

Stabilized Autologous Fibrin-Chondrocyte Constructs for Cartilage Repair in Vivo

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Abstract: Stabilization of fibrin-chondrocyte constructs with fibrinolytic inhibitors has been shown to be a feasible method for the reconstruction of cartilage in vitro. In this study, the method was tested in vivo. Autologous cultures were used to form stabilized fibrin-chondrocyte constructs that were injected into auricular cartilage defects of rabbits. Stabilization was achieved by high doses of fibrinolytic inhibitors. Samples were prepared for magnetic resonance imaging, histology, and immunohistochemistry after 1, 2, 4, and 6 months. Defects of the contralateral ear, which were treated with stabilized fibrin without cells, were used for controlled comparisons. In all cell-fibrin samples, cartilage-like tissue was present. Immunohistochemistry revealed the presence of collagen II. This finding was similar for all observations. In the control samples, only minor new cartilage could be detected at the cut edges. The reconstruction of cartilage in vivo by injecting fibrin-chondrocyte constructs, stabilized with inhibitors of fibrinolysis, is thus possible.

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Cartilage has a very slow turnover at the cellular and molecular levels and therefore has a limited capacity for self-repair. Small, noncritical defects can regenerate in a low percentage of cases, but full-thickness defects of critical size are replaced by tissue of inferior quality.¹ Classic methods of cartilage replacement such as autografting, allografting, and the use of synthetic materials are not ideal. Tissue engineering was therefore proposed as a cartilage replacement

method, because it is able to overcome many of the problems associated with traditional cartilage replacement methods.² In several applications, tissue engineering has already made a successful transition from a scientific method to a clinical procedure.^{3–6} The successful reconstruction of a phalanx by bone tissue engineering in a human has recently been reported.⁷ Successful tissue engineering is dependent on numerous factors, but adequate scaffolds are among the most important prerequisites for stable three-dimensional and histiotypic tissue. Numerous synthetic and natural polymers have been tested as scaffolds for tissue engineering.^{8–12} One natural polymer is fibrin, which is increasingly used for tissue engineering. It can easily be polymerized and molded from its basic constituents. It is noncytotoxic, biocompatible, and biodegradable and has been in clinical use for several years. However, fibrin is unstable and is quickly disintegrated by cells. An in vitro study demonstrated that fibrin is an adequate scaffold for the reconstruction of cartilaginous tissue when stabilized by antifibrinolytic substances.¹³ These fibrin-chondrocyte constructs were stable for 4 weeks in vitro. The cells appeared to be viable and produced an extracellular matrix typical for cartilage. The present study tested the potential of stabilized fibrin-chondrocyte for cartilage reconstruction in an animal model. Auricular cartilage defects in rabbits were created and treated with stabilized fibrin-chondrocyte constructs. Neocartilaginous tissue was present in all treated defects. No to only minor neocartilaginous tissue was found in sham-treated control defects.

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MATERIALS AND METHODS

Experimental Design

Twelve animals were treated with injectable stabilized fibrin-chondrocyte constructs.

Step 1

Surgical procedure 1: Excision of two cartilage segments from one ear of each animal, one with and one without the perichondrium.

Step 2

Control defects: The defects created during surgical procedure 1 were filled with stabilized fibrin gel and served as control defects.

Step 3

Cell culture: From the excised segments without the perichondrium chondrocyte cell cultures were established.

Step 4

Surgical procedure 2: After 12 days, these cultures could be used for treatment. Therefore, a second operation had to be performed. Two new defects were created in each animal in the contralateral ear. One defect was created by leaving the perichondrium intact, whereas the perichondrium was excised in the other. The newly created defects were filled with chondrocytes dissolved in stabilized fibrin gel.

In each animal, four defects subjected to four different treatments could therefore be observed:

- Defect A: No perichondrium, stabilized fibrin, no chondrocytes
- Defect B: Intact perichondrium, stabilized fibrin, no chondrocytes
- Defect C: No perichondrium, stabilized fibrin, chondrocytes present
- Defect D: Intact perichondrium, stabilized fibrin, chondrocytes present

Step 5

Observation period: Three animals were sacrificed after 1, 2, 3, and 6 months after the second operation. Because the control defects were filled with stabilized fibrin gel already in the first surgical procedure, a time gap of 12 days existed between control and cell-treated defects. All defects were analyzed by histology and immunohistochemistry.

