Cartilage reconstruction in head and neck surgery: Comparison of resorbable polymer scaffolds for tissue engineering of human septal cartilage

Nicole Rotter,[1] J. Aigner,[1] A. Naumann,[1] H. Planck,[2] C. Hammer,[3] G. Burmester,[4] M. Sittinger[5]
[1]*Department of Otorhinolaryngology, Head and Neck Surgery, Klinikum Großhadern, Ludwig–Maximilians University of Munich, Marchioninistrasse 15, 81377 Munich, Germany*
[2]*Department of Biomedical Engineering, Institute for Textile and Process Engineering, Denkendorf, Germany*
[3]*Institute for Surgical Research, Ludwig–Maximilians University of Munich, Munich, Germany*
[4]*Department of Medicine III, Charité, Humboldt University of Berlin, Berlin, Germany*
[5]*German Rheumatism Research Centre, Berlin, Berlin, Germany*

Received 5 August 1997; accepted 17 April 1998

Abstract: New cell culture techniques raise the possibility of creating cartilage *in vitro* with the help of tissue engineering. In this study, we compared two resorbable nonwoven cell scaffolds, a polyglycolic acid/poly-L-lactic acid (PGA/PLLA) (90/10) copolymer (Ethisorb) and pure PLLA (V 7-2), with different degradation characteristics in their aptitude for cartilage reconstruction. Chondrocytes were isolated enzymatically from human septal cartilage. The single cells were resuspended in agarose and transferred into the polymer scaffolds to create mechanical stability and retain the chondrocyte-specific phenotype. The cell–polymer constructs were then kept in perfusion culture for 1 week prior to subcutaneous transplantation into thymusaplastic nude mice. After 6, 12, and 24 weeks, the specimens were explanted and analyzed histochemically on the presence of collagen (azan staining), proteoglycans (Alcian blue staining), and calcification areas (von Kossa staining). Furthermore, different collagen types (collagen type I, which is found in most tissues, but not in hyaline cartilage matrix; and collagen type II, which is cartilage specific) were differentiated immunohistochemically by the indirect immunoperoxidase technique. Vascular ingrowth was investigated by a factor VIII antibody, which is a endothelial marker. Quantification of several matrix components was performed using the software Photoshop. Significant differences were found between both nonwoven structures concerning matrix synthesis and matrix quality as well as vascular ingrowth. Ethisorb, with a degradation time of approximately 3 weeks *in vitro,* showed no significant differences from normal human septal cartilage in the amount of collagen types I and II 24 weeks after transplantation. Thin fibrous tissue layers containing blood vessels encapsulated the transplants. V 7-2 constructs, which did not show strong signs of degradation even 24 weeks after transplantation, contained remarkably smaller amounts of cartilage-specific matrix components. At the same time, there was vascular ingrowth even in central parts of the transplants. In conclusion, polymer scaffolds with a short degradation time are suitable materials for the development of cartilage matrix products, while longer stability seems to inhibit matrix synthesis. Thus, *in vitro* engineering of human cartilage can result in a cartilage-like tissue when appropriate nonwovens are used. Therefore, this method could be the ideal cartilage replacement method without the risk of infection and with the possibility of reconstructing large defects with different configurations. © 1998 John Wiley & Sons, Inc. J Biomed Mater Res, 42, 347–356, 1998.

Key words: cartilage transplantation; perfusion culture; tissue engineering; polymer scaffolds; matrix synthesis

INTRODUCTION

In modern head and neck surgery, there is a growing demand for a material that can be used to replace large cartilage defects.[1,2] Traumatic, tumorous, or congenital lesions of the midfacial region or rhinobasis need to be reconstructed carefully to guarantee sufficient aesthetic and functional results. Furthermore, the replacement of larynx[3] and trachea[2] still remains an unsolved problem. Until now, no material had been found that could fulfill all the demands or the main ones of a tracheal equivalent.

Today, autologous rib and ear cartilage is widely used due to the remaining transplant viability and only minor resorption phenomena. Unfortunately, there is rarely enough material. In addition, the risks of a second operation have to be considered. Furthermore, calcification mainly of rib cartilage would have a negative influence on mechanical characteristics.[4]

Correspondence to: Dr. N. Rotter
Contract grant sponsor: Deutsche Forschungsgemeinschaft; Contract grant numbers: BU 755, BU 445/5-1

 CCC 0021-9304/98/030347-10

Allogeneic transplants must be conserved to prevent autolysis. At the same time, antigenic characteristics and the chance of transmitting an infection are eliminated or at least reduced.[1] However, no definite consensus exists regarding whether transmission of infections is possible[5–7] when applying common conservation chemicals such as cialit. Xenogeneic transplants need to be treated for conservation as well. This results in frequent resorption; moreover, the risk of infectiousness cannot be excluded completely.

A new concept of transplantation has been discussed recently.[8,9] Instead of using the whole tissue, single cells could be applied to obtain the original tissue's function. Hepatocytes were used to support insufficient liver function.[10] It might be possible to transplant microencapsulated islet cells to increase pancreatic function in diabetes mellitus.[11,12] Increasing knowledge of chondrocyte differentiation characteristics could open similar new perspectives for cartilage transplantation. Bovine chondrocytes have already been used to grow new cartilage *in vivo.*[13–15] As there are remarkable differences in the characteristics of chondrocytes from different species,[16] in this study only human cells were investigated. Prior work of our research group showed that human chondrocytes in polymer fleece structures and a perfusion chamber are able to produce their own new cartilage matrix.[17–19] The perfusion culture system was designed to grow cells under the most natural conditions possible. Originally, the inventors grew renal cells in the system[20.21]; our research group first used it for chondrocytes.[17,18] Perfusion guarantees a permanent flow of fresh medium. Chondrocytes are encapsulated by agarose, which allows sufficient diffusion of nutrients, and at the same time leads to accumulation of newly synthesized matrix products. We found that the cells were able to retain their differentiated phenotype in this three-dimensional culture system.[22,23] Furthermore, production of typical matrix components such as proteoglycans and collagens including collagen type II was observed *in vitro.*[17,18,22]

Resorbable polymer scaffolds which allow the three-dimensional distribution of cells and offer mechanical stability have been analyzed for their biocompatibility[24] and found to be suitable for cartilage growth *in vitro.* The aim of the present study was to investigate the matrix synthesis of *in vitro* engineered human cartilage after transplantation.

MATERIALS AND METHODS

Polymer scaffolds

We used Ethisorb (Ethicon, Norderstedt, Germany), a nonwoven material consisting of polyglycolic acid (PGA) and poly-L-lactic acid (PLLA) in a 90:10 ratio, and also containing small amounts of polydioxanon (Fig. 1). These ingredients have been used as suture material in surgery for many years. The degradation time of Ethisorb *in vitro* is approximately 3 weeks.[25]

The second nonwoven material was V 7-2 (Institute for Textile and Process Engineering, Denkendorf, Germany) (Fig. 1). It is made of pure poly-L-lactide (PLLA). The degradation time is approximately 9 months.[24]

Both fleeces were obtained as 10 × 10 × 0.2-cm sheets and were cut into 0.5 × 0.5 × 0.2-cm square pieces.

