Original article

Comparison of the Cellular Composition of Two Different Chondrocyte-Seeded Biomaterials and the Results of Their Transplantation in Humans

(tissue engineering of cartilage / autologous chondrocyte transplantation / biopsy and immunohistochemical analysis / HYAFF 11 / fibrin scaffold)

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Abstract. Our study compares the histological and immunohistochemical cellular composition of two different chondrocyte-seeded biomaterials and the results of their transplantation. Our study cohort included 21 patients, comprising 19 men and two women with a mean age of 32 years, who were affected by single chondral lesions of the femoral condyles. These patients were enrolled in our study and treated with arthroscopic implantation of the tissue Hyalograft C and/or Brno culture. Brno culture bioengineered with a fibrin-based scaffold contains round cells showing features of differentiated chondrocytes expressing S-100 protein and α-smooth muscle actin. In contrast, in the case of Hyalograft C, the scaffold was made up of a fibrillar network composed of biomaterial fibres of the esters of hyaluronic acid and cells resembling fibroblasts and myofibroblasts and expressing only α-smooth muscle actin. The average size of the defects was 2.5 cm². Patients were evaluated using the standardized guidelines of the International Knee Documentation Committee. During the comparison of bioptic samples obtained from both patient cohorts, we did not observe any important differences in the histological makeup of the newly formed cartilage. This was predominantly composed of hyaline cartilage with small areas of fibrocartilage. The histological analysis of these two groups of homogeneous patients shows that this bioengineered approach, under proper indications, may offer favourable and stable clinical results over time, in spite of the different matrix and cellular composition of the two transplants used.

Introduction

Articular cartilage defects are a major problem in orthopedic surgery (Magnusen et al., 2008). Since cartilage shows little tendency for self-repair, injuries persist for years, and their healing is very limited and can eventually lead to further degeneration of the joint. Fibrocartilage, which is mechanically and chemically inferior to hyaline cartilage, is the predominant repair tissue found in articular defects. The treatment of isolated defects may therefore prevent or delay the development of osteochondritis. During the past decades, many investigations have pursued techniques to stimulate articular cartilage repair or regeneration (Brittberg et al., 1994; Hendrickson et al., 1994; Frenkel et al., 1997; Lee et al., 2003; Tognana et al., 2007; Pelttari et al. 2008).

Surgical standard techniques such as drilling or microfracturing of subchondral bone, debridement, osteotomy, mosaicplasty, transplantation of autographs from non-weight bearing zones into former defect area, and soft tissue grafts, such as perichondrium, periosteal flap, joint capsule, or fascia (Magnusen et al., 2008) have the potential to stimulate formation of new articular surface and improve the joint function. However, in the majority of cases they are not able to restore normal articular cartilage (Buckwalter and Mankin, 1998a).

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Abbreviations: ACT – autologous chondrocyte transplantation, QC– quality control.

The most modern methods involve the use of autologous chondrocyte transplantation (ACT) by different techniques (Henrickson at al., 1994; Hollander et al., 2003, 2006; Lee et al., 2003; Knutsen et al., 2004; Podškubka et al., 2006; Filardo et al., 2011). The third generation of ACT is based on using autologous chondrocyte-seeded biomaterials as cell carriers. According to the original method described by Brittberg et al. (1994) the chondrocyte suspension was injected into the defect and covered by a periosteal flap. This was not always optimal, because the periosteal flap fixation was not always ideally strong or even water proof, and this resulted in chondrocyte loss. For this reason, the modifications of the original technique consist in improved fixation of cultured chondrocytes using a suitable three-dimensional carrier scaffold. Such a chondrograft is ready for immediate use and convenient for the surgeon because of its mechanical properties. The size and thickness of the graft is possible to adapt according to the cavity size and depth. Hydrogels most closely mimic the natural highly hydrated cartilage. Among the materials used have been demineralized bone, polylactic acid, polyglycolic acid, hydroxyapatite/Dacron composites, fibrin, collagen gels, and collagen fibres (Frenkel et al., 1997). The list of products which are commercially available or entering clinical trials for cartilage engineering are reported by Coates and Fisher (2010).

We had the opportunity of comparing the cell composition of two different chondrocyte-seeded biomaterials and compare the results of their transplantation when different scaffolds for cartilage regeneration were used in two groups of patients. In our study, we also compared the immunophenotype of transplanted cell-seeded biomaterials and chondrocytes in new-formed articular cartilage. The results of our study are summarized in this article, and we are confident that no similar work has been presented in the literature so far.

