Tissue and Cell 37 (2005) 339–348

Tissue&Cell

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Migration pattern, morphology and viability of cells suspended in or sealed with fibrin glue: A histomorphologic study

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Received 20 October 2004; received in revised form 6 May 2005; accepted 9 May 2005
Available online 11 July 2005

Presented in parts at the "Fourth Combined Meeting of the Orthopaedic Research Society" in Rhodes, Greece, June 2001.

Abstract

Introduction: We studied the migration pattern, morphology and viability of cells suspended in five different fibrin glues. Besides this, the behaviour of chondrocytes seeded on porous matrices comprising different collagen types sealed with fibrin glue was investigated.
Material and methods: In an experiment A, cell suspension (0.5×10^6 cells) was incubated with different fibrin glues. Experiment B was set up to evaluate chondrocytes migration either through a collagen I/III (Chondro-Gide®, Geistlich Biomaterials, Switzerland) or collagen II matrix sealed with different fibrin glues in a perfusion chamber system. Analysis were performed by lightmicroscopy (Mayer's hematoxylin-eosin; Masson–Goldner; TUNEL test) and by transmission and scanning electron microscopy. All fibrin glues were measured for TGF-β 1 and 2 with a specific ELISA.
Results: After incubation of cell suspension in autologous fibrin glue, the morphology of cells is chondrocyte-like. Spindly, process-bearing cells were seen in commercial fibrin glue. Cells suspended in commercial fibrin glue revealed a significant higher percentage of TUNEL positive cells compared to fibrin tissue adhesives mixed with autologous serum ($p = 0.006$). The TGF-β 1 and 2 concentration was significantly higher in partial autologous fibrin sealant (PAF) compared to their commercial counterparts ($p = 0.001$). Cells seeded on the collagen I/III matrix retained their chondrocytic morphology, while in the type II collagen matrix the chondrocytes displayed a fibroblastic phenotype. The ratio of TUNEL positive cells for the collagen I/III matrix was significantly surpassed by the values, when a collagen II matrix was used ($p = 0.008$). No ingrowth of cells was seen in any of the experimental conditions.
Conclusion: Partial autologous fibrin glue and collagen I/III matrices are favourable in respect to migration pattern, morphology and viability, but definitive conclusions can only be drawn after in vivo studies. This will be addressed in future animal studies.

Keywords: Cartilage repair; Fibrin glue; Collagen matrices; Apoptosis; Tissue engineering

1. Introduction

Tissue engineering may be a promising approach for the treatment of focal articular cartilage defects (Brittberg, 1994). To deliver and temporarily stabilise the cells in the defect, a resorbable scaffold is used in matrix-associated autologous chondrocyte transplantation (MACT) to allow ingrowth of cells, stimulate matrix formation and to bind new cells and matrix to the host tissue (Paletta et al., 1992; Vacanti et al., 1994; Behrens et al., 1999). During the past decade, scaffolds from natural or synthetic resorbable polymers have been extensively tested for their capability to support the growth of chondrocytes (Fuss et al., 2000; Gugala and Gogolewski, 2000; Frenkel and DiCesare, 2004). Promising results are reported about collagen I/III and II scaffolds in MACT

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doi:10.1016/j.tice.2005.05.004

(Nehrer et al., 1997; Russlies et al., 2002). The matrix can be fixed in the defect by fibrin glue.

By now, only little is known about the influence of fibrin glue on chondrogenic cells. On the one hand, fibrin glue is considered to be suitable as a biological vehicle for cells, because no adverse effects of fibrin glue on chondrocytes were observed in cell culture (Homminga et al., 1993). On the other hand, negative effects are described, making the theory of the fibrin adhesive acting as a scaffold for tissue ingrowth apparently not generally valid (Brittberg, 1994). By now, no data are available allowing to compare the influence of commercial and autologous fibrin glue on chondrogenic cells in vitro. This study comments on the migration pattern, phenotype and vitality of cells either suspended in fibrin glue or grown on a collagen matrix sealed with fibrin glue. We performed this study using different commercial and autologous fibrin sealants.

Fibrin glue may contain aprotinin to prevent early fibrinolysis. But aprotinin can cause allergic reactions and may carry the risk of negative influence on chondrogenic cells, as reported by Beierlein et al. (2000). In this study, we investigated the influence of aprotinin in fibrin glue on chondrogenic cells.

Recent studies have shown that cartilage metabolism is influenced by local growth factors (Trippel et al., 1996; Pecina et al., 2001). Transforming growth factors beta one and two (TGF-β 1 and 2) have effects on osteogenesis and chondrogenesis by stimulating different cell types and plays an important role in repair and remodeling of mesenchymal tissue (Cornell and Lane, 1992). It is well documented that TGF-β stimulates the formation of new cartilage both in vitro and in vivo (Kekow and Gross, 1992; Summer et al., 1995; Baylink et al., 1993). In this study we measure the TGF-β concentration of different fibrin glues in view of the influence on the chondrogenic vitality of cells.

2. Material and methods

In this study we investigated five different types of fibrin glue [Tissucol® Duo S, Baxter Immuno AG, Germany; Tissucol® Kit without aprotinin, Baxter Immuno AG, Germany; Tissucol® Kit with 50% autologous serum, Baxter Immuno AG, Germany; Beriplast®, Centeon Pharma GmbH, Germany, and autologous fibrin glue]. In an experiment A, we tested the influence of different fibrin glues on the morphology and viability of cells. This was performed by light-microscopy after immunostaining and by transmission and scanning electron microscopy. Experiment B was set up to evaluate chondrocytes migration through a collagen I/III or II matrix sealed with different fibrin glues in a perfusion chamber system. All fibrin glues were measured for TGF-β 1 and 2 with a specific ELISA as prior described (Gille et al., 2002a, 2002b; Symkowiak et al., 1995).

