

Stabilization of Fibrin–Chondrocyte Constructs for Cartilage Reconstruction

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Cartilage replacement is a challenging issue in reconstructive surgery. In the past few years, tissue engineering has been tested as a means of cartilage reconstruction. Tissue engineering of cartilage depends on the use of adequate polymers. In addition to several natural and synthetic polymers, fibrin gel has been tested for cartilage reconstruction. However, fibrin is intrinsically unstable. The purpose of this study was to stabilize fibrin by increased fibrinolytic inhibition and to test these preparations for cartilage reconstruction with human nasal septum chondrocytes. Increased fibrinolytic inhibition was achieved with aprotinin and tranexamic acid. Stabilized fibrin–chondrocyte constructs were cultivated for 4 weeks *in vitro* and compared with constructs made of standard, commercially available fibrin gel. The effect of several cell densities on stability, and the production of extracellular matrix components, were assessed on the basis of histology and immunohistochemistry. In contrast to constructs made of standard fibrin gel, stabilized constructs were stable for the entire observation period and demonstrated no or only minor shrinkage. Cells in these constructs appeared to be viable, and an extracellular matrix could be demonstrated in all constructs. The authors conclude that fibrin–chondrocyte constructs stabilized by increased fibrinolytic inhibition could be an adequate tool for cartilage reconstruction.

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Lost or destroyed cartilage causes a number of diseases, especially those of the craniofacial region and the large joints. Because cartilage has virtually no intrinsic repair potential, various replacement methods such as autografting, allografting, or transplanting synthetic materials are used. In the past few years, tissue engineering as a means of cartilage reconstruction has been given considerable attention in plastic and orthopedic surgery.¹ The common approach is to embed isolated chondrocytes

into polymeric scaffolds, in which the cells produce an extracellular matrix that gradually replaces the polymeric structure. In terms of morphology and function, these constructs should closely resemble native tissue. A variety of polymers have been tested so far. These include products made from natural or synthetic polymers, such as alginate,^{2–4} agarose,^{5,6} polyglycolic acid,^{7,8} and poly-L-lactic acid,⁹ or combinations of these.¹⁰ Collagen¹¹ and hyaluronic acid¹² have also been used as scaffolds for chondrocyte seeding. These constructs were either matured *in vitro* or injected into animals. These studies demonstrated that polymer–chondrocyte constructs can be remodeled into cartilaginous tissue. However, disadvantages such as unfavorable mechanical properties or poor biocompatibility have also been reported. Recently, fibrin gel has evoked interest in cartilage reconstruction. Fibrin is a natural substance and can be polymerized and molded easily from its basic constituents. It is noncytotoxic, biocompatible, and biodegradable, and has been in clinical use for several years. These characteristics make it an ideal candidate for cartilage reconstruction. However, fibrin is intrinsically unstable due to fibrinolysis. For successful reconstruction of cartilage, the constructs must be stable during the maturation process. Therefore, increased fibrinolytic inhibition was tested as a means of stabilizing fibrin. The influence of increased fibrinolytic inhibition on construct stability and production of an extracellular matrix was evaluated by histological and immunohistochemical methods.

Materials and Methods

Informed consent was obtained from patients undergoing rhinoplasty, and pieces of surplus septum cartilage from these patients were used for chondrocyte isolation, which was based on

the method described by Klagsbrun.¹³ Cartilage segments were washed twice in Hank's balanced salt solution (Gibco, Paisley, UK) and incubated in a 0.05% trypsin solution (Gibco) for 1 hour. The tissue was then chopped mechanically into approximately 1-mm² pieces. Subsequently the pieces were incubated in a 0.1% collagenase class 2 solution (Worthington Biochemical Corporation, Freehold, NJ) in phosphate-buffered saline without Ca⁺⁺ and Mg⁺⁺ (Gibco), in Erlemeyer tubes (Corning Glass Works, Corning, NY), and shaken 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 per milliliter basic fibroblast growth factor (Boehringer Ingelheim, Ingelheim, Germany) and 20% pooled human serum. Cells were plated on fibronectin (Sigma, St. Louis, MO) precoated T12 culture flasks (Falcon). Cells adhered readily to the flask surface and began to spread and proliferate adequately after a short lag period. Half of the culture medium was changed every other day. After confluence had been achieved, cultures were transferred to T75 culture flasks. Only first-passage cultures were used for experiments.