Cell Harvesting and Cell Culture

After approval by the Austrian Ministry of Science, 12 female New Zealand White rabbits 1 month old were obtained from Charles River and were kept under constant conditions in separate cages. The animals were allowed to acclimate for 3 weeks. Surgery was performed under general anesthesia using 1.5 mL Ketavet (100 mg/mL) and 0.5 mL Rompun (2% solution). In the first operation, two 1-cm² segments of auricular cartilage were removed from one ear. One cartilage segment was removed with the perichondrium, whereas the perichondrium was left intact in the other. The defects were filled with stabilized fibrin glue and surgically closed.

Primary autologous chondrocyte cultures were established from cartilage segments without the perichondrium. The cartilage segments were washed twice in Hank's bal-

anced salt solution (Gibco, Paisley, UK) and incubated for 1 hour in a 0.05% trypsin solution (Gibco). The tissue was then mechanically sliced into pieces approximately 1 mm² in size. These were subsequently incubated in a solution of 0.1% collagenase CLS 2 (Worthington Biochemical Corp., Freehold, NJ) in phosphate buffered saline without Ca⁺⁺ and Mg⁺⁺ (Gibco), shaken in Erlenmeyer tubes (Corning Glass Works, Corning, NY), for 24 hours at 37°C in a shaking water bath.

After incubation, the suspension was filtered through a 100- μ m nylon cell strainer (Falcon, Franklin Lakes, NJ) and centrifuged. The pellet was resuspended in Medium-199 (Gibco), which contained 10 ng/mL bFGF (Boehringer Ingelheim, Ingelheim, Germany) and 20% pooled rabbit serum. The cells were then plated on T12 culture flasks (Falcon) precoated with fibronectin (Sigma, St. Louis, MO). The cells readily adhered to the flask surface and began to spread and proliferate after a short lag period. Half the culture medium was changed every other day. After confluency had been achieved, the cultures were transferred to T75 culture flasks.

Treatment of Defects With Injectable Chondrocyte-Fibrin Constructs

The first-passage autologous chondrocyte cultures were ready for use after 12 days. Defects for treatment were created in a new surgical procedure in the contralateral ear. Two 1-cm² cartilage segments were again excised, one with and one without the perichondrium. These defects were filled with the stabilized fibrin-chondrocyte solution. After polymerization of the solution, the defects were surgically closed. Stabilization of commercially available fibrin glue (Immuno, Vienna, Austria) was achieved by adding 8500 IE/mL aprotinin (Bayer, Leverkusen, Germany) and 15 mg/mL tranexamic acid (Pharmacia, Stockholm, Sweden) to the solution. Cells were enzymatically detached from the culture flask, pelleted, and resuspended in the fibrin component of the glue. Cell density was adjusted to 15×10^6 /mL.

Animals were examined on a regular basis. General health status and wound healing were monitored. Thickness of operation sites was measured. Three animals were sacrificed after 1, 2, 3, and 6 months.

Histology

Samples were fixed in a 7.5% buffered formalin solution and embedded in paraffin. Sections of 4 μ m were stained with hematoxylin-eosin and Alcian blue for proteoglycan detection. Sections were also stained with orcein for detection of elastic fibers. Immunohistochemical staining for collagen II (clone 6B3, Neo Markers, Fremont, CA) was performed on paraffin-embedded material by the avidin-biotin method. The staining reaction was achieved with 3-amino-9-ethylcarbazol (AEC/Ventana NEXES, Strasbourg, France). Finally, the

sections were counterstained with hemalaun (Ventana, Strasbourg, France).

Evaluation of Tissue Formation and Statistics

Histologic samples were digitized with a high-resolution slide scanner. The amount of cartilaginous and osteogenic tissue was calculated from digitized images by using Adobe Photoshop software (Adobe).^{14,15} For group comparisons, the Student unpaired *t* test was performed. Differences were considered significant if $P < 0.05$.

RESULTS

Wound Healing and Thickness of Treated Defects

Wound healing appeared to be uneventful in all animals but one. In this animal, signs of an infection were present in one ear. This animal was successfully treated with antibiotics.

All type A defects (no perichondrium, stabilized fibrin, no chondrocytes) and B defects (intact perichondrium, stabilized fibrin, no chondrocytes) were thinner 6 months after the operation compared with the thickness of the defect area before operation.

All type C defects (no perichondrium, stabilized fibrin, chondrocytes present) and type D defects (intact perichondrium, stabilized fibrin, chondrocytes present) were slightly thicker at 6 months after the operation. (Table 1).