Cartilage samples were collected from six (two female and four male) healthy 50- to 60-year-old patients with informed consent who underwent elective surgery for total hip replacement. In addition, 100 ml autologous blood was drawn in a tube without additives to be processed for cryoprecipitation.

2.1. Cell culture

Cartilage was diced into 1–2 mm^3 pieces and placed into Falcon tubes containing 0.5% hyaluronidase (Sigma H3506) for 15 min at room temperature. After rinsing with PBS, the samples were washed with 0.25% trypsin (Biochrom KG L2133) and then incubated in 0.1% collagenase (Sigma C9891) and 0.25% trypsin for 20 min at 37 °C. Samples were washed again in PBS and transferred into a new Falcon tube containing Iscove's culture medium (Biochrom KG F0465) with 0.2% collagenase (Sigma C9891) and placed onto a shaker rotating at 200 rpm for 8 h at 37 °C. The cell suspension was centrifuged and resuspended in Ham's F-12 culture medium (Biochrom KG FG 0815) containing 10% fetal calf serum (Biochrom KG S015), 50 μl/ml glutamine (Biochrom KG K0282) and 50 μl/ml non-essential amino acids (Gibco BRL 11140-035). Cells were cultured until confluent in a humidified environment containing 5% CO_2 at 37 °C for 4–6 weeks.

2.2. Fibrin glue

2.2.1. Autologous fibrin glue

As mentioned above, 100 ml blood was drawn from each patient and stored within 30 min of collection at 4 °C until centrifuged at 1000 rpm for 15 min. The plasma was frozen in a −80 °C freezer for 12 h and then defrosted in a refrigerator (4 °C). The defrosted plasma was proceeded a second time as described above. Supernatant was removed and the precipitate was collected as cryo. For application of the sealing components a double-syringe applicator (Duploject System, Immuno, Heidelberg, Gemany) was used. For autologous fibrin glue, one syringe was filled with 1 ml of the cryoprecipitate and the other syringe contained 1 ml $CaCl_2$ with 1 mg of dehydrated thrombin (1000 units). For handling Tissucol® and Beriplast® fibrin sealant the instructions of the companies were followed in detail. Using the Tissucol Set® opened the opportunity to resuspend the dehydrated Tissucol® with PBS, leading to an aprotinin-free fibrin glue. For the preparation of partial autologous fibrin sealant, 0.5 ml of the thrombin solution (Tissucol® Kit) was removed and autologous serum was adjusted to the initial volume. The autologous serum was prior achieved by centrifugation of 3 ml blood at 3000 rpm for 15 min.

2.2.2. Experiment A

The cells were trypsinized and counted. Trypan blue is a vital dye. After having added 0.4% Trypan blue staining (Sigma, St. Louis, U.S.A.) for 5 min, cells were noted and counted in a haemocytometer using a light microscope. Cells present in 25 squares of the haemocytometer were evaluated and cell number and viability were calculated for the original

culture. A suspension of 1 million cells per ml was used. The cell suspension was resuspended into 0.5 ml of each of the above mentioned glues and stored on a 12-well plate (Nunc, Wiesbaden, Germany). The sealant-cell suspension was incubated at 37 °C and 5% for 24 h. Each experiment was repeated three times.

2.3. Light microscopy

Specimens were fixed with Bouin's fixation and sections mounted onto poly-L-lysine-coated glass slides. They were dehydrated through a graded ethanol series and stained with Mayer's hematoxylin-eosin and Masson–Goldner. The morphology of cells was described and apoptotic cells were counted. An apoptotic cell was defined as having a condensed, pycnotic nucleus and one of the following attributes: shrunken, deeply eosinophilic cytoplasm and fragmentation of nucleus/cytoplasm.

2.4. Electron microscopy

For transmission electron microscopy, specimens were fixed with 2.5% glutaraldeyde in a 0.06 M sodium cacodylate buffer (pH 7.35) for 48–72 h at 4 °C, rinsed in 0.2 M sodium cacodylate buffer and postfixed with 1% osmium tetroxide at room temperature. Samples were then rinsed in 2.4% sodium chloride solution, washed in 0.2 M sodium acetate buffer (pH 5.0) and block stained with 1% uranyl acetate in 0.2 M sodium acetate buffer (pH 5.0) in the dark for 30 min at room temperature. Routine procedures were followed for dehydration in alcohol and embedding in araldite. Ultrathin sections (50–70 nm) were contrasted with lead citrate and uranyl acetate and viewed using a Philips 400 electron microscope. For scanning electron microscopy, cells were rinsed with warm PBS and fixed with 2% glutaraldehyde and 0.6% paraformaldehyde in 0.06 M sodium cacodylate buffer for 24 h at 4 °C. Samples were then dehydrated in graded series of acetone, dried in a critical-point dryer, sputter-coated with gold-paladium and examined in a Philips SEM 505 scanning electron microscope operated at 30 kV. Results were documented on APX 100 films (Agfa).

2.5. Immunostaining

To detect individual cells undergoing apoptosis, we used the technique of Terminal-transferase dUTP Nick End Labelling (TUNEL, ApopTag®-Kit, Oncor Appligen, Heidelberg, Germany). Samples were fixed in 4% paraformaldehyde at 4 °C overnight and then embedded in paraffin. Sections (4 μm) were mounted on slides and a proteinase K digestion (20 μg/ml) was carried out for 15 min. Endogenous hydrogen peroxidase activity was quenched in 3% hydrogen peroxide. After a series of rinsing, nucleotides labelled with digoxigenin were enzymatically added to the DNA by terminal deoxynucleotidyl transferase enzyme (TdT). The incubation was carried out for 60 min. The labelled DNA was detected using anti-digoxigenin-peroxidase for 30 min. The chromogen diaminobenzidine tetrahydrochloride (DAB) resulted in a brown reaction product that was evaluated by light microscopy and cells were counted. Positive and negative controls were carried out on slides from the same block. Incubation without TdT served as the negative control.