Commercially available fibrin glue (Immuno, Vienna, Austria) was used to form cell-polymer constructs. Components were prepared either according to the supplier's instructions with 3,000 KIU per milliliter aprotinin (group 1, standard fibrin gel) or modified by additional fibrinolytic inhibition (group 2, stabilized fibrin gel). Fibrinolytic inhibition was achieved by adding 8,500 KIU per milliliter aprotinin (Bayer, Leverkusen, Germany) and 15 mg per milliliter tranexamic acid (Pharmacia, Stockholm, Sweden) to the solution. Cells were detached enzymatically from the culture flask, pelleted, and resuspended in the fibrin component of the glue. For each of these two groups (standard fibrin gel and stabilized fibrin gel) 1-cm³ constructs in duplicate were drawn into 24-well culture plates (Costar, Cambridge, MA). Constructs were covered by culture medium and cultured in a humidified incubator at 37°C and 5% carbon dioxide for 4 weeks. Investigated cell densities in both groups included 12.5×10^4 , 25×10^4 , and 1.0×10^6 cells

per milliliter (low cell densities), and 4×10^6 , 8×10^6 , and 20×10^6 cells per milliliter (high cell densities). In each of the two groups, constructs without cells served as control samples. Thus a total of 28 constructs were prepared, 14 in each group. During the culture period the culture medium was changed every other day and macroscopically visible changes were documented.

Samples were fixed in formalin and embedded in paraffin. Four-micrometer sections were stained with hematoxylin-eosin and Alcian blue stains for proteoglycan detection. Immunohistochemical staining for collagen II, which is a good marker of the differentiated phenotype of chondrocytes (clone 6B3, Neo Markers, Fremont, CA) was performed on paraffin-embedded material using the avidin-biotin method. The staining reac-

tion was achieved with 3-amino-9-ethylcarbazol (Ventana, Strasbourg, France). Sections were finally counterstained with hemalum (Ventana). The thickness of constructs was measured microscopically at the edge and in the center using a microgrid. Viability of cells was assessed by applying morphological criteria. Cells were considered viable if no signs of caryolysis, caryorrhexis, cytoplasmic vacuolation, or cytolysis were detected.

Results

Standard fibrin constructs with low cell densities were solid during the entire culture period. After 4 weeks, constructs had a mean thickness of 3.8 ± 1.2 mm, whereas acellular constructs had a mean thickness of 3.7 ± 0.5 mm.

In constructs with standard fibrinolytic inhibition and high cell densities, the first signs of disintegration were visible macroscopically after 3 days in culture. The surface appeared irregular and holes were clearly visible. After 4 weeks these constructs had disintegrated completely, and only remnants of fibrin were visible.

Stabilized constructs with low cell numbers were solid during the entire observation period. No shrinkage was seen and the mean thickness of constructs was 4.0 ± 0.8 mm after 4 weeks compared with 4.2 ± 0.7 mm for acellular controls. Constructs with high cell numbers were

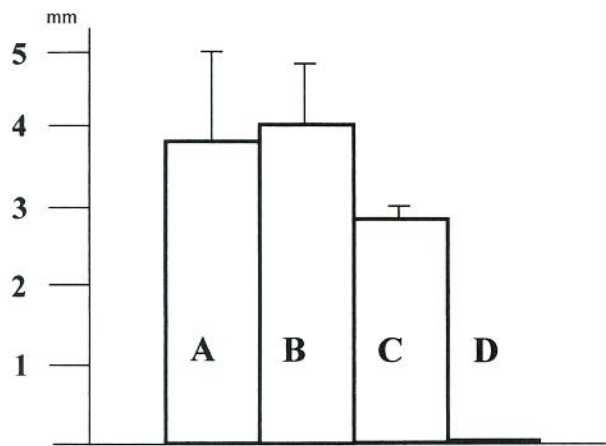


Fig 1. Bar graph of mean thickness of constructs grouped according to fibrin gel and cell density. Note that mean thickness of standard fibrin–chondrocyte constructs with high cell densities (D) was zero because all constructs disintegrated completely within 4 weeks. To the contrary, stabilized fibrin–chondrocyte constructs with high cell densities (C) were stable for 4 weeks, with a mean thickness only moderately less than that measured for stabilized fibrin–chondrocyte constructs with low cell densities (B) and standard fibrin–chondrocyte constructs with low cell densities (A).

also solid during the 4 weeks of cultivation. However, the thickness (2.8 ± 0.1 mm) appeared to be slightly less than that of constructs with low cell densities or acellular constructs (Fig 1).

Constructs made of standard fibrin gel with low cell densities formed partially laminated structures. Although these cells appeared to be viable, no extracellular matrix was detected (Fig 2). Constructs made of standard fibrin gel and with high cell densities disintegrated completely and thus could not be examined histologically. However, the cells of these constructs were viable. They attached to the bottom of the culture plates, where they formed typical cell layers.

Stabilized constructs showed evenly distributed cells embedded in a fine and uniform fibrin meshwork. In constructs with low cell densities, many cells had a dendritic appearance (Fig 3). Where clusters of cells had formed, the cells were round. Adjacent to the cells, an extracellular matrix was identified by Alcian staining. (Fig 4). Only minimal collagen II-positive areas could be detected by immunohistochemical staining. These areas were only found around cell clusters.