Each nonwoven material was coated with a 0.1-mg/mL poly-L-lysine-solution (Biochrom, Berlin, Germany) to improve chondrocyte attachment to the polymer fibers.[24] After they were kept in a solution of about the 10-fold volume of the fleeces for 1 h at room temperature, the fleeces were lyophilized for 2 days.

Isolation of chondrocytes and monolayer culture

Human septal cartilage was obtained from 80 patients (ages 22–64) who had undergone reconstructive surgery of the nasal septum.

As described previously,[19] chondrocytes were isolated enzymatically in a solution of 2 mg/mL collagenase type II (Seromed, Berlin, Germany), 0.1mg/mL hyaluronidase (Serva, Heidelberg, Germany), and 0.15 mg/mL DNAse type II (Pasel, Frankfurt, Germany). Chondrocytes were seeded in monolayer culture flasks (Greiner, Frickenhausen, Germany) in a concentration of 20,000 cells/cm^2. The cells were grown in Dulbecco's minimal essential medium (DMEM) (Gibco, Deisenhofen, Germany) supplemented with 10% fetal calf serum (FCS) (Gibco) and 20 mg/L gentamicine (Merck, Darmstadt, Germany). Medium was changed three times per week. Culture conditions were held constant at 37°C in an atmosphere of 5% CO_2 and 90% humidity.

Tissue engineering and perfusion culture

After 1 week in monolayer culture, the chondrocytes were harvested using a 0.05% w/v trypsin–ethylenediaminetetra-

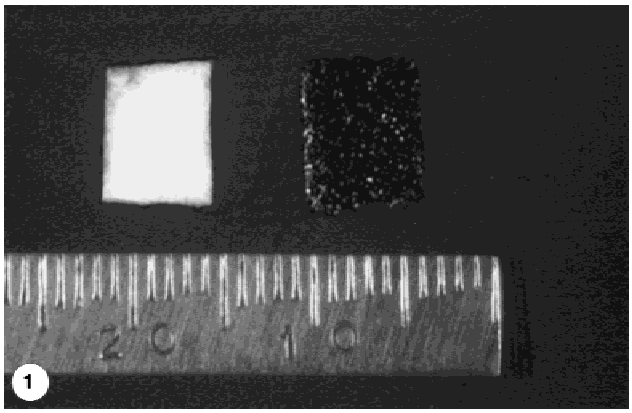


Figure 1. Macroscopic appearance of polymer fleeces prior to cell seeding. White-colored V 7-2 (Institute for Textile and Process Engineering, Denkendorf, Germany) is on the left-hand side, versus the lilac-colored Ethisorb (Ethicon, Norderstedt, Germany) on the right side.

acetic acid (EDTA) solution (Seromed, Berlin, Germany). They were washed three times in DMEM and suspended in 2% w/v ultra-low-melting agarose type IX (Sigma, Munich, Germany). The cell concentration was 1.5×10^7 cells/mL. This chondrocyte–agarose suspension, corresponding to the volume of the fleece squares, was poured over the polymer nonwoven materials until the complete suspension was absorbed into them. Then, the fleeces were put into 4% w/v ultra-low-melting agarose type IX to form a capsule approximately 1 mm thick around the polymer–chondrocyte constructs.

The specimens were placed into perfusion culture chambers[20,21] (Minucells and Minutissue, Bad Abbach, Germany) as described previously in detail.[17,18,23] Briefly, a peristaltic pump was operated in on–off intervals of 30 min to guarantee constant supply of 1 mL fresh culture medium per hour. We used Ham's F12 (Gibco) supplemented with 2% w/v FCS and 50 μg/mL ascorbic acid as culture medium. Fresh medium was taken from a cooled reservoir and used medium was pumped into a waste bottle (Fig. 2). The temperature inside the perfusion culture system was kept up by a heating plate to guarantee a constant temperature of 37°C. The system was operated at room atmosphere. The specimens were kept in perfusion culture for 1 week.

Transplantation of specimens

Male homocygotic nude (athymic) mice, aged 18–19 weeks (CD1, *nu/nu;* Charles River Wiga, Sulzfeld, Germany), served for the investigation of the *in vivo* behavior of our cartilage transplants. The German guidelines (GV-SOLAS) for the care and use of laboratory animals were observed. Ether (Hoechst, Frankfurt, Germany) and pentobarbital (Nembutal®; Wirtschaftsgenossenschaft deutscher Tierärzte, Hannover, Germany) were used for anesthesia. Under aseptic conditions, a subcutaneous pouch was formed in the back, into in which the fleece–chondrocyte constructs were placed. The wound was closed with Seralon sutures (Ethicon, Norderstedt, Germany). After 6, 12, and 24 weeks, the mice were sacrificed by an overdose of pentobarbital. The specimens were removed for macroscopic and microscopic analysis.

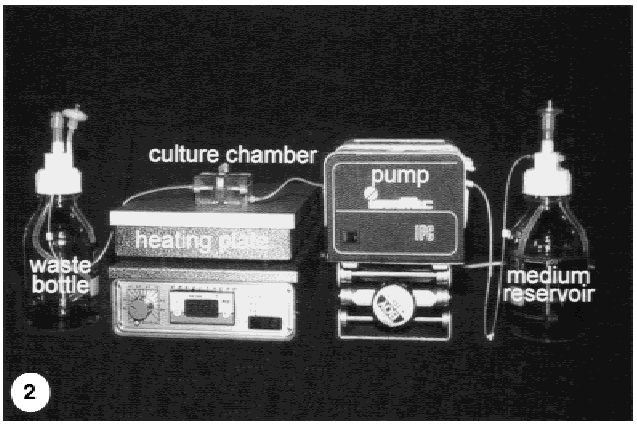


Figure 2. Perfusion culture chamber. The peristaltic pump delivers a constant flow of fresh culture medium from the medium reservoir. The culture chamber is placed upon a heating plate to guarantee the optimal temperature. Used medium is pumped into the waste bottle.

Experimental groups

We investigated three experimental groups (Table I). At 6, 12, and 24 weeks after subcutaneous transplantation, we explanted six specimens into groups I and II, and three specimens in group III. They were placed in OCT compound (Tissue Tek, Hallstadt, Germany) and kept at −80°C until histologic workup.

Sections from each transplant were cut in three different areas: the left and right margins and center. As qualitative assessment did not show major differences in the transplants' histological appearance, we used the central sections for quantitative evaluation only.

Mechanical stability

The transplants' size was measured on graph paper prior to transplantation. After explantation, this procedure was performed again to determine any change in size or configuration. The mechanical testing was performed independently by two experienced surgeons. Reference material was native human hyaline cartilage. We determined whether the material was soft or hard using the following criteria: It was soft when it was squeezable when cut with a scalpel; it was hard when it was hard to cut with a scalpel, but it did not get squeezed.