2.5.1. Experiment B

One million cells were seeded on a 1 cm^2 either collagen I/III (Chondro-Gide®, Geistlich Biomaterials, Wolhusen, Switzerland) or collagen II matrix (Institute of Molecular Medicine, University Lübeck, Germany) for 3 days. For better handling the collagen sponges were placed in plastic setup rings of 13 mm diameter (Minusheet No. 1300). The seeded membrane was given in a modified Boyden chamber with chondrocyte conditioned media as a chemotactic attractant and Thermanox® plastic cover slides on the bottom of the chamber (Frenkel et al., 1997). The different fibrin glues were placed between the media and the seeded side of the membrane on an 8 μm polycarbonate filter ("sandwich-like"). After 6 and 9 days incubation the filter-glue-membrane composite was fixed for light and electron microscopy as described above. Seeded collagen I/III and II membranes not covered by fibrin glue served as controls.

2.6. Data analysis

Data are expressed as mean ± standard deviation.

For light microscopy, each section was divided into 6 microscopic fields at ×40 magnification. Cells (75–100) were counted in each field at ×320 magnification, and the percentage of apoptotic chondrocytes was counted within these defined fields. The same method was used to count the percentage of TUNEL positive cells. We analysed the data with the two sided *U*-test (Mann–Whitney).

3. Results

After an initial harvest of articular cartilage, in vitro expansion of cells was carried out for 4–6 weeks to achieve confluence and a mean total of 4.6 (3.8–5.6) × 10^6 cells. No significant interindividual difference was to be made up between the six articular cartilage samples in respect to cell growth in vitro. These cell populations were processed for experiments A and B.

3.1. Experiment A

The cells were homogenously dispersed throughout the glue with only occasional clusters of cells, probably based on incomplete resuspension of cells. During in vitro expansion, cells had a fibroblast-like morphology. After incubation of cell suspension in commercial and autologous fibrin glue, as mentioned above, the morphology of the cells was chondrocyte-like rather than fibroblast-like. Partially

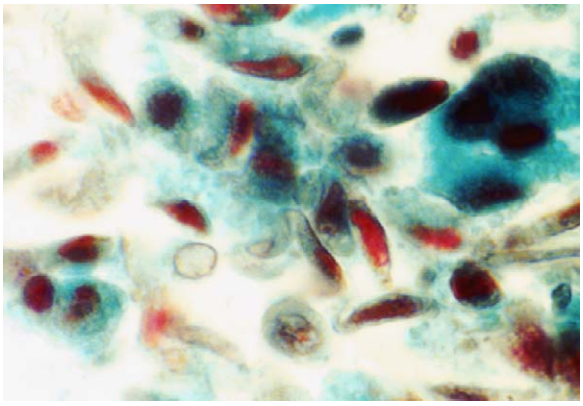

Fig. 1. Representative sections of chondrocytes suspended in commercial fibrin glue (Tissucol®). Light microscopy, Masson–Goldner-stained. Cells show a partially spindly, process-bearing morphology (original magnification ×260).

spindly, process-bearing cells were seen in commercial fibrin glue (Fig. 1) compared to cells suspended in autologous fibrin sealant showing a roundish, chondrocyte-like morphology (Fig. 2). Evaluation by light microscopy after Mayer's hematoxylin-eosin and Masson–Goldner staining revealed a high percentage of vital cells. Cells suspended in Tissucol® without aprotinin showed a morphology consistent with apoptosis with a typical condensed, pycnotic nucleus and deeply eosinophilic, shrunken cytoplasm compared to other fibrin sealants (Fig. 3).

All samples revealed apoptotic cells as determined by the TUNEL technique (Fig. 4). Over all, almost half of cells were positive for TUNEL. Cells suspended in commercial fibrin glue (Tissucol® Duo S, 55% (51–58%); Beriplast® 51% (47–54%)) revealed a significant higher percentage of TUNEL positive cells compared to fibrin tissue adhesives mixed with autologous serum (Tissucol® Kit with 50% autologous serum, 43% (39–45%)) or autologous fibrin sealant (34% (21–46%)). Therefore, the viability of cells by negative TUNEL result was significantly higher in (semi)autologous glue compared to their commercial counterparts. Cells suspended in Tissucol® without aprotinin stained positive for TUNEL in 66% (59–68%), while cells grown in Tissucol® with aprotinin less often revealed apoptotic cells by TUNEL test (55%). The control group revealed a positive TUNEL staining in 17% of the cells (15–21%).

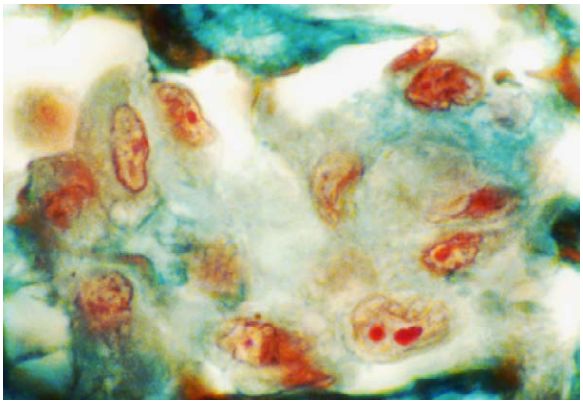

Fig. 2. Representative sections of chondrocytes suspended in autologous fibrin glue. Light microscopy, Masson–Goldner-stained. Cells show roundish, chondrocyte-like morphology (original magnification ×320).