Material and Methods

Patients

A cohort of 21 patients; 19 men and two women with the mean age of 32 years, affected by a single posttraumatic chondral lesion of the femoral condyles, were enrolled and treated with arthroscopic implantation of the two different bioengineered tissues Hyalograft C (Fidia Advanced Biopolymers, Abano Terme, Italy) and Brno Chondrograft (Tissue Bank, University Hospital Brno-Bohunice, Czech Republic) with a fibrin-based scaffold. The average size of the defects was 2.5 cm^2. Patients were evaluated using criteria standardized by the International Knee Documentation Committee (Mainil-Varlet et al., 2002). All patients gave informed consent for arthroscopy and biopsy examinations in accordance with local ethical committee guidelines. Standard serological tests (HIV 1/ HIV 2 Antibody, Hepatitis B Surface Antigen, Hepatitis B Core Antibody, Hepatitis C Antibody, test for Syphilis) were required.

The part of clinical findings of this study has been reported elsewhere, and these have confirmed improvement of knee function and symptoms in more than 90 % of patients (Podškubka et al., 2006).

The samples analysed in our work represent a series of biopsies received in our laboratory from 12 patients treated with Hyalograft C in the Prague Orthopedic Clinic of Bulovka Hospital and nine patients treated with Brno culture in the Orthopedic Clinic of Prague Motol Hospital.

The first step was arthroscopic verification of the articular cartilage defect. During this examination, we obtained samples from the thin layer of residual cartilage filling the traumatic defect of the articular cartilage surface of the distal femur (N = 21). Simultaneously, a small piece from the low-weight-bearing femoral cartilage was removed for culture.

Chondrocytes isolated from the low-weight-bearing cartilage of patients were expanded in a monolayer culture, seeded onto Hyalograft C scaffolds, and cultured for a further 14 days as previously described (Grigolo et al. 2002; Tognana et al., 2007) in an Italian laboratory. The second type of culture was a fibrin-based scaffold prepared in the Brno laboratory. Cartilage tissue was cleaned and cut into small pieces under laminar air flow hood conditions. The fragments of cartilage were treated with 0.2% trypsin and 0.2% collagenase to digest the matrix and to isolate the chondrocytes. The released chondrocytes were propagated by conventional monolayer technique. The chondrocytes suspended in Ham's F12 medium (Gibco, Life Technologies, Carlsbad, CA) supplemented with 12% foetal bovine serum and gentamycin (Sigma-Aldrich, St. Louis, MO) were inoculated into the flasks and cultured in a carbon dioxide (environment) atmosphere at 37 °C. During successful primocultivation, the cell monolayer was formed via subculturing the primoculture, and the required number of cells was obtained.

Proliferation of chondrocytes was monitored using inverted phase contrast microscopy. The quality control (QC) of the process was done periodically and included QC of cultured chondrocytes (morphology and viability) and QC of the culture medium (microbiological controls). The combination of chondrocytes with fibrin glue resulted in a solid graft formation.

The cell-loaded scaffold was implanted into traumatic cartilage lesions in the knee which were first debrided as necessary. Most were at the medial femoral condyles, though some were at the lateral condyles.

During second-look arthroscopy, which was performed 10–12 months after autologous-chondrocyte transplantation, samples from the newly formed cartilage at the site of the posttraumatic chondral defect were obtained (N = 21).

We evaluated the quality of the repair tissue generated *in vivo* following implantation of autologous chondrocytes seeded onto hyaluronic acid-based scaffolds (Hyalografts C) or fibrin glue scaffolds in humans.

Tissue processing

Histopathological examination

Samples of the articular cartilage obtained during the first and second arthroscopy, and the remaining scaffolds used for transplantation were processed without decalcification. Following fixation in buffered 10% formalin for 72 h, the samples were processed with paraffin and sectioned into 5-micron sections. Each section was stained with haematoxylin and eosin to evaluate the general morphology and cell organization. Staining with safranin O (Fluka Chemie GmbH, Sigma-Aldrich Chemie, Steinheim, Germany) enabled evaluating the content of proteoglycans. Polarized light was used to examine the collagen organization of the cartilage samples.

Each biopsy was scored histologically and the cartilage tissue was classified as hyaline, fibrocartilage, or mixed tissue, according to the guidelines of ICRS (Mainil-Varlet et al., 2002). For each biopsy, we set out to determine: 1) the histological organization; 2) the relative proportions of hyaline and fibrocartilage; 3) the abundance of extracellular matrix; 4) the immunophenotype of cartilage cells; 5) the presence of the residual scaffold.