Histochemistry

Sections were stained with Alcian blue for the investigation of proteoglycan synthesis, with azan for collagen synthesis. For the identification of calcification areas, we carried out von Kossa's staining technique.[26]

Immunohistochemistry

For the indirect immunoperoxidase technique, the following polyclonal antibodies were applied:

TABLE I
Experimental Groups

Group (No. of Animals)	Fleece	Monolayer Culture (wk)	Perfusion Culture (wk)
I (18)	Ethisorb	1	1
II (18)	V7-2	1	1
III (9)	Agarose	1	1

PS-48 (Monosan, Uden, Netherlands): rabbit–anti-human collagen type II.[27,28] The dilution was 1:20; the incubation time was18 h.
PS-47 (Monosan, Uden, Netherlands): rabbit–anti-human collagen type I.[27,28] The dilution was 1:50; the incubation time was 18 h.
AO 82 (Dako, Hamburg, Germany): rabbit–anti-human factor VIII, which can be found in the endothelial basal membrane.[29–31] The dilution was 1:3000; the incubation time was 30 min.

The 5-μm cryostat sections were fixed with acetone (Merck) and hydrogen peroxide, then incubated with one of the primary antibodies. After a 10-min wash in Tris base solution, detection was carried out with a peroxidase-conjugated anti-rabbit antibody (Dako) for 30 min. Visualization was performed with the AEC (3-amino-9-ethylcarbazol) (Sigma) method.[32] For negative controls, the primary antibody was omitted according to the immunohistochemical protocol.

The dilutions were optimized with dilution series on native cartilage and skin specimens.

Quantification of matrix synthesis

The software Adobe Photoshop was used on a Macintosh Quadra 800 computer to perform quantification of matrix synthesis.

Stains were evaluated only when they differed from the positive control (septal cartilage) < 5%. Septal cartilage was also used to set the standards for the different staining methods.

Each specimen was photographed in a 50-fold magnification and transfered to a CD-ROM. The whole area of the transplants was determined. Afterward, the positively stained sections were measured. The quotient of positively stained area and the whole transplant area resulted in the positively stained area as a percentage.

Statistical analysis

The nonparametric Kruskal–Wallis test was applied because it does not require Gaussian distribution as a prerequisite; this means it is suitable for small sample sizes as in this experiment. Afterward, Dunn's multiple comparison test, which is a modification of the Bonferroni test, was applied. All results are shown as box plots. The median is marked with a thick bar; the 25th and 75th percentile ranks are the respective borders of the boxes.

RESULTS

Perfusion culture

After 1 week in perfusion culture, collagen was detected by azan staining in all specimens of each experimental group. With the indirect immunoperoxidase method, we identified large portions of the whole collagen to be cartilage-specific type II collagen. Type I was found in small amounts as well. There were only slight differences between the groups.

Proteoglycans were identified with Alcian blue staining. In summary, they were less frequent than collagens. There were no major differences between the experimental groups.

Transplantation

Macroscopic appearance and stability of size and configuration

In group I, complete resorption of four specimens was observed. Two samples could not be explanted after 12 weeks, and another two samples after 24 weeks. In all other transplants of group I, there was no remarkable change in size [Fig. 3(a)]. Mechanical stability was similar to cartilage, with slight elasticity revealed by gross examination. There was no resorption or change in size in group II [Fig. 3(b)]. In comparison to group I, the specimens were slightly harder. All grafts of group III showed a decrease in size [Fig. 3(c)]. Mechanical characteristics were clearly softer than in both other groups.

Histologic appearance

Samples varied among groups. In group I, 14 of 18 samples showed typical histological features of native cartilage with a homogenous matrix, embedding roundly configurated cells in chondron-like structures. However, transplants also included areas, mainly in the periphery of the samples, with a fibrous matrix similar to fibrous regenerating tissue. Both other experimental groups were less inhomogenous in their histologic appearance. Group II samples mainly contained fibrous cartilage-like tissue. Areas with few cells and poor matrix formation appeared in the samples' periphery. Group III transplants showed homogenous matrix in some parts, while areas without matrixproduction were observed in others.

Collagen synthesis

Collagen was detected in all explanted specimens by azan staining (Fig. 4). Quantitative analysis revealed significant differences among the experimental groups [Fig. 5(a–c)]. Large portions of collagen were detected, especially in group I. After 24 weeks of

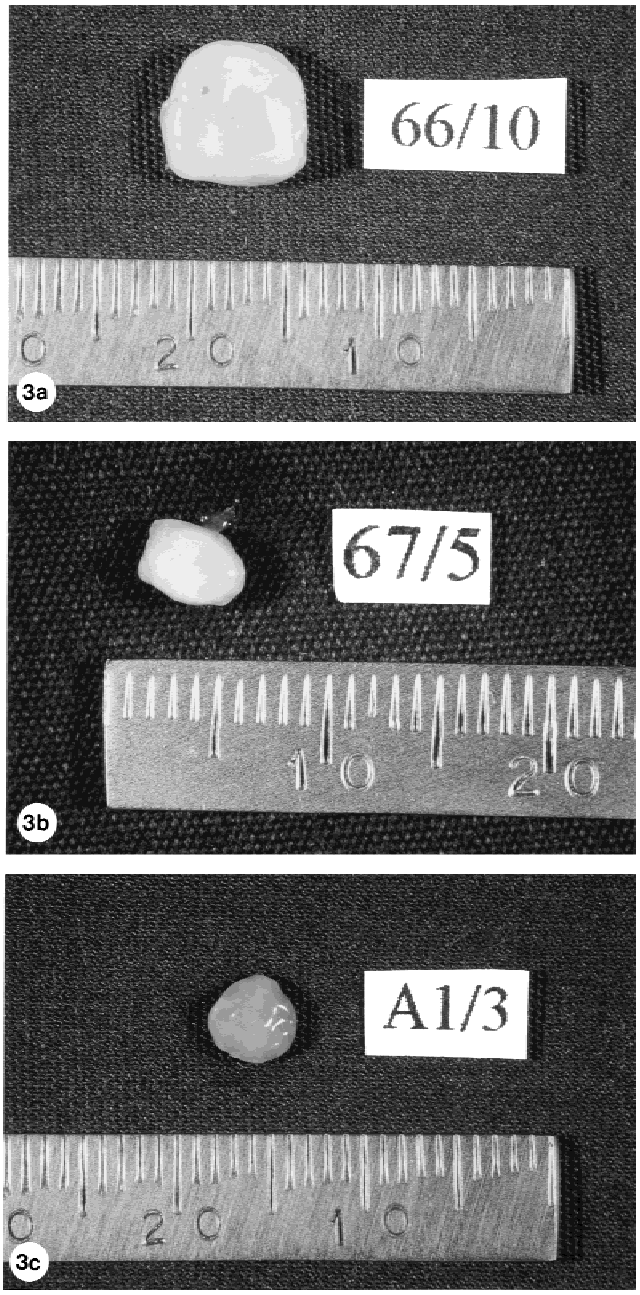


Figure 3. Macroscopic appearance of neocartilage 24 weeks after transplantation: (a) group I specimen; (b) group II specimen; (c) group III specimen.

transplantation, no significant difference between the content of collagen in our neocartilage (92%) and the content in native human septal cartilage (95%) existed. Clearly lower amounts were found in the other groups. Collagen reached a maximum of 70% of the whole transplant area in group II 6 weeks after transplantation, and a maximum of 63% in group III 12 weeks after transplantation.