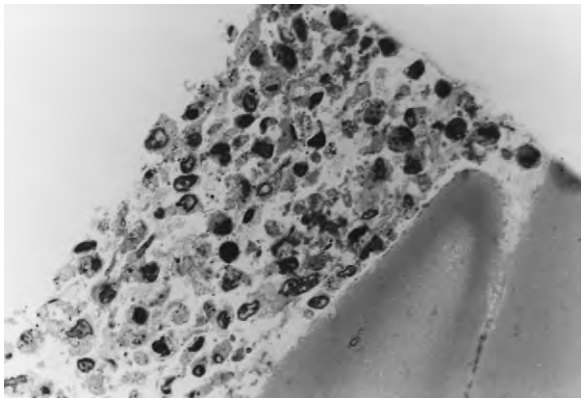

Fig. 3. Representative sections of chondrocytes suspended in commercial fibrin glue (Tissucol® without aprotinin). Light microscopy, Masson–Goldner-stained. A condensed, pycnotic nucleus and deeply eosinophilic, shrunken cytoplasm is consistent with apoptosis. Some cells appear to be surrounded by a white halo in which no matrix staining appeared, indicating pericellular glue dissolution (original magnification ×320).

In ultrastructural studies, cells suspended in (semi)autologous glue (Tissucol® Kit with 50% autologous serum and autologous fibrin sealant) are characterised by their roundish, chondrocyte-like morphology with an ovoid nucleus, extensive rough endoplasmatic reticulum and mitochondria (Fig. 5). Scanning electron micrographs yield mainly round cells with microvilli on their surface and processes wrapping around the fibrils (Fig. 6). Cells suspended in commercial fibrin sealants have a flattened and extensive aspect (Fig. 7). They showed an even condensation of nuclear chromatin with loss of nuclear membranes and cytoplasmatic swelling with swelling and loss of organelles; i.e. features of necrosis (Fig. 8). Besides this, we found

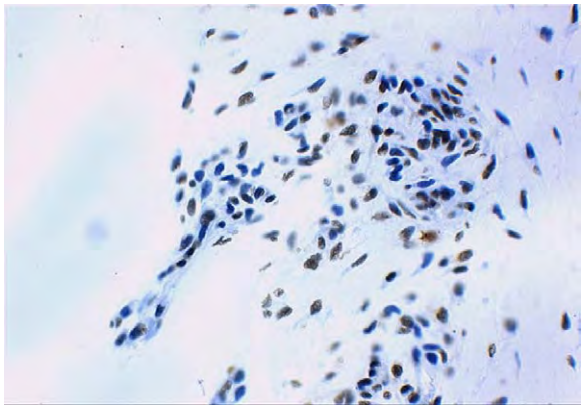

Fig. 4. Representative section of chondrocytes suspended in commercial fibrin sealant (Beriplast®). Light microscopy, TUNEL staining (ApopTag®-Kit) for detection of DNA fragmentation. The TUNEL positive cells have a pycnotic, shrunken appearance. (Original magnification ×100).

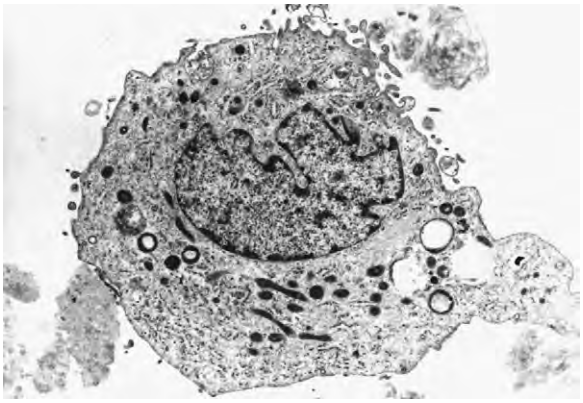

Fig. 5. Transmission electron microscopy (×6000) of a chondrocyte suspended in autologous fibrin glue. Note the roundish, chondrocyte-like morphology with an ovoid nucleus, extensive rough endoplasmatic reticulum and mitochondria.

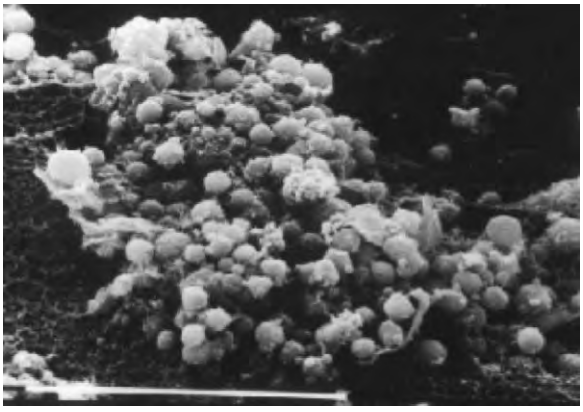

Fig. 6. Scanning electron micrograph (×2100) of a chondrocyte suspended in autologous fibrin sealant. The cells are round and have microvilli on their surface. The cells adhere firmly to the underlying matrix with their membrane processes.

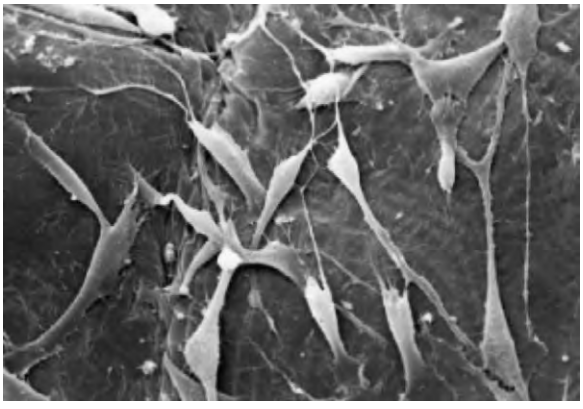

Fig. 7. Scanning electron micrograph (×2100) of a chondrocyte suspended in commercial fibrin sealant (Tissucol®). Cells suspended in commercial fibrin sealants yield a flattened and extensive morphology.