Histology and Immunohistochemistry

After 1 month, histology already revealed cartilaginous tissue in defects treated with stabilized fibrin-chondrocyte (C and D). The newly formed tissue showed dense cellularity and was directly adjacent to the native cartilage. The cells were situated in their lacunae and were surrounded by an extracellular matrix (Fig. 1). Moreover, the tissue appeared to be tightly connected to the native tissue (Fig. 2). The amount of cartilaginous tissue was independent of harvesting date and did not significantly differ between defects of type C and type D (Fig. 3; $P > 0.05$). The reconstructed cartilaginous tissue filled between 35% and 90% of type C and D defects (Table 2).

TABLE 1.

Defect	Thickness Before Surgery	Thickness 6 Months After Surgery
A	1.27 ± 0.03	0.98 ± 0.02
B	1.07 ± 0.08	0.78 ± 0.13
C	1.29 ± 0.15	1.35 ± 0.28
D	1.10 ± 0.1	1.19 ± 0.16



FIGURE 1. Histology of defects of type C (no perichondrium, stabilized fibrin, chondrocytes present) and D (intact perichondrium, stabilized fibrin, chondrocytes present): Alcan staining. Cut edges are marked by arrows. In defects C and D, the reconstructed cartilage (rc) is clearly visible between the native cartilage (nc). Digitally enlarged by 50% from scanned Alcan-stained slides.

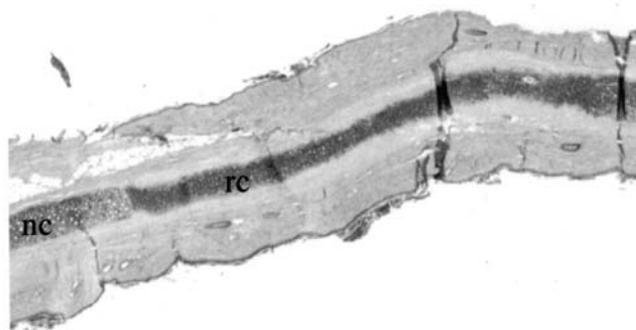


FIGURE 2. The reconstructed cartilage (rc) resembled native cartilage (nc) histologically and was tightly connected to it. Digitally enlarged by 50% from scanned hematoxylin-eosin-stained slides.

In defects of type A (control defects subjected to only sham treatment), no neocartilaginous tissue formation could be detected. In only three animals, there were small areas of neotissue formation found exclusively at the cut edges of the defects. The three animals belonged to different analytical groups (1, 2, and 6 months), and the tissue formation did not appear to be increasing over time (Figs. 3 and 4, Table 2). Only minor signs of tissue formation were found in defects of type B. In this group, areas of tissue formation were found in six animals at the cut edges of the defects. Of these, only two animals showed smaller areas of neocartilage formation in the center of defects, and these animals again belonged to different analytical groups (1, 2, and 6 months). Smaller areas