Collagen types I and II

The indirect immunoperoxidase technique distinguished between cartilage-specific type II collagen and the nonspecific type I. Type I is typical of many body tissues, but regarding cartilage, this type is part of the perichondrium only.

All explanted specimens contained collagen type II. In group I, the content reached 80%, which is close to native cartilage 24 weeks after transplantation (Fig. 6). After 6 and 12 weeks, the amounts were markedly lower [Fig. 7(a)] and varied from 19% to 41%. In group II, very low levels of type II collagen [Fig. 7(b)] were detected, with a maximum of 13%. No changes were observed with increasing observation time. Group III showed an enlargement in collagen type II content from a minimum of 5% after 6 weeks to a maximum of 50% after 24 weeks, but could not reach cartilage-like contents [Fig. 7(c)].

Collagen type I was observed in all grafts. In group I, small amounts were detected which were not significantly higher than the content in native cartilage, owing to the perichondrium (8%). A minimal value of 4% was observed after 6 weeks; however, larger amounts up to 38% were found in other transplants of this group. Strikingly, collagen type I appeared mainly in the transplants' periphery [Figs. 8 and 9(a)]. Both other experimental groups showed higher contents of collagen type I, up to 79% in group II and 60% in group III [Fig. 9(b,c)]. Furthermore, it was found in varying areas of the neocartilage samples, including central and marginal areas.

Comparison between collagen type II and type I staining on serial sections revealed that some areas were positive for type II as well as for type I.

Proteoglycan synthesis

Proteoglycans are typical contents of the cartilage matrix. Alcian blue staining showed the presence of these macromolecules in all explanted samples. As this staining was very bright, we did not perform a quantitative analysis. Qualitative assessment revealed that there were large areas containing protoglycans, especially in group I. These amounts increased with time, which means that the largest areas were found 24 weeks after transplantation (Fig. 10). Unlike in collagen type II, proteoglycans were detected abundantly in both other experimental groups as well.

Calcification

Von Kossa's stain was applied to all specimens to detect calcification. One such area existed in one graft of group II after 12 weeks. This area measured approximately 25% of the whole transplant area and was localized in the middle of the sample with an oval

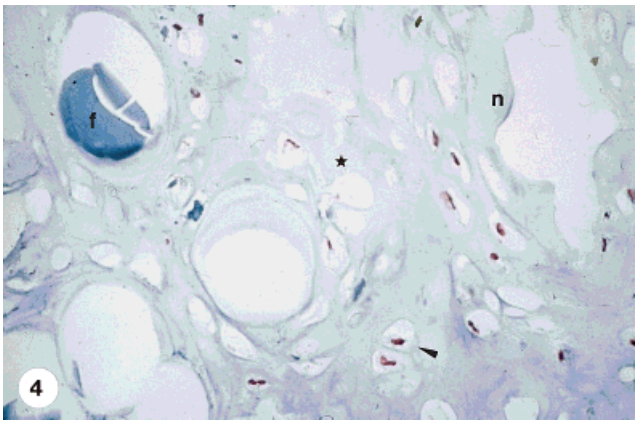


Figure 4. Azan staining of a group I specimen 24 weeks after transplantation. There are only very few remnants of Ethisorb left (f). Spaces which used to be filled with fleece fibers are now partially filled with newly synthesized cartilage matrix (n). * = Collagen-containing matrix; ▶ = chondron-1 like cell arrangement. Original magnification ×460.

configuration (Fig. 11). Calcification was not observed in any other transplant.

Vascular ingrowth

The thin fibrous tissue layers encapsulating all transplants contained abundant small vessels (Fig. 12). The specimens of group I contained few vessels in peripheral areas (Fig. 12) where cartilage-like matrix had not yet developed. In central parts of the transplants, no vessels could be detected. Inside the transplants of both other experimental groups, vessels were observed throughout the whole sample.

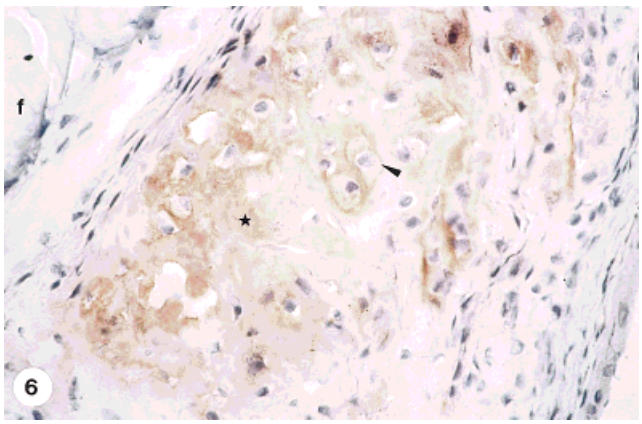
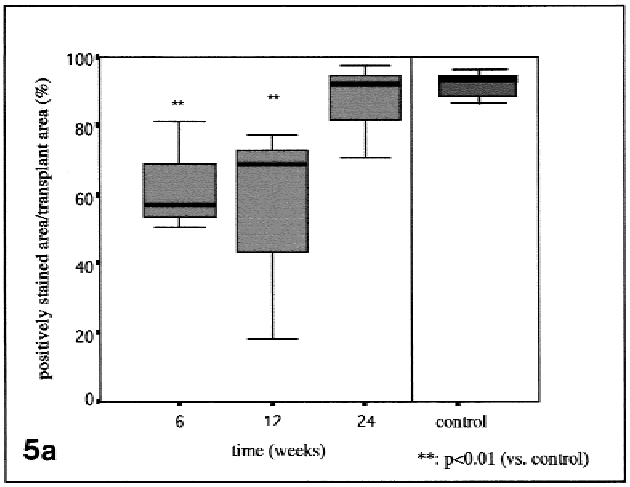
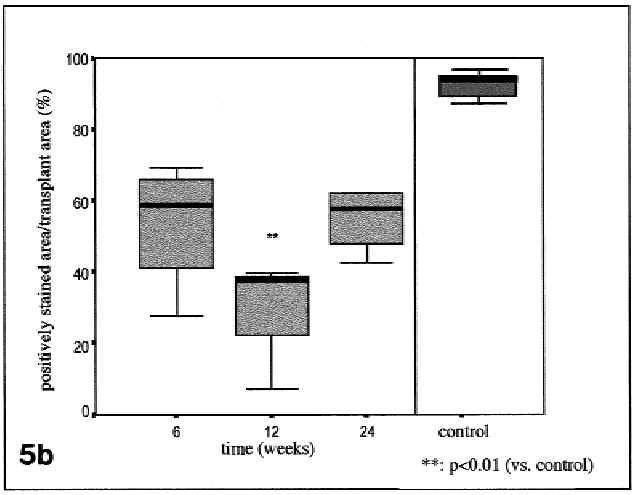


Figure 6. Indirect Immunoperoxidase staining for collagen type II. Group I neocartilage 12 weeks after transplantation. * = Collagen II–positive area; f = fleece fiber; ▶ cell nucleus. Original magnification ×400.





