TISSUE ENGINEERING
Volume 12, Number 6, 2006

Differential Cell Viability of Chondrocytes and Progenitor Cells in Tissue-engineered Constructs Following Implantation into Osteochondral Defects

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ABSTRACT

Animal studies in cartilage tissue engineering usually include the transfer of cultured cells into chondral or osteochondral defects. Immediately at implantation, the cells are exposed to a dramatically changed environment. The aim of this study was to determine the viability of two cell types currently considered for cellular therapies of cartilage defects–chondrocytes and progenitor cells–shortly after exposure to an osteochondral defect in rabbit knees. To that end, autogenic chondrocytes and periosteal cells were labeled with CM-DiI fluorochrome, seeded or cultured in PEGT/PBT scaffolds for periods up to 2 weeks, transferred into osteochondral defects, harvested 5 days postimplantation, and analyzed for cell viability. In order to further elucidate factors effecting cell viability within our model system, we investigated the effect of serum, 2) extracellular matrix surrounding implanted cells, 3) scaffold interconnectivity, and 4) hyaluronan, as a known cell protectant. Controls included scaffolds with devitalized cells and scaffolds analyzed at implantation. We found that the viability of periosteum cells (14%), but not of chondrocytes (65–95%), was significantly decreased after implantation. The addition of hyaluronan increased periostium cell viability to 44% ($p < 0.05$). Surprisingly, cell viability in less interconnected compression-molded scaffolds was higher compared to that of fully interconnected scaffolds produced by rapid prototyping. All other factors tested did not affect viability significantly. Our data suggest chondrocytes as a suitable cell source for cartilage repair in line with clinical data on several chondrocyte-based therapies. Although we did not test progenitor cells other the periosteum cells, tissue-engineering approaches using such cell types should take cell viability aspects into consideration.

INTRODUCTION

THE POSSIBILITIES FOR TREATMENT of cartilage defects have improved considerably due to progress in the field of tissue engineering (TE). Recently studied and applied methods for cartilage repair, such as autogenic perichondrium transplantation,[1,2] autogenic periosteum transplantation,[3] autogenic chondrocyte implantation (ACI),[4] and mosaic plasty,[5,6] are all examples of TE according to the definition of Langer.[7]

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At present substantial research focuses on the transplantation of cultured cells with chondrogenic potential seeded into biodegradable scaffolds. Such scaffolds facilitate cellular transfer to and retention within the defect and, if appropriately selected, may provide mechanical functionality.[8] A major question in tissue engineering is the selection of a viable cell source. Both chondrocytes and progenitor cells are currently considered for cellular therapies of cartilage defects. Progenitor cells from bone marrow have been widely used. However, animal-derived bone marrow cells, in particular from rabbit origin, have shown a highly variable chondrogenic potential.[9] Previously, it was established that periosteal cells are a potential source of chondro-progenitor cells.[10,11]

Our aim in this study was to test the hypothesis that progenitor cells and chondrocytes react differentially upon transfer into osteochondral joint defects. This hypothesis is based on the differential *in vivo* histories of these cell types: progenitor cells are in close vicinity to vasculature, and thus have sufficient nutrient supply, including oxygen, while chondrocytes are not.[12,13]

The effectiveness of any cellular repair approach will depend on the retention of cell viability early after implantation.[14,15] Surprisingly, little is known of the survival of transplanted cells.[16] To our knowledge, we report here for the first time a detailed analysis of cell viability in the initial osteochondral repair phase.

At implantation, the cell environment changes considerably. Cell culture conditions have been optimized for either proliferation or (re)differentiation of cells. Once transplanted in an osteochondral wound bed, cells are exposed to the hostile conditions of the primary wound healing reaction, including polymorphonuclear cells, catabolic enzymes, and deleterious cytokines (TNFα, IL-1). Other potentially harmful factors are mechanical forces and changes in the supply of nutrients and oxygen.

Factors that may affect cell viability upon transplantation include: 1) immunological, due to traces of foreign body proteins from culture media (i.e., fetal bovine serum [FBS]), 2) the presence of extracellular matrix, which may protect the cells and keep them well differentiated, and 3) interconnectivity of pores within the scaffold, which will determine the nutrient and oxygen supply.

In this study we addressed these factors, by 1) comparing chondrocytes with progenitor cells from periosteum, 2) comparing chondrocytes and periosteal cells cultured in media containing FBS with cells cultured in media containing autogenic serum (AS), 3) comparing chondrocytes that were seeded and implanted with chondrocytes that were seeded, cultured for 2 weeks, and implanted, and 4) comparing chondrocytes seeded on scaffolds produced by compression molding and salt leaching (CM scaffold) with chondrocytes seeded on scaffolds with increased interconnectivity produced by rapid prototyping (RP scaffold). In addition, we tested whether hyaluronan could be used as a cell-protective agent by injecting it intraarticularly after closure of the joint.[13,17–22]

To distinguish transplanted cells from endogenous ones, the cells to be transplanted were labeled with the fluorochrome chloromethyl-benzamidodialkylcarbocyanine (CM-DiI). This fluorescent marker becomes intercalated within the cell membrane lipid bilayer and is useful for tracing cells in cell survival studies.[16,23,24] The dye has been demonstrated not to effect cell metabolism or viability for a number of different cell types.[16,25–27] Possible transfer of the dye to endogenous cells[16] was tested *in vitro* by a filter assay and *in vivo* after implantation of cell-scaffold constructs in nude mice.

