycolic acid are attractive candidates for fabricating tissue-engineered scaffolds. Polymer fleece structures offer high mechanical stability and a minimum amount of biomaterial per volume. According to the porosity, the handling of the cell distribution within the polymer fleece is difficult and results in an inhomogeneous cell distribution, which leads to transplant instability and loss of functionality. Furthermore, the adhesion of cells to the surface of polymer fibers differs and could lead to a loss of cells during the time of in vitro cultivation.

In the search for an alternative gel carrier to agarose gel, fibrin glue was investigated for its use in cartilage engineering. Fibrin glue is a well-investigated medical device and has been used for over 20 years in clinical and laboratory medicine. Fibrin glue also is generally considered to be biodegradable and biocompatible [17, 18, 19]. The described results emphasize that fibrin glue matrices provide the cells with a microenvironment that allows them to synthesize their own peri- and extracellular matrix. Furthermore, gel structures based on fibrin glue com-

ponents are also appropriate as culture media, providing homogenous and stable three-dimensional cell distribution. This enables the chondrocytes to re-differentiate and form their pericellular matrix. Different authors describe the successful employment of fibrin glue combined with chondrocytes [5, 6, 7]. In contrast to gel structures like agarose, fibrin glue guarantees full biocompatibility and a high level of security against infections.

Despite all of the described advantages of fibrin glue carriers, the exclusive use of fibrin glue components to form well-defined and thin, anatomically shaped structures is limited. Fibrin glue does not offer enough morphological stability during the process of in vitro cultivation. Therefore, the use of a combination of fibrin glue and synthetic polymer fleece for a three-dimensional scaffold was investigated. The combination of polymer fleece and fibrin glue scaffolds takes advantage of the benefits of both scaffolds. Polymer fleece guarantees the initial conservation of shape, and fibrin glue offers stable, homogenous three-dimensional cell distribution. The observed increase in the in vivo formation of pericellular matrix components, such as collagen II and proteoglycanes, leads to a homogenous, stable cartilage transplant with pleasing mechanical properties.

Because of the clinical need for a well-defined, auricular-shaped cartilage transplant for auricular reconstruction that is engineered in vivo, the malleability of tissue-engineered cartilage was investigated. Based on the described methods, the creation of auricular-shaped cartilage transplants was performed within a silicone cylinder. Cultivating the transplants under stable conditions for up to 6 weeks in vito leads only to a limited formation of cartilage matrix compared to in vivo maturation. Both techniques, in vivo and in vitro maturation, guarantee maintenance of the transplant shape, but with different results in the formation of the pericellular cartilage matrix. This represents the main factor concerning cartilage quality, guaranteeing three-dimensional stability and flexibilty. Stem-cell technologies using multipotent cells may avoid these problems during re-differentiation that are observed when performing tissue engineering with adult-differentiated cells [20].

Macroscopic and microscopic examination of the implanted chondrocyte transplants reveals neocartilage formation that is similar to hyalin cartilage with chondron-like chondrocyte formation and collagen type II, proteoglycan and chondroitin sulfate expression. Unlike the in vivo experiments, transplants of in vitro-cultivated cartilage showed only a limited number of pericellular cartilage components, which explains their lack of three-dimensional stability and flexibility. Besides these factors, in vito cultivation carries the risk of iatrogen infection during the culture process with the resulting loss of the transplant.

The experiments presented here emphasize the advantages of an engineering approach that uses a combination of all carriers. The process of cell amplification and the transplant shaping in vitro were strictly performed using biodegradable and biocompatible biomaterials, followed by the in vivo maturation of the cartilage transplants after subcutaneous implantation. However, nude mice with a more or less functional cellular immune system only mimic an autogenous transplantation because of their lack of a functional b- and t-cell immune system. Therefore, the validity of the described experiments has to be confirmed by experiments performing a complete autogenous transplantation using a complete immuno-competent animal model, such as a rabbit or pig.

Conclusion

The established surgical methods of external ear reconstruction using autogenous tissue represent the current state of the art. In the near future, new methods of biotechnological tissue engineering may avoid the disadvantages of the present established surgical technology. On the way to clinical application, experiments using adult human cartilage and biocompatible, bioresorbable biomaterials are necessary to resolve the conflicts established by recent studies using juvenile animal chondrocytes. In this context, experiments using stem-cell technologies could resolve problems observed in adult differentiated cells. The presented study focuses on these conflicts, using adult human hyalin cartilage chondrocytes and biocompatible, bioresorbable biomaterials. Summarizing the results of the presented experiments, it can be demonstrated that combined in vitro and in vivo engineering of human cartilage allows for the formation of pre-shaped, vital autologous cartilage, based on the combination of autologous chondrocytes, bioresorbable polymer fleeces and fibrin glue. This could offer new perspectives not only in auricular reconstruction, but also in the broader field of reconstructive head and neck surgery.

Before clinical application, further investigations in a complete immuno-competent animal model dealing with the long-term results after implantation are required.

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