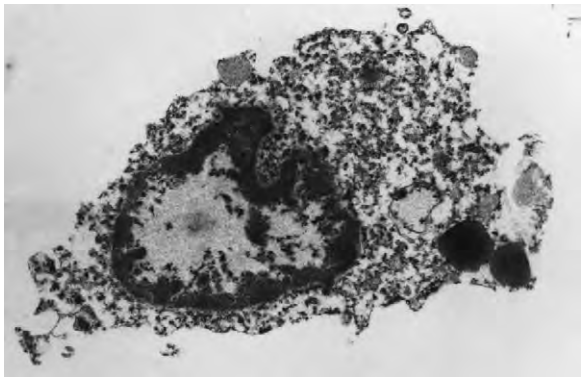

Fig. 8. Transmission electron microscopy of a chondrocyte suspended in commercial fibrin glue (Tissucol®). Note an even condensation of nuclear chromatin with loss of nuclear membranes and cytoplasmatic swelling with swelling and loss of organelles; i.e. features of necrosis.

no bona fide apoptotic bodies accompanied by external membrane blabbing, but we did observe cells with chromatin condensation and volume shrinkage consistent with apoptosis.

3.2. Experiment B

This study shows that chondrogenic cells can be cultivated on a collagen II or I/III sponge sealed with different fibrin glues under perfusion chamber conditions. Staining with Mayer's hematoxylin-eosin and Masson–Goldner allows the evaluation of the scaffold and cells by light microscopy. Neither the handling of the cell-matrix biocomposite nor the procedures for fixation could destroy the scaffold or the cell sheet adhering firmly to the scaffold. While the collagen II matrix is a monolayer scaffold, the collagen I/III matrix has two different sides. One side is rough and porous for better attachment of cells seeded onto the scaffold, the other side is smooth and impermeable. Cells invade the superficial areas of the matrix, but do not traverse it completely. Cells grown on a collagen II membrane have a fibroblast-like aspect with a round or ovoid nucleus (Fig. 9). A high percentage of cells showed morphologic changes consistent with apoptosis. Chondrocytes grown on the type I/III collagen scaffold form an apical sheet with partially fibroblast-like, spinocellular cells and partially roundish, chondrocyte-like cells (Fig. 10). Analysis of cellular morphology by light microscopy indicate that only some cells died by an apoptotic process. Underneath the apical layer, cells invade the superficial areas of the matrix, forming a loose reticular cell sheet. No cells did traverse the matrix–fibrin glue interface in all samples determined by light microscopy. In this point of view we did not observe differences regarding the type of collagen matrix or fibrin glue; no ingrowth of cells was seen in any of the experimental conditions when cell seeded matrices were sealed with different fibrin glues.

All samples revealed apoptotic cells as determined by the TUNEL technique. After 6 days of incubation on a collagen

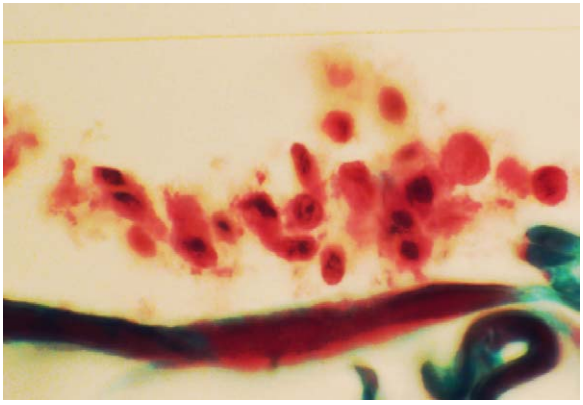

Fig. 9. Representative sections of chondrocytes grown on a collagen II scaffold. Light microscopy, Masson–Goldner-stained. Cells have a fibroblast-like appearance and form a loose reticular sheet. Note cells with condensed, pycnotic nucleus and deeply eosinophilic, shrunken cytoplasm (original magnification ×320).

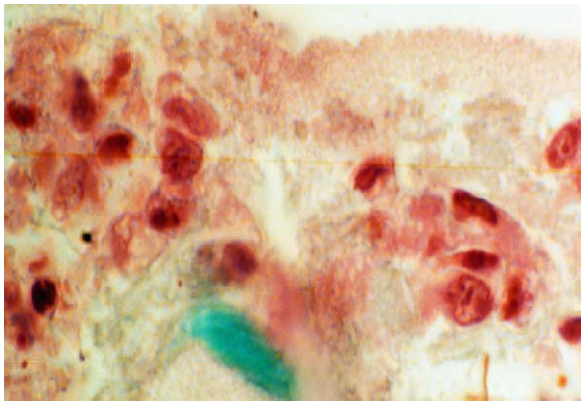

Fig. 10. Representative sections of chondrocytes grown on a collagen I/III scaffold. Light microscopy, Masson–Goldner-stained. Cells form an apical sheet with mainly roundish, chondrocyte-like cells. Underneath the apical layer, cells invade the superficial areas of the matrix, do not traverse the matrix–fibrin glue interface (original magnification ×320).