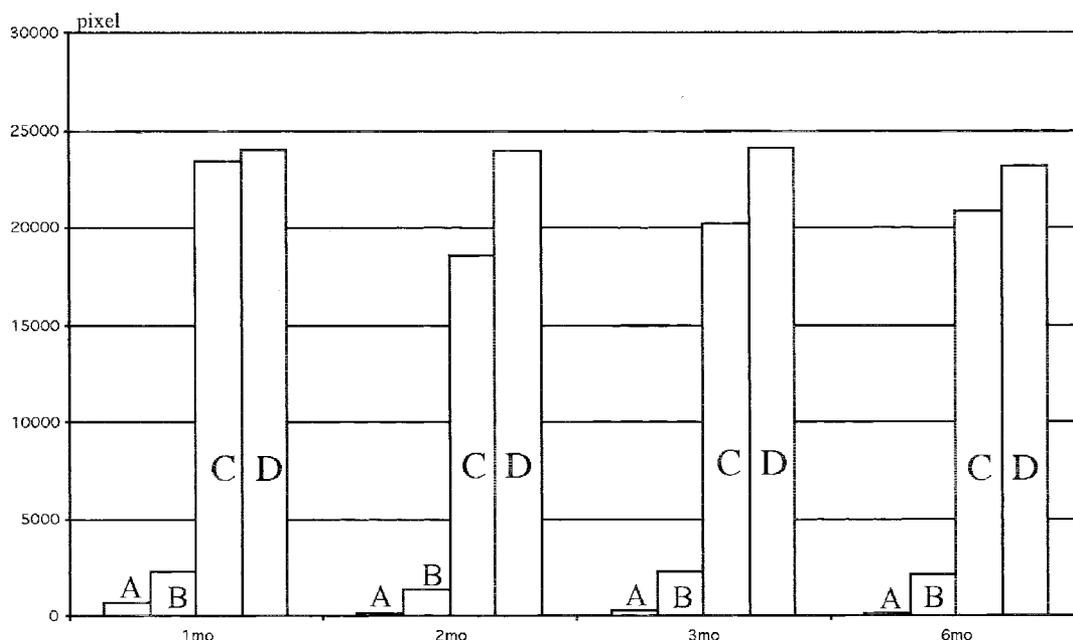


FIGURE 3. Amount of reconstructed cartilage expressed in number of pixels for defects of type A (no perichondrium, stabilized fibrin, no chondrocytes), B (intact perichondrium, stabilized fibrin, no chondrocytes), C (no perichondrium, stabilized fibrin, chondrocytes present), and D (intact perichondrium, stabilized fibrin, chondrocytes present). The amount of reconstructed tissue in defects C and D appears to be the same at 1, 2, 3, and 6 months. There is no statistically significant difference ($P > 0.05$) between C and D.

TABLE 2. Percentage of New Tissue Formation

Animal	Defects, %			
	A	B	C	D
1 Month				
1	2	0	87	52
2	0	5	63	85
3	0	5	88	90
2 Months				
4	0	5	79	81
5	1	0	35	72
6	0	0	80	82
3 Months				
7	0	0	48	90
8	0	3	82	85
9	0	0	65	84
6 Months				
10	0	7	85	85
11	0	0	90	90
12	2	5	81	87

of ossification could be detected in some samples from month 2 onward in samples of types B, C, and D (Figs. 3 and 4, Table 2).

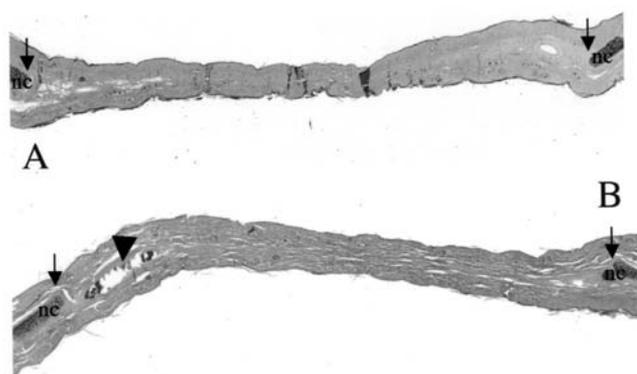


FIGURE 4. Histology of defects of type A (no perichondrium, stabilized fibrin, no chondrocytes) and B (intact perichondrium, stabilized fibrin, no chondrocytes). Alcan staining. Cut edges are marked by arrows. No neotissue can be seen in defects A and B. In some defects of type B, smaller ossified areas could be detected (arrowhead). Digitally enlarged by 50% from scanned Alcan-stained slides. nc = Native cartilage.

The reconstructed cartilaginous tissue in defects treated with stabilized fibrin-chondrocyte constructs stained positive for collagen II, which was abundant in type C and D defects after 1, 2, 3, and 6 months (Fig. 5). In defects undergoing

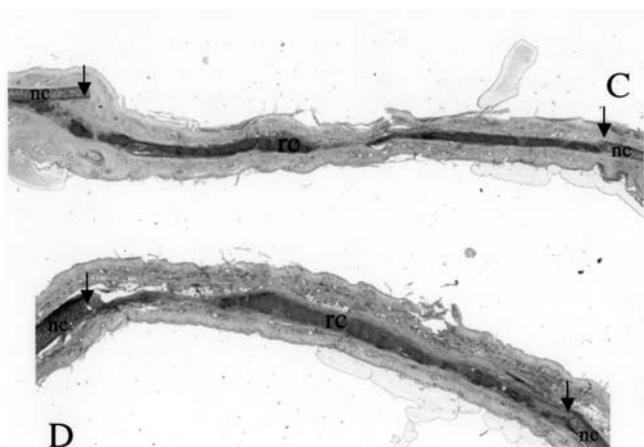


FIGURE 5. Immunohistochemistry (Ab vs. collagen II) of the reconstructed cartilage of defects of type C (no perichondrium, stabilized fibrin, chondrocytes present) and D (intact perichondrium, stabilized fibrin, chondrocytes present). Collagen II is abundant in reconstructed cartilage (rc) and native cartilage (nc). Digitally enlarged by 50% from scanned slides.

sham treatment only, areas exhibiting collagen II could be detected only in subjects with limited spontaneous neocartilage tissue formation.

