Behavior of tissue-engineered human cartilage after transplantation into nude mice

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Cartilage lacks the ability to regenerate structural defects. Therefore, autologous grafting has been used routinely to replace cartilaginous lesions. Because tissue engineering of human cartilage with the help of bioresorbable polymer scaffolds is possible in experimental models, the demand for the clinical application grows. In this study we present an analysis of the behavior of transplants made of human chondrocyte pools, agarose and the resorbable polymer scaffold Ethisorb and a preliminary comparison with transplants made of single patients' cells and Ethisorb but without the additional ingredient agarose.
Chondrocytes were isolated from the matrix of human septal cartilage by enzymatic digestion. The pool cells were kept in monolayer culture for 2 weeks, the single patients' cells for 3–4 weeks. Chondrocyte pools were suspended in agarose and seeded into the resorbable polymer scaffold Ethisorb. Single patients' cells were seeded without agarose. All cell–polymer constructs were kept in perfusion culture for 10–14 days and transplanted subcutaneously into thymusaplastic nude mice. Additionally we implanted Ethisorb fleeces embedded in agarose without chondrocytes. After 6, 12 and 24 weeks the animals were sacrificed and the specimens were explanted and analyzed histochemically and immunohistochemically.
Polymer scaffolds not seeded with chondrocytes did not show cartilage formation. Resorption was complete after 12 weeks *in vivo*. Transplants from cell pools remained mechanically stable over 24 weeks apart from four transplants that were resorbed completely. Cartilage formation was observed in all pool-specimens with the presence of chondronic structures and a homogeneous matrix containing hyaline cartilage-specific matrix molecules such as collagen type II. Single patients' transplants showed hyaline cartilage matrix synthesis and mechanical stability as well.
Chondrocyte pools are a suitable method to study cartilage engineering of human cells *in vitro* and *in vivo* in experimental models. Under clinical conditions it is, however, necessary to study the generation of cartilage from single patients' cells. We showed that it is possible without additional ingredients such as agarose. However, variations in the preliminary results show that the clinical application with human cells is more difficult than one would expect when using human chondrocyte pools. Further studies need to be performed to find out which individual factors influence the *in vitro* engineered cartilage's fate *in vivo*.

1. Introduction

As cartilage lacks the ability to replace damaged tissue with newly synthesized cells and extracellular matrix [1] cartilage grafting has been used widely to reconstruct skeletal defects of the nose, ear and trachea. Autologous grafting has proved to be an appropriate technique [2]. However this method has certain disadvantages, such as donor site morbidity, limited amounts of material and transplant degeneration leading to formation of fibrous tissue [3]. Therefore, in recent years different experimental strategies have been followed to reconstruct cartilage defects. A free periosteal flap covering a suspension of chondrocytes was used successfully to treat defects in the knee [4]. Nevertheless, this method is not suitable for the treatment of defects in the head and neck region because only preformed defects can be filled.

A different concept is the *in-vitro* growth of bioartificial cartilage with the help of resorbable polymer

scaffolds. Engineered cartilage from animal cells has been studied by different research groups in recent years [5–7]. Our research group demonstrated recently [8, 9] that *in vitro* grown human chondrocyte–polymer constructs form cartilage-like tissue *in vivo* after transplantation. These constructs had the typical appearance and histological characteristics of hyaline cartilage.

In the present study we focus on transplant structure, vascular ingrowth and the degradation of the polymer scaffold of *in vitro* engineered cartilage made of human chondrocyte pools. Furthermore, we present preliminary results from transplants made of single patients' cells without additional ingredients to experimentally fulfill all requirements for the clinical application.

2. Material and methods

2.1. Cell preparation

Chondrocytes were isolated enzymatically from human septal cartilage in a solution of $2\,mg\,ml^{-1}$ collagenase type II (Seromed, Berlin, Germany), $0.1\,mg\,ml^{-1}$ hyaluronidase (Serva, Heidelberg, Germany) and $0.15\,mg\,ml^{-1}$ DNAse type II (Paesel, Frankfurt, Germany) [15]. Chondrocytes were seeded in monolayer culture flasks (Greiner, Frickenhausen, Germany) in a concentration of 20 000 cells cm^{-2}. The cells were grown as chondrocyte-pools in Dulbecco's minimal essential medium (DMEM) (Gibco, Deisenhofen) supplemented with 10% fetal calf serum (FCS) (Gibco, Deisenhofen) and $20\,mg\,ml^{-1}$ gentamicin (Merck, Darmstadt) for 2 weeks. Single patients' cells were kept in monolayer culture for 3–4 weeks, autologous serum was added instead of FCS. Medium was changed three times per week. Culture conditions were constantly held at 37 °C in an atmosphere of 5% CO_2 and 90% humidity.