Immunocytochemical staining

For the purposes of immunohistochemical studies, we used the avidin-biotin complex (ABC) technique. Primary monoclonal antibodies against S-100 protein (1 : 600, DAKO, Glostrup, Denmark); S-100 A1 and A2 protein (Labvision Corp. Neomarkers, Westinghouse, Fremont, CA); S-100 proteins A4 and A6 (1 : 200, Neomarkers); S-100 protein P (1 : 40, RnD Systems, Minneapolis, MN); S-100 protein A10 (ready to use, Neomarkers); (α-smooth muscle actin (1 : 100, Sigma, St. Louis, MO); muscle-specific actin HHF35 (1 : 100, DAKO); desmin (1 : 200, DAKO); CD68 (1 : 50, DAKO); CD34 (1 : 50, DAKO); Ki67 (1 : 50, DAKO); and anti-human collagen type II (1 : 100, Santa Cruz, CA) were applied. Negative controls were performed by substituting the primary antibody with non-immune mouse serum.

Results

Defective cartilage removed before transplantation

In our cohort, we examined samples of residual chondral tissue of the chondral defects. In some samples the original structure of hyaline cartilage was partly preserved (Fig. 1a). The base of the defects was composed of a deep cartilage layer including the residua of calcified cartilage tissue. In other cases, there were regions composed of cellular fibrous cartilage (Fig. 1b) with an admixture of original articular hyaline cartilage showing features of degeneration consistent with that of osteoarthritis. In such cases, we observed degenerative changes including superficial fibrillation, cartilage fragmentation, and chondrocytes in the process of clonal grouping.

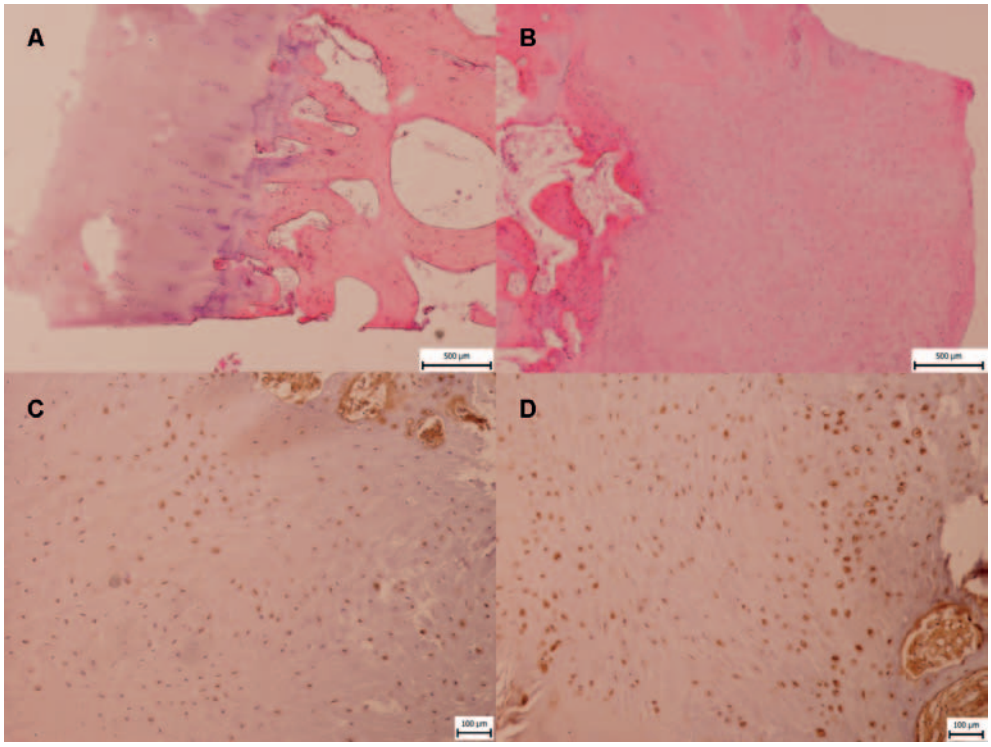

Fig. 1. Biopsy specimens of the residual chondral tissue removed from the chondral defects before transplantation. Residual cartilage was only composed of deep layers of the normal structure (a). In some cases original hyaline cartilage was substituted with predominant fibrous cartilage (b) HE × 40. Chondrocytes of the fibrous cartilage expressed α-smooth muscle actin and S-100 protein (c, d) × 100.