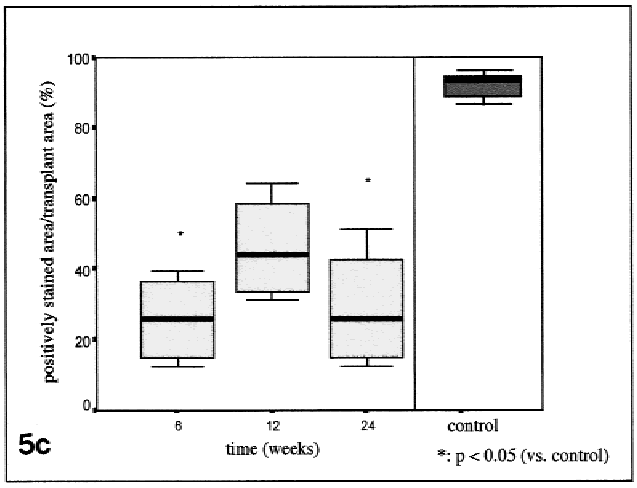


Figure 5. Quantitative analysis of the azan stains. (a) Group I: 6 and 12 weeks after transplantation, the collagen content was significantly lower than in the original hyaline cartilage. After 24 weeks, no significant difference was found. (b) Group II: 12 weeks after transplantation, the collagen content was significantly lower than in the control group. After 6 and 24 weeks, we found only clear differences. (c) Group III: differences between the transplanted and the control cartilage's collagen content were significant 6 and 24 weeks after transplantation. After 12 weeks, the differences were not as distinct.

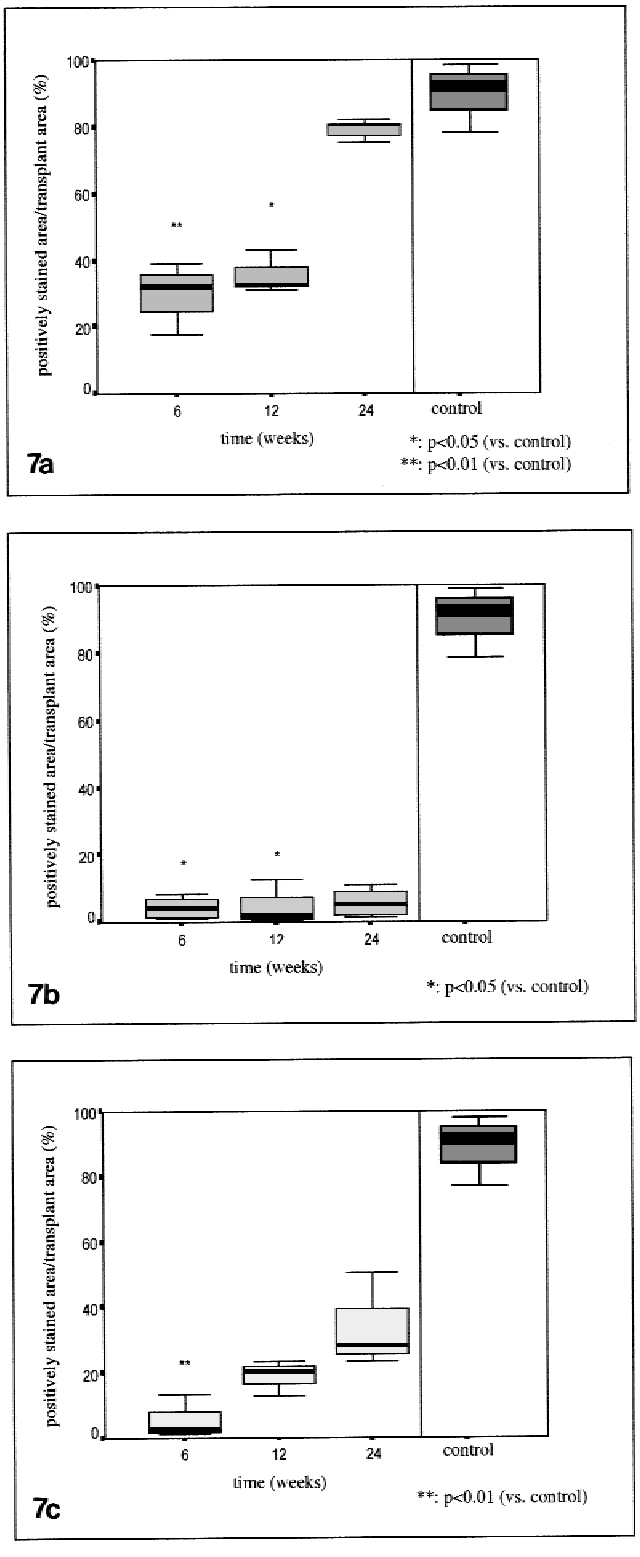


Figure 7. Quantitative analysis of the collagen type II stains. (a) Group I: After 6 and 12 weeks, the amount of collagen type II was significantly lower in the engineered cartilage than in the control. (b) Group II: The differences in collagen type II content were obvious at all times, although there was no statistical significance after 24 weeks. (c) Group III: There was a clear enlargement in collagen type II content with increasing transplantation time.

DISCUSSION

Agarose used as a scaffold alone (group III) was obviously not able to maintain the original configuration of the transplant, because we observed a clear decrease in size. Furthermore, handling of the very soft and weak transplants would be extremely inconvenient for the surgeon. Although a suspension of autologous chondrocytes injected under a periostal flap was recently used successfully to repair cartilage defects in the knee,[33] our results emphasize the necessity of using an additional scaffold that can offer better mechanical stability for the transplantation procedure and a specific form corresponding to the cartilage defect. Several characteristics need to be met by the optimal material.[17,18,22,23] It has to be biocompatible and biodegradable, as it is only a temporary scaffold and should be replaced by cartilage tissue as soon as possible. It should support cell adhesion and cell function. Besides the material's structural characteristics should allow a three-dimensional and homogenous distribution of the cells.[18] As nonwoven fleece structures such as Ethisorb and V 7-2 with high internal surface areas, and at the same time low amounts of biomaterial, accomplish most of these aspects, they are suitable cell carriers for cartilage engineering.

Recent studies by our group[18,24,33] showed that PGLA and PLLA nonwoven materials are suitable for chondrocyte growth *in vitro.* The cells tolerate even high concentrations of the degradation monomers glycolic acid and L(+) lactic acid with a better tolerability to L(+) lactic acid. However, under perfusion culture conditions, different characteristics were observed. Ethisorb degrades rapidly with bulky releases of degradation products. This process leads to the death of the whole cell culture after 3 weeks *in vitro.* In contrast, V 7-2 is significantly more stable in perfusion culture. Thus, for long-term growth of *in vitro* cartilage, V 7-2 is a more suitable material.