2.2. Tissue engineering and culture

The fully resorbable polymer fleece Ethisorb (Ethicon, Norderstedt, Germany) was cut into squares measuring $0.5 \times 0.5\,cm$. It contains fibers of poly-L-glycolic-acid (PGA) and poly-L-lactid-acid (PLA) in a 9 : 1 ratio and some polydioxanon adhesions. After monolayer-culture the chondrocytes were harvested using a 0.05% trypsin-ethylendiamine tetra-acetic acid (EDTA) solution (Seromed, Berlin, Germany). The chondrocyte-pools were washed three times in DMEM and then suspended in 2% ultra-low-melting agarose type IX (Sigma, Munich, Germany). The cell-concentration was 1.5×10^7cells ml^{-1}. This chondrocyte–agarose suspension was poured over the polymer fleeces until the complete suspension was absorbed into the fleeces. Then the fleeces were put into prepolymerized 4% ultra-low-melting agarose type IX to form a capsule of approximately 1 mm thickness around the polymer–chondrocyte constructs. Chondrocytes from single patients were loaded into the polymer scaffolding without agarose. The specimens were placed into perfusion culture chambers (Minucells and Minutissue, Bad Abbach, Germany) for 10–14 days. A peristaltic pump was operated in on/off intervals of 30 min and guaranteed constant supply with 1 ml fresh culture medium per hour. We used DMEM supplemented with 2% FCS and $50\,\mu g\,ml^{-1}$ ascorbic acid as culture medium. Fresh medium was kept in a cooled reservoir, used medium was pumped into a waste bottle.

2.3. *In vivo* experimental model

Male homozygotic nude (athymic) mice (CD1, nu/nu; Charles River Wiga, Sulzfeld, Germany) were operated under aseptic conditions. The fleece–chondrocyte constructs were placed into a subcutaneous pouch in the dorsum of the mice. Pentobarbital (Nembutal, Wirtschaftsgenossenschaft deutscher Tierärzte, Hannover, Germany) was used for anaesthesia. Eighteen nude mice received transplants of chondrocyte pools, agarose and Ethisorb fleece, nine mice received Ethisorb soaked with agarose only and seven mice (until now) received transplants of two single patients (chondrocytes, Ethisorb fleece, but no agarose). After 12 and 24 weeks the mice were sacrificed by an overdose of pentobarbital. The specimens were removed for macroscopic and microscopic analysis.

2.4. Histochemistry and immunohistochemistry

To investigate proteoglycan synthesis Alcian Blue and Toluidine Blue staining were performed. For collagen synthesis azan staining was used [10].

The following polyclonal antibodies were applied in indirect immunoperoxidase technique: PS-48 (Monosan, Uden, Netherlands) for the detection of collagen type II [11, 12]. PS-47 (Monosan, Uden, Netherlands) for the detection of collagen type I [11, 12].

3. Results

3.1. Perfusion culture

All specimens maintained their original size and shape after 10 days in perfusion culture. The constructs still appeared in their original violet color, indicating the presence of the polymer scaffold. Histological evaluation revealed that only slight production of matrix products took place *in vitro*. Collagens were found in small amounts only.

3.2. Gross morphology

As the specimens were transplanted subcutaneously in the dorsum of the nude mice, their macroscopic structure could be monitored during the whole experimental time and not only after explantation. Ethisorb fleeces that were soaked with agarose only showed a reduction in size starting immediately after implantation. Already after 6 weeks there were only small macroscopic remnants of the material left. After 12 and 24 weeks no remnants were detected. There were no signs of cartilage formation.