I/III matrix, 75% (71–77%) of the cells sealed with Tissucol® without aprotinin were TUNEL positive. This apoptotic ratio was increased compared to the results, when the other fibrin glues were used. Their results showed comparable values, as charted in Fig. 11 [(Tissucol® Duo S with aprotinin 68% (66–72%); Beriplast® 68% (65–73%); Tissucol® Kit with 50% autologous serum 73% (66–69%); autologous fibrin sealant 57% (54–61%); control 59% (58–61%)]. The mentioned apoptotic cell ratio for the collagen I/III matrix was significantly surpassed by the values, when a collagen

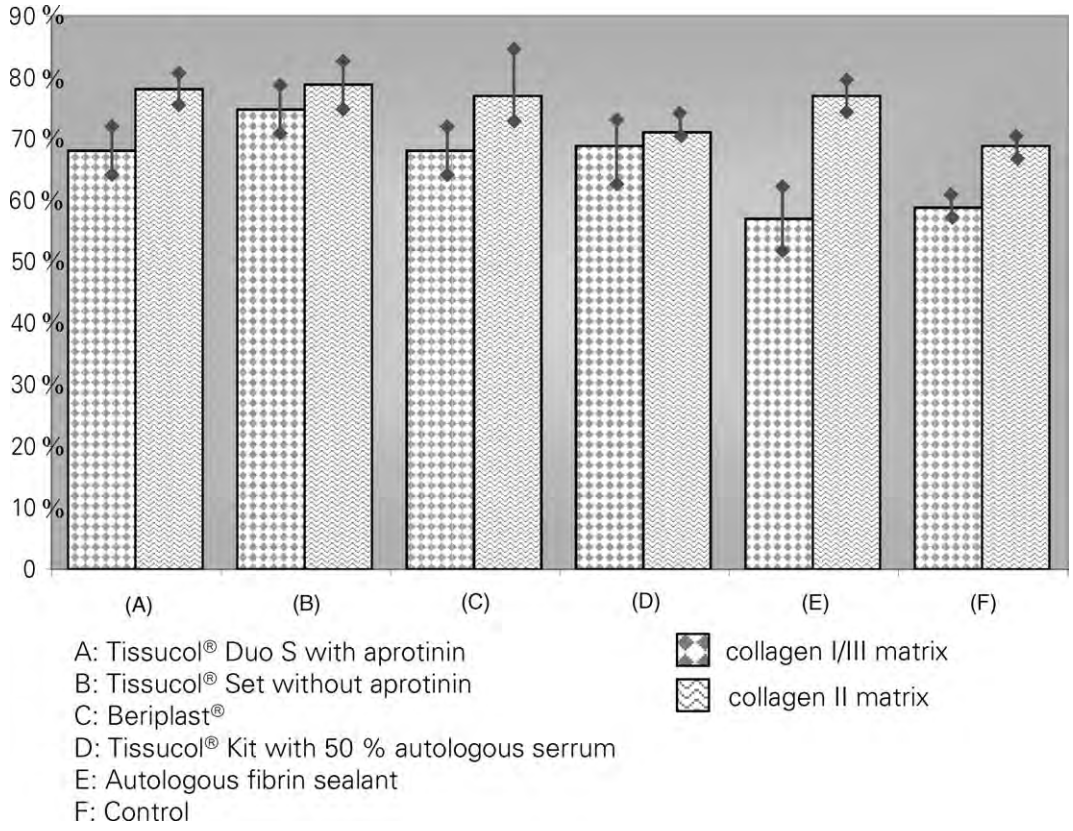

Fig. 11. Ratio of TUNEL (ApopTag®-Kit) positive cells seeded on porous matrices comprising different collagen types sealed with fibrin glue (bars mark the standard error of mean). The apoptotic cell ratio for the collagen I/III matrix was significantly surpassed by the values, when a collagen II matrix was used ($p = 0.008$). For detailed information see chapter "Results".

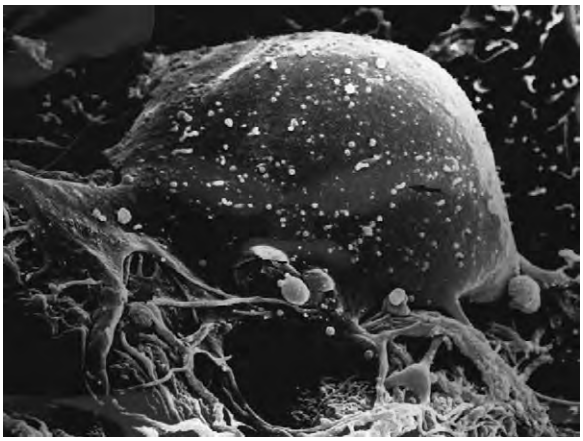

Fig. 12. Scanning electron micrograph (×9000) of a chondrocyte grown on the Chondrogide® matrix sealed with autologous fibrin glue. The cell is round and has microvilli on its surface. The cell adheres firmly to the underlying matrix with its membrane processes.

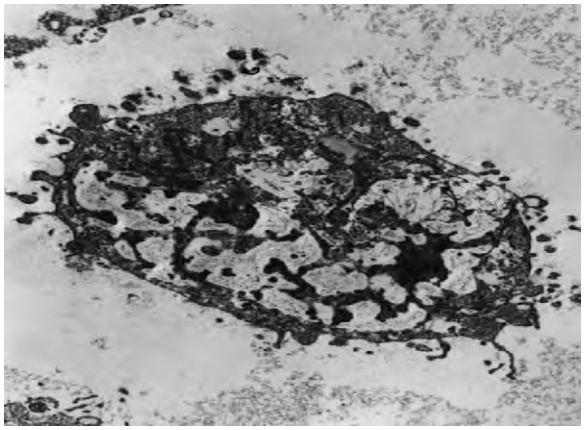

Fig. 13. Transmission electron microscopy (×7800) of a chondrogenic cell incubated on a collagen II membrane sealed with commercial fibrin glue (Beriplast®). There is nuclear chromatin condensation, increased cytoplasmatic electron lucency and swelling and loss of organelles. The nuclear membrane is degenerating and the chromatin shrunken and fragmented centrally.

II matrix was used ($p = 0.008$). In the control group 69% (68–70%) of the cells were TUNEL positive. The TUNEL technique did not indicate a significant difference in commercial (Tissucol® Duo S with aprotinin 78% (76–81%); Beriplast® 77% (72–85%); Tissucol® without aprotinin 79% (77–82%)) or (semi)autologous fibrin glue (Tissucol® Kit with 50% autologous serum 71% (71–75%); autologous fibrin sealant 77% (74–79%).