Prolonged *in vitro* development of the engineered cartilage could result in a more distinct matrix formation. This could inhibit vascular and cellular ingrowth. Both factors can be expected to have a negative influence on the transplant's mechanical characteristics. Vascularization is known to be the crucial step leading to calcification and ossification of cartilage. Thus, transplants could become very hard, lacking the typical flexible mechanical stability. Ingrowth of immunocompetent cells, on the other hand, might destroy the growing cartilage matrix and would thus lead to weak transplants unable to keep up the original size and configuration. Supporting the hypothesis of a positive influence of a strong development of cartilage matrix *in vitro* is the fact that we did not find vessels and host cells in areas with a well-developed cartilage-like matrix. Furthermore, cartilage is known to synthesize an angiogenesis inhibitory factor.[34,35] Puelacher and co-

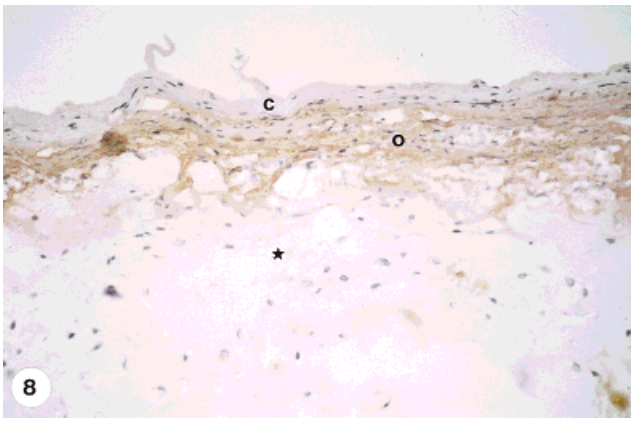


Figure 8. Indirect immunoperoxidase staining for collagen type I. Group I neocartilage 24 weeks after transplantation. ○ = Collagen I–positive area in the transplant periphery; * = central parts of the transplant which do not contain any collagen type I; C = fibrous capsule around the transplant. Original magnification ×200.

workers[13,14] found bovine neocartilage to be avascular 12 weeks after transplantation, which could well be a result of sufficient production of the angiogenesis inhibitory factor. On the other hand, reasons for avascularity in these studies could be a rapid matrix synthesis caused by the application of the cells of newborn calves or the shorter observation time compared to our experiments.

A distinct matrix synthesis *in vitro* is also important because intercellular matrix is a crucial factor for cell function[36] which influences cellular proliferation, migration, and cellular metabolism. Antigenic characteristics which are expressed on isolated chondrocytes[38,39] are masked in intact cartilage.

Comparing the PGLA and the PLLA nonwoven materials regarding matrix synthesis *in vivo*, PGLA obvi-

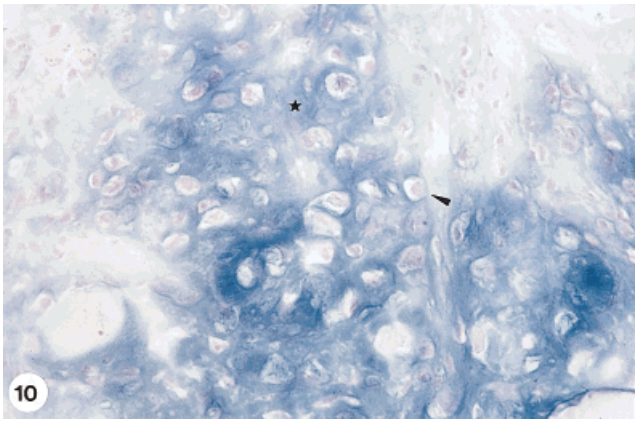
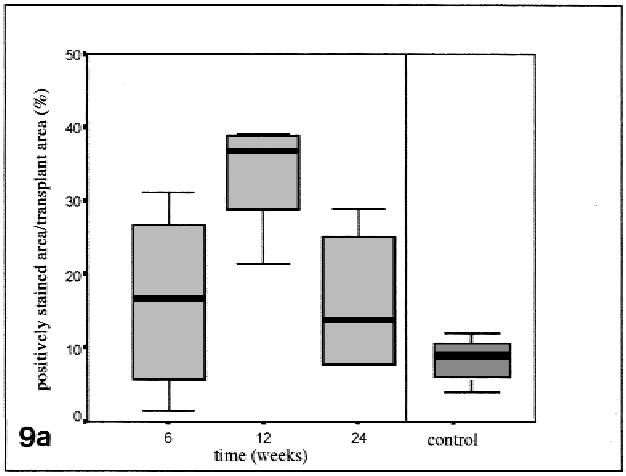
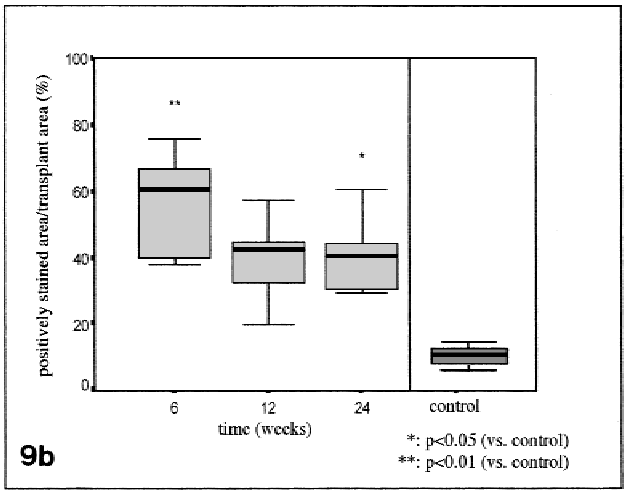


Figure 10. Alcian blue stain. Group I specimen 24 weeks after transplantation. * = Wide areas of matrix with cartilage-like configuration were stained positively for proteoglycans: ▶ = Cell nucleus. Original magnification ×400.





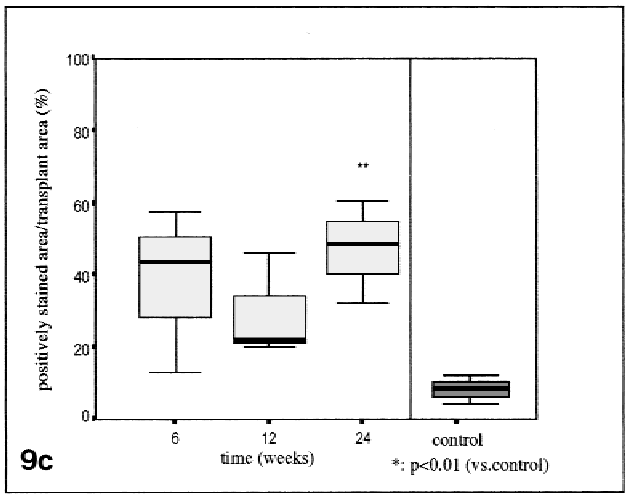


Figure 9. Quantitative analysis of the collagen type I stains. (a) Group I: No significant differences in collagen type I content existed at all times. (b) Group II: The amount of collagen type I was markedly higher in group II than in the control cartilage. Statistical significance was found after 6 and 24 weeks. (c) Group III: The content of cartilage-unspecific type I collagen was clearly higher than in the control group and significantly higher after 24 weeks of transplantation.