Fourteen of 18 specimens of Ethisorb loaded with chondrocyte pool cells did not show remarkable changes in size or configuration. Two specimens started to be resorbed after 6 weeks and could not be explanted after 12 weeks. Another two specimens started decreasing in size from about 10 weeks on and were completely

resorbed after 24 weeks. After explantation all the transplants looked like hyaline cartilage in a beige–white color with no macroscopic remnants of the polymer scaffold (Fig. 1). Mechanical stability was firm with slight elasticity. Three of the seven single patients' transplants kept up a constant configuration and size and were of beige–white color as well; four transplants decreased in size and were of soft consistency.

3.3. Histological appearance and ingrowth of blood vessels

Around every explanted specimen that contained chondrocytes was a thin fibrous capsule, separating the neocartilage from the surrounding murine tissue. Within the capsule we found vessels abundantly (Fig. 2a). Inside the transplanted tissue some vessels were observed especially in areas where only little matrix formation had taken place and in areas close to remnants of fleece fibers. In the single patients' transplants that were prepared without agarose, we found vessels in the same pattern as in the chondrocyte pool transplants (Fig. 2b).

3.4. Degradation of the polymer scaffolds

Obviously, the degradation of Ethisorb fleece fibers surrounded by chondrocytes with or without agarose suspensions was a lot slower than the degradation of the fleece soaked with agarose alone.

The degradation of the pool chondrocyte-loaded fleece was evident already 6 weeks after transplantation. After 12 weeks the fleece fibers were completely fractured, however large portions of the material were still visible. After 24 weeks we still found very few remnants of Ethisorb resulting from the polydioxanon adhesions although the degradation seemed to be almost complete at that time (Fig. 3a, b). The single patients' transplants showed no remarkable changes in the degradation time of the polymer fleece compared to the pools transplants.

3.5. Synthesis of extracellular matrix components

The histochemical stainings revealed the presence of collagens and glycosaminoglycans in all cell-loaded transplants. In the single patients' transplants the distribution of this matrix component was not equal. In contrast, we observed peripheral areas containing large amounts of collagens and at the same time almost no glycosaminoglycans. However, central regions contained both collagens and glycosaminoglycans abundantly (Fig. 4 a, b).

In all transplants (chondrocyte-pools and single patients' specimens) some parts of the collagen were identified as collagen type II, although collagen type I was synthesized as well in the same region. Collagen type II was mainly found in the central parts of the transplants but it was observed in some more peripheral regions as well. Vice versa, collagen type I was found predominantly in the transplants periphery and only rarely in central parts of the transplants. The areas with typical hyaline cartilage-like appearance and the chondrocytes arranged in chondron-like structures contained typically type II collagen.

Concerning matrix synthesis in general, three single patients' transplants closely resembled the chondrocyte pool transplants while the other four single patients' transplants only showed small areas of typical hyaline cartilage matrix and only few chondrocytes inside the transplant.

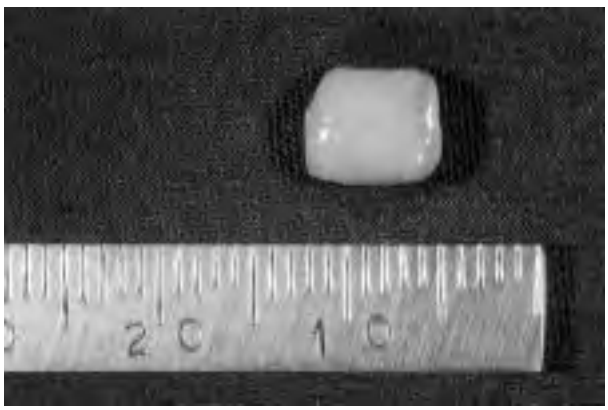
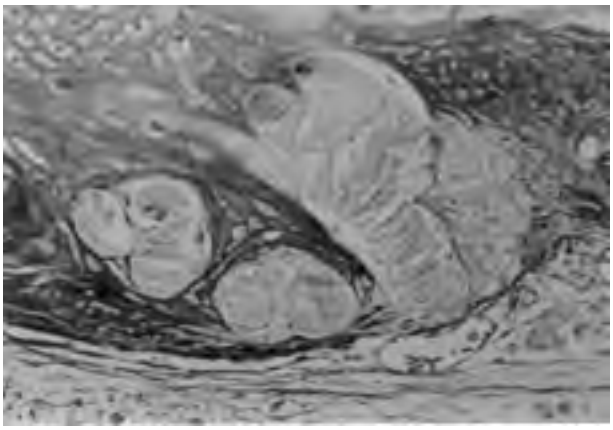
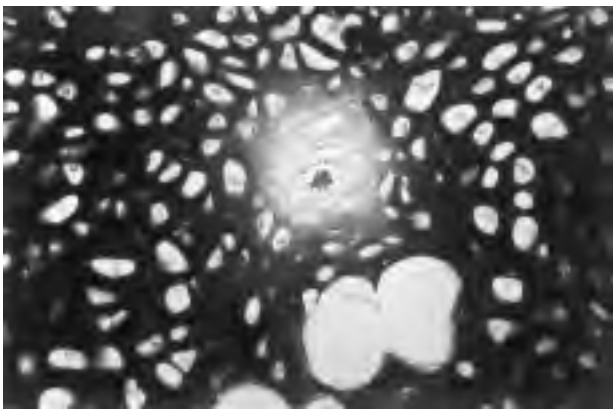