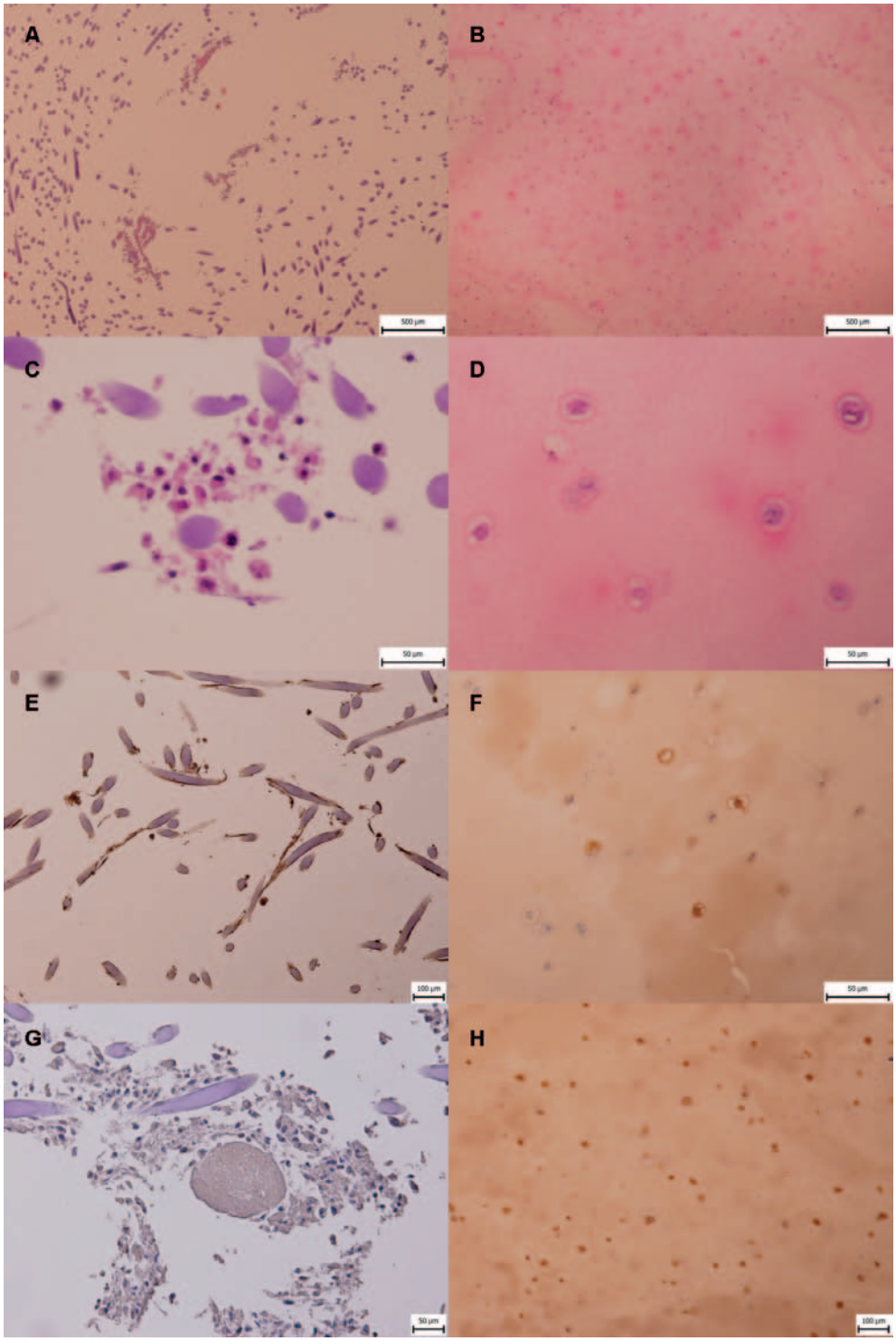

Fig. 2. Two scaffolds used in our study had a different histological structure.
The scaffold Hyalograft C was made up of a fibrilar network composed of biomaterial fibres of the esters of hyaluronic acid and clusters of small cells (a, c), HE, × 40 and × 400. The cells seeded onto the fibers expressed smooth muscle actin only (e), S-100 protein was negative (g) × 100. Brno Chondrograft culture was composed of a fibrin scaffold and round cells rather resembling differentiated chondrocytes (b, d), HE, × 40 and × 400. The cells of Brno Chondrograft culture expressed smooth muscle actin and different isoforms of the S-100 protein (f, h) × 100.

The main difference, when compared with normal articular cartilage, was the predominance of chondrocytes expressing smooth muscle actin (Fig. 1c). It was also possible to stain the chondrocytes with antibodies against different isoforms of S-100 protein (Fig. 1d). The most intensive staining was observed when antibodies against S-100 A6 protein were used.