DISCUSSION

Fibrin-chondrocyte constructs have been tested for cartilage reconstruction in vitro and in vivo. In vitro, it has been shown that fibrin is quickly degraded by chondrocytes.¹⁶ In vivo, fibrin-chondrocyte constructs polymerized from cryoprecipitated autologous fibrinogen have been used by some groups for cartilage reconstruction with favorable results,^{17,18} whereas other groups saw extensive shrinkage^{19,20} or early resolution of the fibrin glue and replacement by fibrous tissue.²¹ In an earlier in vitro study by this author,¹³ it was found that fibrin-chondrocyte constructs were dissolved too early, before cartilaginous tissue formation could occur. It could thus be demonstrated that stabilization of chondrocyte-fibrin constructs by the addition of high concentrations of antifibrinolytic substances is a feasible method for three-dimensional formation of cartilaginous tissue in vitro. The use of high concentrations of fibrinolytic inhibitors for stabilization slows degradation to an extent that provides enough time for matrix production. Potential systemic and adverse effects of aprotinin and tranexamic acid include anaphylactic reactions, thrombosis, and local thrombophlebitic events. We did not observe any of these events in our animals. Moreover, similar high doses of aprotinin and tranexamic acid have been used clinically for the reconstruction of peripheral arteries³ by tissue engineering approaches.

It has also been shown that degradation can be slowed by a higher fibrinogen concentration.²² However, the in vitro

study also demonstrated that constructs with higher fibrinogen concentration but low fibrinolytic inhibition were not stable. Constructs with a higher fibrinogen concentration and higher fibrinolytic inhibition were stable, but matrix production was reduced compared with constructs with low fibrinogen concentrations and high fibrinolytic inhibition.

The current in vivo study therefore used stabilized fibrin-chondrocyte constructs. Cartilaginous tissue was found in all samples derived from defects treated with stabilized fibrin-chondrocyte constructs. Reconstructed tissue resembled native elastic cartilage both histologically and immunohistochemically. The newly formed cartilage appeared to be directly adjacent to the native cartilage.

A recent study demonstrated that cartilage segments joined by a fibrin-chondrocyte solution exhibit substantially more tensile strength than those held together by fibrin glue alone.²³ This fact is of particular importance in therapeutic applications, in which the tissue is exposed to continuous mechanical forces as in articular, auricular, or nasal septal cartilage repair.

Between 35% and 90% of the defected area was filled by cartilaginous tissue. Practical surgical experience shows that this is most likely a result of an uneven distribution of the solution at the site of the defect. A more reliable injection method is therefore required to ensure that an even reconstruction is achieved.

No regeneration or only minor regeneration was observed in defects undergoing sham treatments, even when the perichondrium was left intact. The perichondrium also had no obvious effect on defects treated with stabilized fibrin-chondrocyte constructs. The amount and morphologic appearance of the reconstructed tissue was identical in samples with and without the perichondrium.

A few samples taken during the study exhibited evidence of ossification. These samples were derived from defects treated either with stabilized fibrin-chondrocyte constructs or from defects that underwent only sham treatments but retained an intact perichondrium. No ossification was observed in defects in which the perichondrium was removed or in defects that did not have chondrocytes in the fibrin solution.

The potential of transplanted perichondrium to form bone has been documented,^{24,25} and it has also been shown that isolated chondrocytes can form calcifying cartilage when injected into the muscle of animals.²⁶ Bone formation has also been seen in immunosuppressed animals after injection of allogeneic cultured chondrocytes.²⁷ A recent publication also suggests that hypertrophic chondrocytes can differentiate into osteoblast-like cells contributing to bone formation, but only if they are located at the border of the osteogenic tissue.²⁸

Stabilized fibrin-chondrocyte constructs may therefore be useful not only for cartilage reconstruction but also for the

reconstruction of bone. The evaluation of factors influencing the different pathways of tissue formation in fibrin-chondrocyte constructs will be a challenging topic for future studies.

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