Figure 1 Chondrocyte-pool–Ethisorb–agarose construct 24 weeks after transplantation. The size and configuration is preserved. The mechanical stability is firm–elastic.

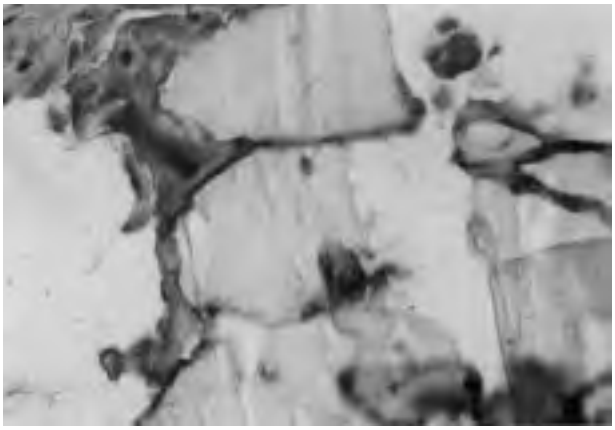


(a)

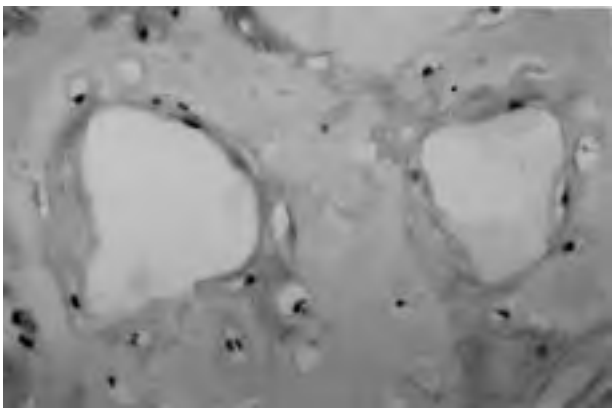


(b)

Figure 2 (a) Blood vessels are found in the fibrous capsule surrounding the engineered cartilage *in vivo*. Single patients' transplant after 12 weeks. Azan staining. (b) Vessels are also found in areas with less production of extracellular matrix molecules than in the surrounding areas. Single patients' transplant after 12 weeks. Azan staining.

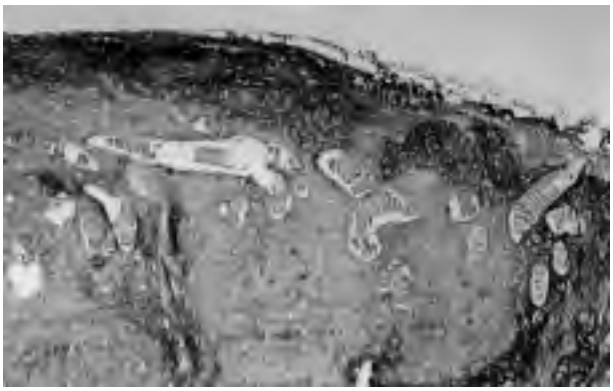


(a)



(b)

Figure 3 Degradation of the polymer scaffold Ethisorb after 12 (a) and 24 (b) weeks. (a) The fleece fibers are fractured into small pieces. Some fibers have disappeared. Collagen I staining. (b) After 24 weeks the material is almost completely degraded. Areas containing fleece fibers at the beginning are now partially filled with newly synthesized cartilage matrix. Azan staining.