Ultrastructurally, some cells grown on a collagen I/III or II matrix appeared to contain several golgi zones and areas of rough endoplasmatic reticulum, partly filled with dense secretory material. The ultrastructure of the cells grown on Chondro-Gide® is chondrocyte-like (Fig. 12). But within some cell clusters, the cell shape had changed from oval to spindle-cell. These dedifferentiated cells missed to express signs of active secretion like microvilli and secretory vesicles. Cells contained nuclear chromatin condensation and volume shrinkage consistent with apoptosis (Fig. 13).

To measure the TGF-β 1 and 2 concentration, an ELISA was performed three times of each commercial and partial autologous fibrin glue and the median values were calculated, as charted in Fig. 14. The TGF-β 1 concentration was significantly higher in partial autologous fibrin sealant (11.9 ng/ml) compared to their commercial counterparts (Tissucol® 1.2 ng/ml; Beriplast® 2.1 ng/ml) ($p = 0.001$). Similar results were obtained when measuring the TGF-β 2 concentration. Commercial fibrin glues (Tissucol® 16.8 ng/ml; Beriplast® 14.3 ng/ml) showed a three times lower TGF-β 2 concentration than partial autologous fibrin sealants (59.7 ng/ml)

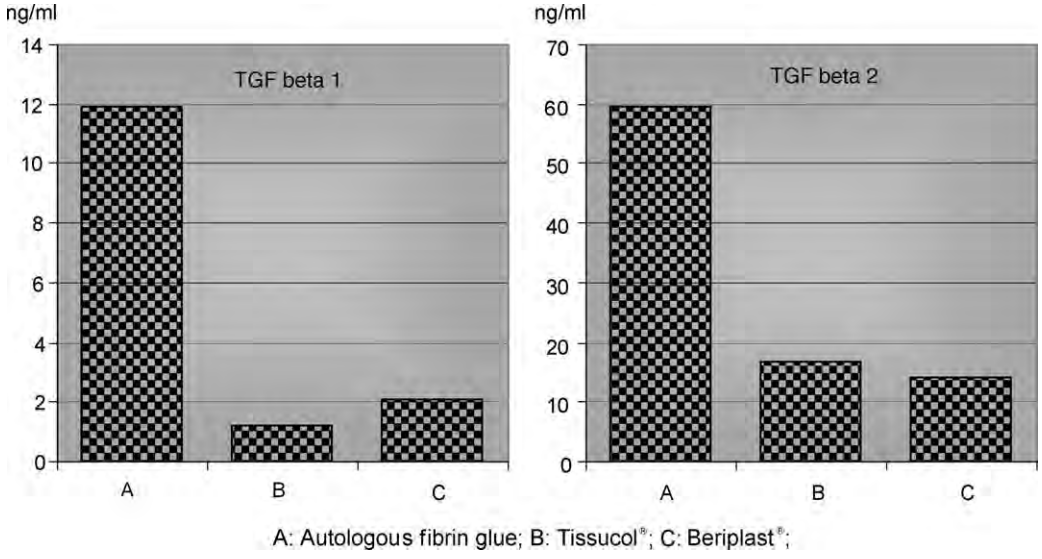

Fig. 14. TGF-β 1 and 2 concentration (nanograms per millilitre) in commercial and (semi)autologous fibrin sealants.

4. Discussion

Several types of fibrin glue are commercially available and are widely used as a biological adhesive in surgical operations (Schlag and Redl, 1988; Yoshida and Kamiya, 1998). The choice of fibrin is not new in orthopaedic surgery: previous uses have included "gluing" of osteochondral defects, reattachment of cartilage flaps and repair of cartilage lacerations (Hendrickson et al., 1994). Autologous grafts or reimplantation of loose fragments using fibrin glue showed excellent clinical results in articular cartilage defect therapy (Passl et al., 1979). But patients in whom commercial fibrin glue is used are placed at risk for infection by thermostable pathogenicities (Knight and Collins, 2001) and for allergic reactions to subtype components of the glue (Suzuki et al., 1994). To prevent these adverse effects, cryoprecipitate produced from the patient's own autologous plasma is recommended to use in fibrin sealant (Yoshida et al., 1999). To our knowledge the current study is the first to comment on migration pattern, morphology and viability of cells suspended in or sealed with different commercial and (semi)autologous fibrin glues. Different authors have investigated the influence of commercial fibrin sealant on cells and chondrocyte behaviour in fibrin glue in vitro (Homminga et al., 1993; Brittberg et al., 1994). These results have been similar and are in accordance with our results regarding chondrogenic cells incubated in different fibrin glues. Our data endorse the fact that after incubation of cell suspension in commercial and autologous fibrin glue, the morphology of the cells is chondrocyte-like rather than fibroblast-like. More partially spindly, process-bearing cells were seen in commercial fibrin glue compared to cells suspended in autologous fibrin sealant showing a more roundish, chondrocyte-like morphology. It can be suggested that the physical and chemical characteristics of the scaffold determine growth, morphology and phenotype of the chondrocytes.

Light microscopy revealed some cells in an apoptotic process. More cells suspended in Tissucol® without aprotinin showed a typical condensed, pycnotic nucleus and deeply eosinophilic, shrunken cytoplasm compared to other fibrin sealants. This was a puzzling observation since aprotinin bears the risk to cause allergic reactions and may, therefore, act as a premium movens in programmed cell death (Beierlein et al., 2000). On the other hand, aprotinin, a proteinase inhibitor, inhibits early fibrinolysis and therefore stabilizes the fibrin clot (Beierlein et al., 2000). Our data indicate that too rapid degradation of the fibrin clot may influence the viability in a negative way. This is in accordance with previous findings demonstrating chondrocytes cultured in fibrin glue, who retained their phenotypic expression as long as they were surrounded by the glue (Homminga et al., 1993). In conclusion, the fibrin glue-matrix may provide a trophic signal for the cells, or the altered fibrin matrix may influence the availability of factors that regulate apoptosis.