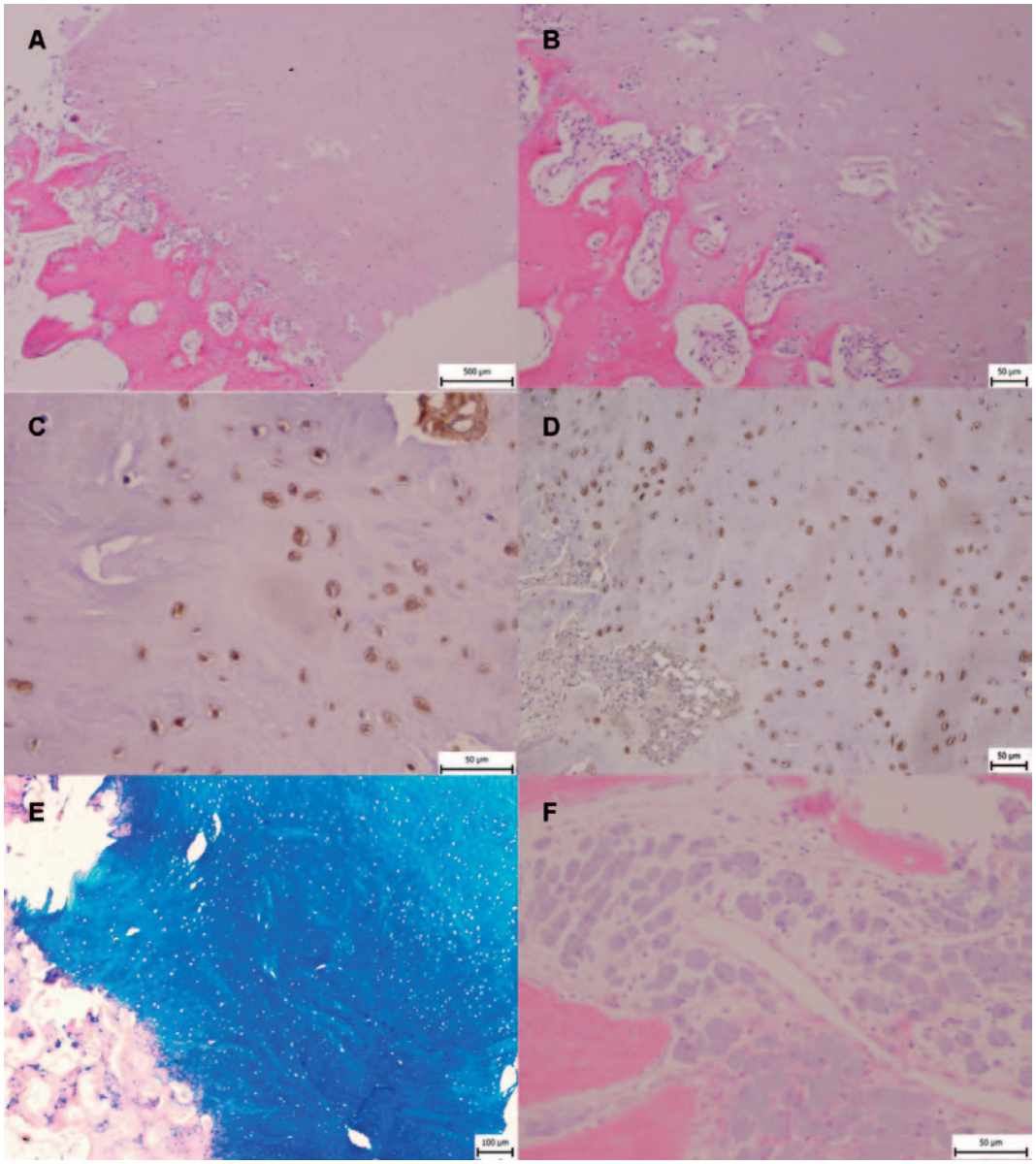

Fig. 3. The newly formed cartilage after autologous-chondrocyte transplantation of Hyalograft C after 10 months had a predominantly hyaline cartilage. Columnar distribution of cells in the deep zone was irregular and the zone of calcified cartilage as well as the subchondral bone plate were incomplete (a, b), HE, × 40 and × 200.
The cells of the newly formed cartilage expressed α-smooth muscle actin and S-100 protein (c, d) and intercellular matrix was alcian blue positive (e) × 100 and × 400.
In the intertrabecular spaces of subchondral bone there was focal accumulation of macrophages with blue-coloured cytoplasm containing residual phagocytized material from the scaffold composed of the esters of hyaluronic acid.

Histology of cell-seeded scaffolds

Histological examination of both types of cell-seeded scaffolds used during the transplantation showed markedly significant differences (Fig. 2 a-h).

In the case of Hyalograft C, the composition of the transplanted scaffold was remarkably different. The scaffold was made up of a fibrillar network composed of biomaterial fibres of the esters of hyaluronic acid. The distribution of cells was completely irregular and their concentration differed from site to site. Clusters of small cells around the fibres were observed (Fig. 2a, c, e, h). The cells seeded onto the fibres resembled fibroblasts and myofibroblasts not only by their spindle shape, but also by their expression of smooth muscle actin (Fig. 2e). All cells were completely negative during immunohistochemical examination with antibodies against different isoforms of S-100 protein (Fig. 2g). It was supposed that these cells may represent immature mesenchymal cells with features resembling stem mesenchymal cells and myofibroblasts. The proliferation index was not evaluated because the staining with antibody against Ki67 gave unclear results.