(a)

(b)

Figure 4 (a) Azan staining of one single patients' transplant after 12 weeks *in vivo*. The whole transplant contains collagens, the staining is more intense in the periphery. (b) Toluidine blue staining of the same specimen. Glycosaminoglycans are preferably found in the central region of this transplant.

4. Discussion

Tissue engineering as a new concept of transplantation opens up new horizons for patient treatment. Referring to Langer and Vacanti [13] an estimated 1 million patients per year could benefit from these new techniques in the US alone. Including such different kinds of tissues as liver, kidney, pancreas, heart muscle or even nervous tissue engineering could offer a more effective and cheaper treatment. Cartilage grafting has certain disadvantages; therefore, improved concepts are necessary in this clinical field as well.

Engineering of human cartilage transplants was performed recently with human chondrocyte pools in the nude mouse model [9]. As cartilage grafting is planned to be performed in an autologous system, studies with single patients' cells are unequivocable. Our preliminary results show clear variations in the *in vivo* development of the transplants. As the prerequisites such as age of patient, culturing of chondrocytes, application of polymer scaffolding were identical, the reasons for the inconsistency of the transplants are still unclear. One reason could be the degradation of the polymer scaffold Ethisorb. *In vitro*, bulky releases of degradation products lead to the death of the whole cell culture [9]. This phenomenon might also be the cause for some of the complete resorptions that have been observed. However, in most engineered cartilage specimens the material degradation did not have a negative influence on the development of cartilaginous matrix *in vivo*. So, another possible reason why resorption took place in some constructs might be an uneven cell distribution. Originally, we used agarose to increase the ability of chondrocyte redifferentiation [14] and to get a better homogeneity of cell distribution inside the fleece [15]. The preliminary results from single patients' cells without agarose show though, in accordance with literature [16], that the influence of agarose on cell distribution and cartilage matrix development is not essential. For clinical application the renunciation of additional ingredients such as agarose can reduce the risk of possible immunological reactions to the transplants which cannot be studied in our nude mouse model.

The distribution of cartilage-specific matrix components inside the transplants was uneven. However, areas that morphologically resembled hyaline cartilage, with a homogeneous matrix and chondron formation, always contained type II collagen and glycosaminoglycans comparable to the native tissue. The fact that collagens type I and II were both found in some areas is a point of discussion. The chondrocytes could have switched from type I to type II synthesis sometime after transplantation,

and the synthesized collagen type I is not replaced by type II collagen but remains stable in the extracellular matrix. Another reason could be the cosecretion of type I and type II collagen as demonstrated by von der Mark [17]. However, the general agreement in literature is that chondrocytes either secrete type II or type I collagen depending on their state of differentiation. Further investigations need to be performed to clarify this phenomenon.

Vascular ingrowth was observed in the fibrous capsule surrounding the transplants. Furthermore, vessels were found in areas with poorly developed matrix and near polymer fleece fibers. As the vascularization is known to be an initial step leading towards calcification of the transplant a further differentiation of the specimens into bone seems to be possible, although no calcification was found in our transplants (application of von Kossa's staining, data not shown). Whether vascular ingrowth is the reason for poor matrix formation, or whether poor matrix formation leads to vascular ingrowth remains to be studied. *In vivo* microscopy to study the vessel formation dynamically could give an answer to this question and is going to be performed in our laboratory in the near future.

In summary, chondrocyte pools are a suitable method to study cartilage engineering of human cells *in vitro* and *in vivo* in experimental models. The engineering of cartilage from single patients' cells is possible without additional ingredients such as agarose. However, variations in the preliminary results show that the clinical application with human cells is more difficult than one would expect when using human chondrocyte pools. As the possibility of generating human cartilage *in vitro* has already been demonstrated, the tissue engineering studies of the future should be performed under clinical conditions with human cells from single patients and without adding ingredients that are not suitable for the clinical application like, for example, FCS. These further studies need to be performed to clarify which individual factors influence the *in vitro* engineered cartilage's fate *in vivo*.

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