In constructs with high cell densities, only typically rounded cells situated in lacunae were visible. An extracellular matrix was found both



Fig 2. Fibrin–chondrocyte construct made of standard fibrin gel with a low cell number (25×10^4 cells per milliliter) after 4 weeks in culture. Note the lamellalike appearance. Cells are rounded but no extracellular matrix is detectable. Alcian blue, original magnification $\times 500$ before 54% reduction.

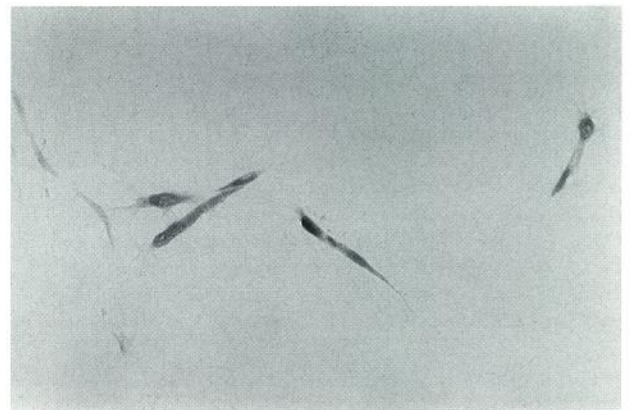


Fig 3. Fibrin–chondrocyte construct made of stabilized fibrin gel with a low cell number (25×10^4 cells per milliliter) after 4 weeks in culture. Cells are distributed evenly in a fine, reticular fibrin mesh. The majority of cells have a dendritic appearance. H&E, original magnification $\times 500$ before 54% reduction.

close to the surface as well as in the depth of the constructs. Again, an extracellular matrix was present, especially where clusters of cells had formed (Fig 5). Additionally, large areas, especially those close to the surface, stained positive for collagen II (Fig 6).

Discussion

The reconstruction of cartilage is a challenging issue in reconstructive surgery. Classic methods of cartilage replacement such as autografting,

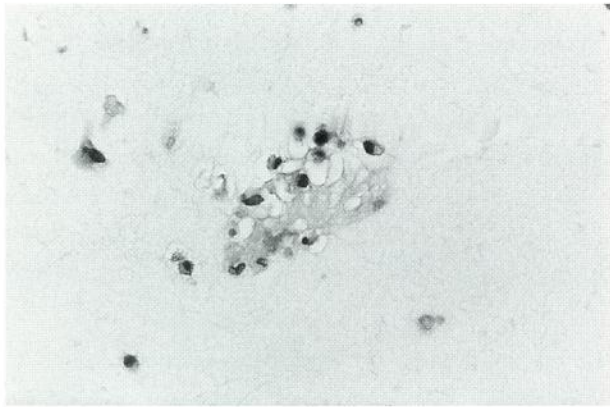


Fig 4. Fibrin-chondrocyte construct made of stabilized fibrin gel with a low cell number (25×10^4 cells per milliliter) after 4 weeks in culture. When cell clusters were formed, an extracellular matrix was present. Alcian blue, original magnification $\times 500$ before 54% reduction.

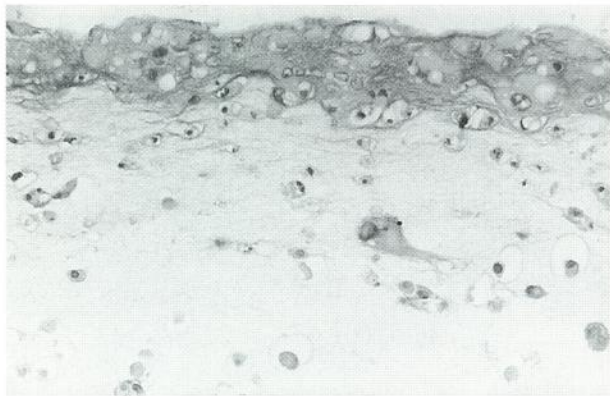


Fig 5. Fibrin-chondrocyte construct made of stabilized fibrin gel with a high cell number (4×10^6 cells per milliliter) after 4 weeks in culture. Large areas are filled with extracellular matrix. Alcian blue, original magnification $\times 500$ before 54% reduction.

allografting, or the use of synthetic materials are not ideal. In the past, tissue engineering has been proposed as a method that overcomes many problems associated with traditional cartilage replacement. Tissue engineering aims to create histiotypic tissue by combining isolated chondrocytes and polymers. One natural polymer that is a candidate for this method is fibrin. Fibrin is a natural polymer with substantial biological functions. Commercially available fibrin glue is used clinically for a variety of applications. Several studies have tested various preparations of fibrin gels as scaffolds for cartilage reconstruction.¹⁴⁻¹⁷ It was shown that commercially available fibrin