Brno culture was composed of a fibrillar fibrin scaffold and round cells relatively regularly distributed that rather resembled differentiated chondrocytes (Fig. 2b, d,

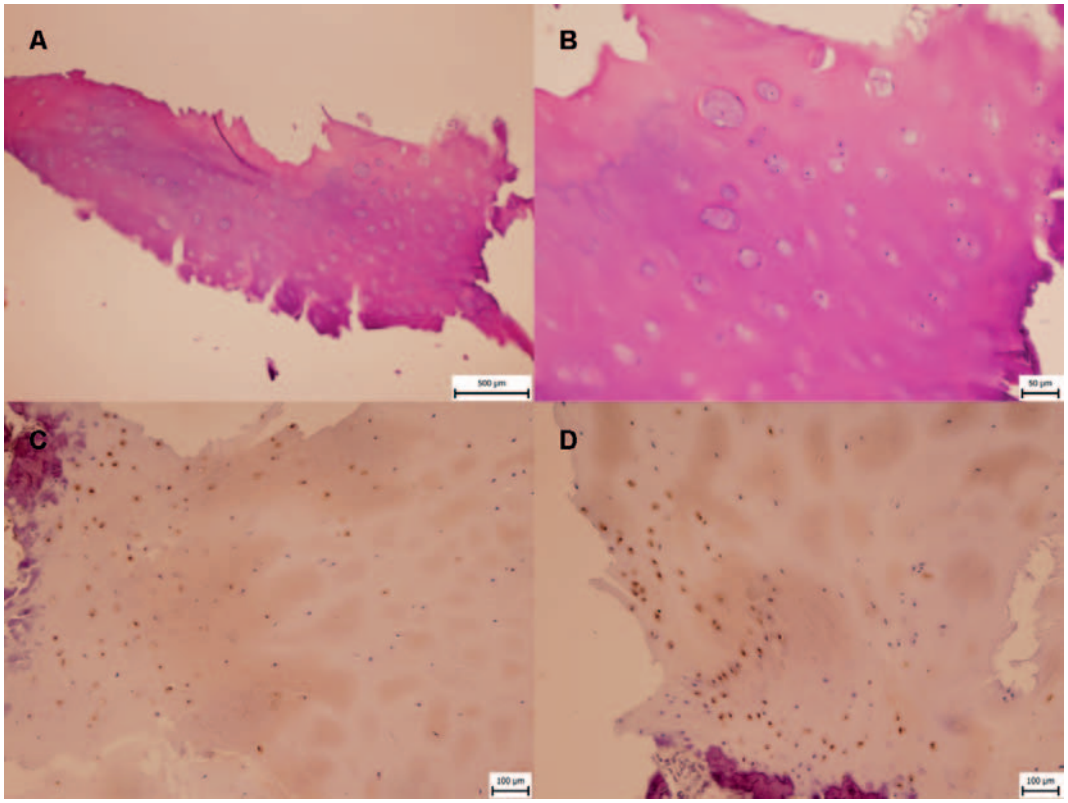

Fig. 4. The newly formed cartilage 10 months after transplantation of Brno Chondrograft culture was mature and predominantly composed of lacunar cells (a, b), HE, × 40 and × 200. Chondrocytes expressed smooth muscle actin and S-100 protein (c, d) × 100.

f, h). The cells of this culture also expressed α-smooth actin (Fig. 2f). The nature of the chondrocytes was confirmed immunohistochemically, because the cells expressed different isoforms of S-100 protein (Fig. 2h). The most intensive staining was observed when antibodies against S-100A6 protein were used. The proliferation index was rather high, around 5 %. We used antibodies against the Ki67 protein.

Cartilage after autologous-chondrocyte transplantation

The newly formed cartilage obtained 10 months after autologous-chondrocyte transplantation of Hyalograft C had a predominantly hyaline character (Fig. 3 a-e).

The distribution of chondrocytes in the cartilage was rather irregular (Fig. 3a, b). All cells were viable and exhibited a clearly delineated nucleus. The columnar distribution of cells in the deep zone was irregular. This finding indicated incomplete maturation of the newly formed cartilage tissue.

The new cartilage contained proteoglycans which were verified upon staining with alcian blue (Fig. 3e) and safranin O. Immunohistochemical staining for collagen II was also positive, but for this examination we only had formol-fixed material. However, polarized light microscopy demonstrated randomly oriented collagen bundles in smaller areas of fibrocartilage. In contrast, the areas of newly formed hyaline cartilage did not differ from normal hyaline cartilage.