At first glance, the immunostaining results seem to be disappointing, being over all almost half of the cells positive for the TUNEL method. Cells suspended in commercial fibrin glue revealed a significant higher percentage of TUNEL positive cells compared to fibrin tissue adhesives mixed with autologous serum or autologous fibrin sealant. While an explanation for this phenomenon is awaited at present, it could be that natural fibrin gel releasing growth factors and cytokines may regulate cell morphology and viability while commercial fibrin adhesives lack many of these stimulating factors. However, one should consider the following arguments in answering the question of whether a positive TUNEL test means irrefutably programmed cell death. In situ detection of DNA fragmentation is susceptible to both positive and negative artefacts. False positive results can be caused by all procedures leading to DNA breaks; e.g. pretreatment with proteinase K (Labatmoleur et al., 1998; Negoescu et al., 1996). Digestion with proteinase K at a concentration that resulted in specific detection of apoptotic cells in rat ileum resulted in labelling of almost every cell in embryonic chick growth plate cartilage (Roach et al., 1995a, 1995b). On the other hand, it has been reported that proteinase K pretreatment resulted in a restricted staining of chondrocytes consistent with the morphological identification of cells undergoing apoptosis (Gibson, 1998). The reason for the difference between the percentage of apoptotic cells seen by morphologic criteria and TUNEL staining remains unclear. It may be based on the fact that the proteinase K digestion of the tissue may have given inconsistent results and have led to positive staining of viable cells. At least some of the TUNEL positive cells were viable by morphologic criteria. A 10-fold difference in the frequency of apoptotic cells between light microscopy examination and TUNEL stain has been reported before (Kim et al., 2000) and confirms our results.

In many cases we confirmed apoptosis by electron microscopy. Taking the dynamics of apoptotic cell death and limitations of the examination field with electron microscopy into account, there is nothing surprising about the partial absence of bona fide apoptotic bodies. We observed many cells with typical chromatin condensation and volume shrinkage.

Several synthetic and natural polymers have been seeded with chondrocytes in vitro and in vivo (Nehrer et al., 1997; Frenkel and DiCesare, 2004). Questions remain as to the most suitable chemical composition to be used in the fabrication of cell-seeded implants to treat defects in the articular surface (Lynn et al. 2004). This study directly compared the behaviour of chondrocytes in porous matrices comprising different collagen types sealed with fibrin glue. There was a significant difference in migration pattern, morphology and viability of cells seeded on collagen II and I/III matrices. Our results show that cells seeded onto a matrix can be investigated by light microscopy (Mayer's hematoxylin-eosin, Masson–Goldner and Trypan blue), electron microscopy and the TUNEL method (ApopTag®-Kit). Neither the handling of the cell-matrix biocomposite nor the procedures for fixation could destroy the scaffold or the cell sheet adhering firmly to the scaffold. Cells grown on a collagen II membrane have a

fibroblast-like aspect with a round or ovoid nucleus. In contrast, chondrocytes grown on the type I/III collagen scaffold form an apical sheet with mainly roundish, chondrocyte-like cells. Underneath the apical layer, cells invade the superficial areas of the matrix, forming a loose reticular cell sheet. A high percentage of cells grown on a collagen II matrix showed morphologic changes consistent with apoptosis. Investigation concerning programmed death of cells grown on a collagen I/III matrix indicate that only some cells died by an apoptotic process and are in accordance with (Gille et al., 2002a). Buma et al. (2003) performed a study with full-thickness defects in rabbits in which the defects were filled with cross-linked type I and II collagen matrices. Type I collagen matrices seems more favourable to guide progenitor cells from subchondral bone in vivo. For a better understanding of the regulatory events of chondrocytes seeded on a matrix, further studies will be necessary. An optimal scaffold will determine the growth, morphology and phenotype of the chondrocytes by its physical and chemical characteristics (Lynn et al., 2004).

No cells did traverse the matrix–fibrin glue interface in all samples determined by light microscopy. It has already been demonstrated that Tisseel® fibrin adhesive revealed cells growing on the surface but not invading into the commercial fibrin glue (Brittberg, 1994). Conversely, when using homologous fibrin clots the cells readily started to grow into the clot (Brittberg et al., 1997). The present study does not support this observation. One explanation for the superiority of the partial autologous fibrin clot to stimulate cell ingrowth compared to commercial fibrin sealant is that a natural fibrin gel could release growth factors and cytokines with the ability to regulate cell migration into the clot while the commercial fibrin adhesives lack many of these stimulating factors. It is open to debate whether or not cytokines present in the autologous fibrin glue (shown by ELISA) function as chemotactic stimuli. This study revealed a significantly higher TGF-β 1 and 2 concentration in partial autologous fibrin sealant compared to their commercial counterparts. The remaining question is whether the data of in vitro studies can be extrapolated to predict in vivo release kinetics and its influence on cell migration pattern, morphology and viability. Further in vivo studies will be necessary to elucidate this fact.

There is ample scope for continued experimental and clinical research. However, it is clear that biological tissue sealing has acquired a firm place in cartilage surgery and, no doubt, has exciting clinical potentialities. Partial autologous fibrin glue and collagen I/III matrix are favorable for focal articular defect therapy, but definitive conclusions can only be drawn after in vivo studies.

Acknowledgements

The authors gratefully acknowledge the skilled technical assistance of Mrs. Erika Meinecke and Mr. Christo Örun. We are indebted to P.D. Dr. Rer. Physiol. H.-J. Friedrich for assistance in statistical analysis.

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