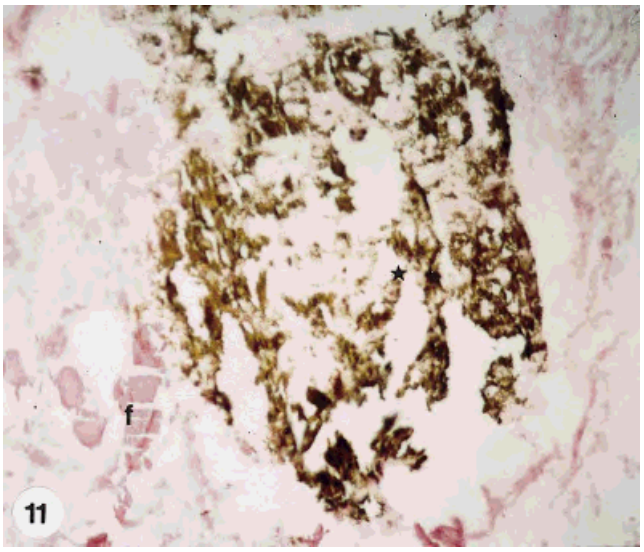


Figure 11. Von Kossa stain of group II specimen 24 weeks after transplantation. * = Calcification in the middle of the transplant; f = fleece fibers. Original magnification ×100.

ously had a positive influence on the stability of the chondrocyte phenotype. Collagen type II, the marker for differentiated chondrocytes generally indicating cartilage formation, was found in amounts comparable to native cartilage when PGLA was used. On the other hand, collagen type I was found only in small amounts. Transplants containing the PLLA scaffold showed significantly higher amounts of collagen type I and lower amounts of collagen type II, which indicates formation of fibrocartilage but not of hyaline cartilage. These results imply that the long-term stability of V 7-2 *in vivo* has a negative influence on the formation of hyaline cartilage which was not detectable when using Ethisorb fleeces. Coating with poly-L-lysine, which is known to stabilize the differentiated chondrocyte phenotype,[39] was not able to inhibit this effect. Certain areas containing both type I and type II collagen were observed as well. This implies that matrix synthesis switched from type I to type II sometime after transplantation. Coproduction or a switch from type II to type I could also be reasons for this observation.

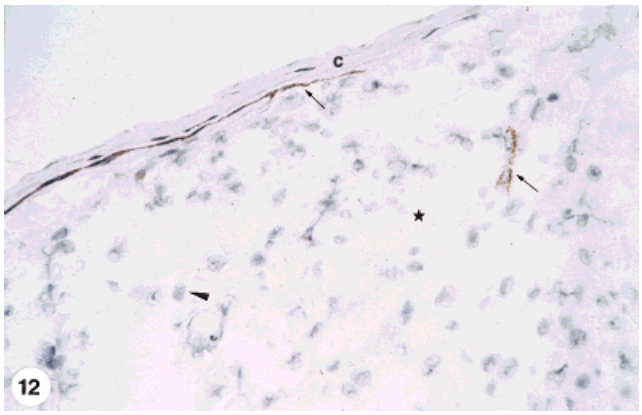


Figure 12. Indirect immunoperoxidase staining for factor VIII. Group I specimen after 24 weeks. The transplants contain vessels (→) mainly in the fibrous capsule (C) and in the periphery in areas around the fleece fibers (f). ► = Cell nucleus. Original magnification ×260.

A still unsolved problem is the homogenous distribution of cells inside the polymer scaffold. If there are transplant areas with very few chondrocytes, only minor quality cartilage-like matrix would develop. Chondrocyte clusters, on the other hand, would also result in unsatisfactory matrix synthesis. The major problem is to create a completely homogenous cell carrier in which the cell distribution could be as homogenous. The development of biomaterials with suitable mechanical characteristics is crucial for the improvement of cartilage engineering for transplantation.

This study clearly shows that nonwoven resorbable polymer scaffolds are suitable to grow a transplantable chondrocyte–polymer construct *in vitro.* Although further investigations concerning especially the biomechanical properties of the neocartilage need to be performed, the results of this study strongly encourage this new way of cartilage replacement with the help of tissue engineering.

This work is dedicated to PD Dr. J. Bujia for many valuable discussions and generous idealistic support. Ethisorb was kindly provided by Dr. H. J. Hoepttner (Ethicon, Norderstedt, Germany). The authors thank K. Lempart and A.-M. Allmeling for excellent technical assistance. Dr. N. Rotter is a recipient of the ''Progress in Medicine'' Award 1996 from the German Physicians Congress.

References

1. E. R. Kastenbauer, ''Preservation and application possibilities of allogenic (homologous) transplants in the ear, nose and throat region,'' *HNO,* **31**, 371–380 (1983).
2. C. Herberhold, ''Transplantation of larynx and trachea in man,'' *Eur Arch. Otorhinolaryngol.,* **1(Suppl.),** 247–255 (1992).
3. G. H. Zalzal, ''Rib cartilage grafts for the treatment of posterior glottic and subglottic stenosis in children,'' *Ann. Otol. Rhinol. Laryngol.,* **97,** 506–511 (1988).
4. C. Hammer and J. Bujia, ''Immunology of vital and preserved transplants,'' *Eur. Arch. Otorhinolaryngol.,* **1(Suppl.),** 3–26 (1992).
5. E. Wilmes, L. Gurtler, and H. Wolf, ''Transmissibility of HIV infections via allogenic transplants,'' *Laryngol. Rhinol. Otol.,* **66**, 332–334 (1987).
6. W. A. Dickson and T. J. Inglis, ''Cialit preserved cartilage: failure to guarantee sterility,'' *Br. J. Plast. Surg.,* **41**, 408–409 (1988).
7. J. Bujia, H. Meyer, C. Hammer, E. Wilmes, and L. Gurtler, ''Human immunodeficiency virus cannot productively infect freshly cultured human cartilage cells,'' *ORL J. Otorhinolaryngol. Rel. Spec.* **55**, 222–225 (1993).
8. A. Atala, J. P. Vacanti, C. A. Peters, J. Mandell, A. B. Retik, and M. R. Freeman, ''Formation of urothelial structures in vivo