The zone of calcified cartilage was also incomplete, as well as the subchondral bone plate (Fig. 3a, b). In all biopsies, there was nearly perfect integration of cartilage to the bone. In subchondral bone tissue, we observed features of remodelling represented by the presence of osteoclasts showing positivity in reaction with antibody against CD68 and by the presence of osteoblast rims on the surface of bone trabecules.

In other areas fibrocartilage predominated. The majority of the newly formed cartilage cells contained α-smooth muscle actin (Fig. 3c) and were S-100 protein positive (Fig. 3d). Other immunohistochemical reactions such as for CD 34, desmin, and muscle-specific actin were negative.

Unusual findings were observed in two cases. In both of these cases, we observed focal accumulation of macrophages with blue-coloured cytoplasm in newly formed cartilage and in the intertrabecular spaces of subchondral bone (Fig. 3f). We suppose that macrophages contain residual phagocytized fibrillar material from the scaffold composed of the esters of hyaluronic acid in their cytoplasm. Such an interpretation is supported by their positivity when stained with alcian blue.

Results of the transplantation of the second type of scaffold from Brno were verified in nine patients by second-look biopsy (Fig. 4 a-d). The cartilage was a little more mature and predominantly composed of lacu-

nar cells (Fig. 4a, b) in contrast with the first group of patients. Fibrocartilage was identified in small areas of the bioptic samples. Immunohistochemical staining of α-smooth muscle actin and S-100 protein were similar to that observed in the Hyalograft C group. No residual scaffold was identified.

Discussion

Cartilage tissue obtained from the surface of articular defects is composed of an admixture of hyaline cartilage and fibrocartilage. Many chondrocytic cells contained α-smooth muscle actin as we have described previously (Povýšil et al., 2008).This finding suggests that chondrocytes may have an adaptation mechanism enabling such transformation of the immunophenotype of cartilage depending on different situations. The chondrocytes probably adopt smooth muscle features during the healing process to produce the initial fibrocartilage step. Based on this finding, we presume that the chondrocyte phenotype is variable and can be modulated by microenviromental stimuli (Povýšil et al., 2008). Such stimuli include mechanical pressure. The mechanisms regulating the expression of α-smooth muscle actin and other cytoskeletal proteins in chondrocytes are only poorly understood.

However, it is well known that the structure of chondrocytes and the composition of extracellular matrix vary with tissue depth. The chondrocyte phenotype varies considerably by zone, and it is supposed that phenotypically stable zonal cell populations are maintained in articular hyaline cartilage (Coates and Fisher, 2010). Zonal differences in the matrix organization and content are largely due to variations in cellular activity (Aydelotte and Kuettner, 1988; Aydelotte et al., 1988; Coates and Fisher, 2010). Cells of the different zones display differences in morphology, density, and metabolic activity. Superficial zone cells are the smallest, and manifest as elongated and thin cells oriented parallel to the articular surface. A portion of them contain smooth muscle actin in their cytoplasm (Povýšil et al., 2008) and they are responsible for producing large glycoprotein proteoglycans occurring in the synovial fluid. The large cells of the deep zone, which are oriented perpendicularly to the articular surface, serve to anchor the articular cartilage to the calcified layer below. Chondrocytes of various zones secrete several other proteins, but their functions are not completely known (Coates and Fisher, 2010).

Recently, the newly developed treatment modality of autologous chondrocyte transplantation has shown great promise, and is now widely used for the treatment of cartilage defects (Brittberg et al., 1994). Tissue engineering combines cells, scaffolds and bio-active factors, which represent one of the most promising approaches to the restoration of damaged tissues. The main purpose of our study was to compare cartilage repair tissue at the femoral condyle after matrix-associated autologous chondrocyte transplantation using two different scaffolds.

Hyalograft C is a tissue-engineered product composed of autologous chondrocytes grown on a 3D scaffold made of HYAFF11 (Brun et al., 1999; Grigolo et al., 2002; Girotto et al., 2003; Hollander et al., 2006; Tognana et al., 2007), which is a benzyl ester of hyaluronic acid. This biodegradable and biocompatible 3D product has been repeatedly used for treatment of articular cartilage defects, and the results of the transplantation were excellent. This technique with its inherent safety profile has already been introduced into clinical practice. Immunohistochemical studies as well as RT-PCR analysis *in vitro* confirmed that chondrocytes seeded on HYAFF 11 express and produce type II collagen and aggrecan (Girotto et al., 2003; Hollander et al. 2003), but the cytological characteristics of the cell populations were not mentioned in these reports. A similar situation is evident in the case of Brno culture based on the fibrin glue composition of the carrier. The cellular composition of these two products was completely different in our study. Despite having been cultivated on 3D scaffolds, the cell differentiation on both transplant types did not reach the levels of native cartilage. Hyalograft C was made up of a fibrillar network composed of biomaterial fibres of the esters of hyaluronic acid. The cells seeded on the fibres may represent immature mesenchymal cells resembling fibroblasts and myofibroblasts primarily by their shape and expression of smooth muscle actin. No signs of chondrocyte differentiation were observed. The cell components of Brno culture had features native to chondrocytes because they had a round shape and expressed S-100 proteins, and also displayed a high proliferation index when we used antibodies against Ki67.