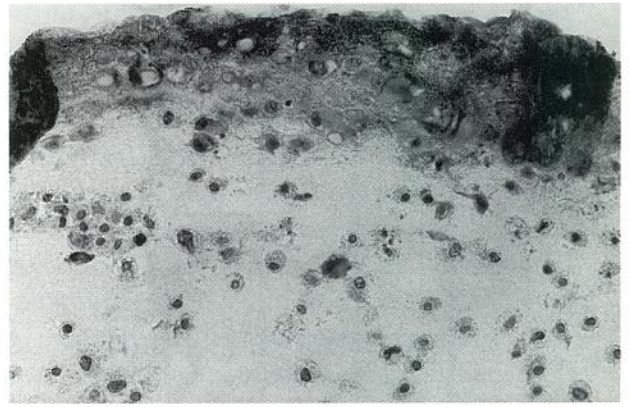


Fig 6. Fibrin-chondrocyte construct made of stabilized fibrin gel with a high cell number (4×10^6 cells per milliliter) after 4 weeks in culture. Large areas are stained positive for collagen II, mainly close to the surface. Monoclonal antibody versus collagen II, original magnification $\times 500$ before 54% reduction.

glue-chondrocyte constructs tend to disintegrate after 3 days in culture and will be completely disintegrated after 7 days. It was also demonstrated that the extent of disintegration depended on cell density.¹⁸ These findings are quite similar to our own results. Standard fibrin-chondrocyte constructs with high cell numbers disintegrated completely within 4 weeks of cultivation. In our study, constructs with low cell numbers shrank moderately and were solid for 4 weeks in culture. This is in accordance with stable standard fibrin-chondrocyte constructs in cell culture after 2 weeks with a cell density of 10^6 —a feature that has been demonstrated previously as well.¹⁹ In a recent study¹⁹ one autologous fibrin-chondrocyte construct consisting of 25×10^6 cells, which was not inhibited fibrinolytically, was cultured for 4 weeks in vitro prior to implantation into a nude mouse. After the in vitro period, the construct had shrunk by 75%. After 4 weeks in vivo additional shrinkage was observed. Only 12% of the original volume was observed at the end of the experiment.²⁰ All these data clearly emphasize the role of cell density for the stability of constructs made of unstabilized fibrin gels. High cell numbers, which are necessary for cartilage reconstruction, disintegrate fibrin gels (which are not or only mildly inhibited fibrinolytically) too early to allow successful remodeling of constructs.

In contrast to constructs made of standard fibrin glue, stabilized fibrin-chondrocyte constructs were solid for the entire in vitro

observation period when both low and high cell numbers were used. Cells appeared to be viable, as documented by the lack of caryolysis, caryorrhexis, cytoplasmic vacuolation, and cytolysis. They were distributed evenly throughout the entire construct. Proteoglycans and collagen II, which is the predominant and characteristic type of collagen in cartilage, could be detected after 4 weeks in culture. Levels of these matrix components were dependent on cell density and cell contact. This indicates that increased concentrations of aprotinin and tranexamic acid inhibited the fibrinolytic activity of chondrocytes effectively but did not influence the viability of the cells or their ability to produce specific matrix components.

The fact that an extracellular matrix was found predominantly close to the surface of constructs might be due to a more favorable nutritional situation and metabolic waste clearance in this area compared with areas in the depth of the constructs. Indeed, it has been shown that more extracellular matrix was found in perfused three-dimensional polymer–chondrocyte cultures than in static cultures.⁸ However, close cell contact might be an additional enhancement for extracellular matrix production, because we found distinct extracellular matrix areas in the depth of the constructs where cells formed clusters.

We conclude that stabilized fibrin is an adequate scaffold for three-dimensional cartilage reconstruction. Cells are viable in strongly fibrinolytically inhibited fibrin gels and produce a cartilage-specific extracellular matrix. Polymeric density and stability may be fine-tuned to any desired extent. Stabilized fibrin may also improve fixation of constructs to native tissue, and high contents of aprotinin might inhibit the penetration of constructs by adjacent tissue.

Stabilized fibrin–chondrocyte constructs for cartilage reconstruction may have several advantages compared with conventional methods. In our study we used commercially available fibrin glue, but autologous fibrin, prepared from the patient's own plasma, may be used as well. Together with autologous chondrocytes and autologous serum for cell culture, a fully autologous neocartilage could be formed. This would avoid the risk of transmitting infectious diseases or triggering an immune response. Moreover, con-

structs are moldable and injectable, and could be used clinically with a minimal invasive procedure. Clinical applications could include reconstruction of nasal septum cartilage, auricular cartilage, and articular cartilage. It could also be used for sealing defects of the forehead or skull base. However, additional studies are necessary to verify these preliminary in vitro results in an in vivo setting.

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