from dissociated cells attached to biodegradable polymer scaffolds in vitro,'' *J. Urol.,* **148**, 658–662 (1992).
9. R. Langer and J. P. Vacanti, ''Tissue engineering,'' *Science,* **260**, 920–926 (1993).
10. P. M. Kaufmann, K. Sano, S. Uyama, T. Takeda, and J. P. Vacanti, ''Heterotopic hepatocyte transplantation: assessing the impact of hepatotrophic stimulation,'' *Transplant. Proc.,* **26**, 2240–2241 (1994).
11. A. M. Sun, I. Vacek, Y. L. Sun, X. Ma, and D. Zhou, ''In vitro and in vivo evaluation of microencapsulated porcine islets,'' *ASAIO J.,* **38**, 125–127 (1992).
12. Z. P. Lum, M. Krestow, I. T. Tai, I. Vacek, and A. M. Sun, ''Xenografts of rat islets into diabetic mice: An evaluation of new smaller capsules,'' *Transplantation,* **53**, 1180–1183 (1992).
13. W. C. Puelacher, D. Mooney, R. Langer, J. Upton, J. P. Vacanti, and C. A. Vacanti, ''Design of nasoseptal cartilage replacements synthesized from biodegradable polymers and chondrocytes,'' *Biomaterials,* **15**, 774–778 (1994).
14. W. C. Puelacher, J. Wisser, C. A. Vacanti, N. F. Ferraro, D. Jaramillo, and J. P. Vacanti, ''Temporomandibular joint disc replacement made by tissue-engineered growth of cartilage,'' *J. Oral Maxillofac. Surg.,* **52**, 1172–1177 (1994).
15. C. A. Vacanti, R. Langer, B. Schloo, and J. P. Vacanti, ''Synthetic polymers seeded with chondrocytes provide a template for new cartilage formation,'' *Plast. Reconstr. Surg.,* **88**, 753–759 (1991).
16. L. E. Freed and G. Vunjak-Novakovic, ''Tissue engineering of cartilage,'' in *The Biomedical Engineering Handbook,* J. D. Bonzino (ed.), CRC Press, Boca Raton, FL, 1995.
17. M. Sittinger, J. Bujia, W. W. Minuth, C. Hammer, and G. R. Burmester, ''Engineering of cartilage tissue using bioresorbable polymer carriers in perfusion culture,'' *Biomaterials,* **15**, 451–456 (1994).
18. J. Bujia, M. Sittinger, W. W. Minuth, C. Hammer, G. Burmester, and E. Kastenbauer, ''Engineering of cartilage tissue using bioresorbable polymer fleeces and perfusion culture,'' *Acta Otolaryngol.,* **115**, 307–310 (1995).
19. J. Bujia, P. Pitzke, E. Wilmes, and C. Hammer, ''Culture and cryopreservation of chondrocytes from human cartilage: Relevance for cartilage allografting in otolaryngology,'' *ORL,* **54**, 80–84 (1992).
20. W. W. Minuth and U. Rudolph, ''A compatible support system for cell culture in biomedical research,'' *Cytotechnology,* **4**, 181–189 (1990).
21. W. W. Minuth, G. Stöckl, S. Kloth, and R. Dermietzel, ''Construction of an apparatus for perfusion cell cultures which enables in vitro experiments under organotypic conditions,'' *Eur. J. Cell Biol.,* **57**, 132–137 (1992).
22. J. Bujia, ''In vitro engineering of autologous cartilage tissue for reconstructive surgery: Possibilities and limitations,'' *Laryngo-rhino-otology,* **74**, 205–210 (1995).
23. M. Sittinger, J. Bujia, N. Rotter, D. Reitzel, W. W. Minuth, and G. R. Burmester, ''Tissue engineering and autologous transplant formation: Practical approaches with resorbable biomaterials and new cell culture techniques,'' *Biomaterials,* **17**, 237–242 (1996).
24. M. Sittinger, D. Reitzel, M. Dauner, H. Hierlemann, C. Hammer, E. Kastenbauer, H. Planck, G. R. Burmester, and J Bujia, ''Resorbable polyesters in cartilage engineering: affinity and biocompatibility of polymer fiber structures to chondrocytes,'' *J. Biomed. Mater. Res. Appl. Biomater.,* **33**, 57–63 (1996).
25. M. Sittinger, O. Schulz, G. Keyszer, and W. W. Minuth, ''Artificial tissues in perfusion culture,'' *Int. J. Artif. Org.,* **20**, 57–62 (1997).
26. M. Arnold, *Histochemie,* Springer, Berlin, 1968.
27. B. V. Shekhonin, S. P. Domogatsky, V. R. Muzykantov, G. L. Idelson, and V. S. Rukosuev, ''Distribution of type I, III, IV and V collagen in normal and atherosclerotic human arterial wall: Immunomorphological characteristics,'' *Coll. Rel. Res.,* **5**, 355–368 (1985).
28. P. S. Amenta, S. Gay, A. Vaheri, and A. Martinez Hernandez, ''The extracellular matrix is an integrated unit: Ultrastructural localization of collagen types I, III, IV, V, VI, fibronectin, and laminin in human term placenta,'' *Coll. Rel. Res.,* **6**, 125–152 (1986).
29. A. Bukh, J. Ingerslev, and S. Stenbjerg, ''The multimeric structure of plasma F VIII: Rag studied by electroelution and immunoperoxidase detection,'' *Thromb. Res.,* **43**, 579–584 (1986).
30. J. Cejka, ''Enzyme immunoassay for factor VIII-related antigen,'' *Clin. Chem.,* **28**, 1356–1358 (1982).
31. M. Sehested and K. Hou Jensen, ''Factor VIII related antigen as an endothelial cell marker in benign and malignant diseases,'' *Virchows Arch. Pathol. Anat.,* **391**, 217–225 (1981).
32. R. C. Graham, ''The histochemical demonstration of monoamine oxidase activity by couplet peroxidatic oxidation,'' *Histochem. Cytochem.,* **13**, 150 (1965).
33. M. Brittberg, A. Lindahl, A. Nilsson, C. Ohlsson, O. Isaksson, and L. Peterson, ''Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation [see comments],'' *N. Engl. J. Med.,* **331**, 889–895 (1994).
34. M. A. Moses, J. Sudhalter, and R. Langer, ''Identification of an inhibitor of neovascularization from cartilage,'' *Science,* **248**, 1408–1410 (1990).
35. M. A. Moses, J. Sudhalter, and R. Langer, ''Isolation and characterization of an inhibitor of neovascularization from scapular chondrocytes,'' *J. Cell Biol.,* **119**, 475–482 (1992).
36. B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson, *Molecular Biology of the Cell,* Garland Publishing, New York, 1994.
37. J. Bujia, E. Wilmes, F. Krombach, C. Hammer, and E. Kastenbauer, ''Detection of class II antigens on human nasal cartilage,'' *Am. J. Otolaryngol.,* **11**, 339–344 (1990).
38. J. Bujia, S. Alsalameh, M. Sittinger, C. Hammer, E. Wilmes, and G. Burmester, ''Antigen presenting cell function of class II positive human nasal chondrocytes,'' *Acta Otolaryngol. Stockholm,* **114**, 75–79 (1994).
39. A. Kahn, L. A. Pottenger, J. G. Albertini, A. D. Taitz, and E. J. Thonar, ''Chemical stabilization of cartilage matrix,'' *J. Surg. Res.,* **56**, 302–308 (1994).