Both methods of transplantation had acceptable short-term clinical results. There were no significant differences in the macroscopic or histological results between the two treatment groups. All patients showed clinical improvement, and the macroscopic appearance of repair tissue at arthroscopy was either normal, or nearly normal in all cases.

Biopsies of the repair tissue were available for 21 of these patients and were analysed according to standard histological and immunohistochemical techniques used for chondrocyte morphology and presence of proteoglycans. The following major conclusions can be drawn from our study. Chondrocytes engineered *in vitro* onto these two different scaffolds produce new cartilage with similar properties after transplantation into cartilage defects. However, it seemed that after transplantation with Brno culture, the newly formed cartilage was a little more mature and predominantly composed of lacunar cells.

We uphold the results of previous works (Grigolo et al., 2002; Girroto et al., 2003; Hollander et al., 2003; Podškubka et al., 2006) that demonstrate that tissue-engineered cartilage implanted into knees matures into an articular cartilage structure as early as 11 months after transplantation of the Hyalograft C. We also observed very similar results in the patients transplanted with Brno culture.

In both groups of patients with transplanted autologous chondrocytes, the newly formed cartilage had a partly hyaline character with an admixture of fibrocartilage. Chondrocyte distribution in the cartilage matrix was rather irregular. All cells were viable and exhibited a clearly delineated nucleus. The columnar distribution of cells in the lower zone was irregular. This finding indicated an incomplete maturation of the new cartilage tissue. The zone of calcified cartilage, as well as the subchondral bone plate, was also incomplete in both groups.

In subchondral tissue, we observed features of bone remodelling with the presence of osteoclasts and osteoblasts on the surfaces of nonlamellar bone trabeculae. The majority of cartilage cells contained α-smooth muscle actin. It seems that actin-positive cells in normal hyaline cartilage, with the exception of the surface layer, occur in association with arthrotic changes (Wang et al., 2001; Povýšil et al., 2008) and predominantly during the process of reparation (Povýšil et al., 2008). This includes new formation of the cartilage after transplantation. Chondrocytes containing actin, or so-called myochondrocytes (Povýšil et al., 1997), may utilize the contractile actin isoform in manipulating the extracellular matrix of articular cartilage (Povýšil et al., 2008). It is also possible that actin-containing chondrocytes have a higher potential for regeneration in contrast to chondrocytes that do not contain this contractile material in their cytoplasm.

However, transformation into hyaline cartilage was not complete in both groups. It may be caused by the fact that the second-look biopsy was performed too soon. We may suppose that complete restoration of the newly formed cartilage could develop later. This would have been seen if the second-look biopsy were performed after a longer time of patient follow up with this type of the treatment. We do not know what are the limits of the maturation process of newly formed cartilage after transplantation.

It is well known that a clear method for retaining and/or creating phenotypically stable zonal cell populations has not been established to date. Zonal differences in matrix organization and content are largely due to variations in cellular activity. Preliminary reports have indicated that this may be possibly due to zonal differentiation in these cells. It is supposed that such cultures will utilize progenitor cell populations, and therefore new commercial products in cartilage tissue have been analysed (Pelttari et al., 2008; Coates and Fisher, 2010). Future investigations will need to ascertain the factors that contribute to the maturational process of the cartilage after transplantation.

Conclusion

Our study was primarily aimed to compare the histological and immunohistochemical cellular composition of two different chondrocyte-seeded biomaterials and the results of their transplantation. Brno culture contained round cells showing features of differentiated chondrocytes expressing S-100 protein and smooth muscle actin. The proliferation activity was rather high, around 5 %. In contrast, in the case of Hyalograft C, the scaffold was made up of a fibrillar network composed of biomaterial fibres of the esters of hyaluronic acid. The cells seeded onto these fibres resembled fibroblasts and myofibroblasts and expressed only smooth muscle actin. No signs of chondrocyte differentiation were observed. Therefore, they may represent pre-chondrocytes or immature mesenchymal cells with features resembling stem mesenchymal cells. During the comparison of both patient cohorts, we did not observe any important differences in the histological makeup of the newly formed cartilage. It is surprising that the results of transplantation were very similar because the cellular composition of the two cultures was completely different in terms of the degree of cell differentiation. On the basis of such findings, it is possible to conclude that more highly differentiated cells with the typical S-100 protein-positive phenotype of chondrocytic cells also have a sufficient growth potential for the healing of cartilage defects.

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