To assess whether transplanted cells survived transplantation, Sytox Green was applied immediately after explantation to stain dead cells.[28] Sytox Green has been used in several cell viability studies[29,30] and can be combined with 4′,6-diaminodino-2-phenidole (DAPI) to detect viable and non-viable cells.

MATERIALS AND METHODS

Scaffolds

PEGT/PBT (PolyActive) scaffolds from IsoTis OrthoBiologics (Bilthoven, The Netherlands) were used. PEGT/PBT is an elastomeric, segmented multiblock copolymer comprised of poly(ethyleneglycolteraphthalate) and poly(butylene teraphthalate).[8] The molecular weight of the PEG in the polymer used for this study was 300 D, and the PEGT/PBT weight ratio was 55:45. This co-polymer has been shown to be biocompatible and biodegradable.[31,32] Scaffolds were made by the compression molding and salt leaching method (CM scaffold) or by rapid prototyping (RP scaffold).[8] Three-dimensional microcomputed tomography (μCT) was used to characterize scaffold architecture.[33] The scaffolds had a comparable porosity of approximately 80%. The average pore sizes for the CM and RP scaffolds were 182 and 525 μm, respectively. The accessible pore volume (measure for interconnectivity) at a pore size of 200 μm was 20% for the CM scaffold. By contrast, the RP scaffolds had an accessible pore volume of 98%. Scaffolds were sterilized by γ-irradiation at 25 kGy.

Isolation of autogenic blood, cartilage, and periosteum

The European and Dutch regulations on animal experimentation were strictly followed throughout the study. The study was approved by the local committee for animal experiments. Eighteen adolescent female New Zealand white rabbits (age 6 months, weight 3–4.5 kg) were used. Fourteen days prior to the first operation, 10

days after the first operation, and 10 days prior to the second operation 15 mL of arterial blood was collected from the ear. Autogenic serum was isolated and complement-inactivated by heating at 56°C for 50 min.

Surgical procedures were performed using sterile techniques under general anesthesia. Anesthetic induction was obtained with 35 mg/kg Ketamine hydrochloride and 5 mg/kg Xylazine hydrochloride i.m. and maintained by a mixture of 2% halothane and oxygen/nitrous oxide. Preoperatively all rabbits received antibiotics (10 mg/kg ceftiofur sodium; Pharmacia & Upjohn, Woerden, The Netherlands). Postoperative analgesia was provided with 0.05 mg/kg buprenorfine i.m.

In both knees an arthrotomy was performed: the patella was luxated laterally and the joint surfaces were exposed. A cartilage-only defect with a diameter of 4 mm was created with a sharp dermal punch on the medial and lateral femoral condyles of both knees. The removed cartilage was collected. Then cartilage from the patellar groove was dissected, sparing the rims of the groove, and harvested as well. After closure of the joint a periosteal flap of about 7×15 mm was harvested from the proximal medial tibia of both legs. The wounds were closed in layers using resorbable sutures.

Cell isolation

Articular cartilage was transported to IsoTis S.A. in transport medium: Dulbecco's modified Eagle's medium (DMEM) containing HEPES as buffer, 0.2 mM ascorbic acid-2-phosphate, 0.4 mM L-proline, 0.1 mM nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. At IsoTis Orthobiologics, half of the samples were digested overnight with 300 U/mL collagenase type II in HEPES-buffered DMEM for isolation of chondrocytes, which were expanded for 2–3 passages (18 days), then isolated, labeled with CM-DiI, and seeded on to scaffolds. These cells-scaffold constructs were subsequently cultured for 2 weeks (cultured groups). The other half of the samples were left in transport medium in a CO_2 incubator for 2 weeks, and the medium was refreshed three times a week. Then the chondrocytes were isolated, expanded for 2–3 passages (18 days), labeled, and, 3 days prior to implantation, seeded onto scaffolds (seeded groups).

Expansion of chondrocytes was performed in monolayer cultures in culture medium: DMEM supplemented with 0.2 mM ascorbic acid-2-phosphate, 0.4 mM L-proline, 0.1 mM nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, and either 10% AS or 10% FBS, in 2–3 passages for 18 days.

Isolation of periosteum cells was performed in the research laboratory of the Department of Orthopedics in Maastricht. Periosteum was incubated with 300 U/mL collagenase type II (Invitrogen, Breda, The Netherlands) in DMEM-Hepes medium supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin for 3 h at 37°C. Subsequently cells were allowed to grow in culture flasks in DMEM/Ham's F12 (1:1) medium (Invitrogen) supplemented with 10% FBS, 0.1 mM nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. After 1 week, this medium was changed to MEM-D-valine supplemented with nonessential amino acids, 10% FBS, and antibiotics to avoid fibroblast overgrowth.[34] Due to the limited amount of AS and the poor growth of periosteal cells in medium with AS, we decided to expand periosteal cells of the AS group in medium with FBS and to use AS medium only in the final passage and the seeding procedure.

Cell labeling

Before labeling, the cells were counted in a hemocytometer. Chondrocytes and periosteal cells were labeled in a solution of 40 μM CM-DiI in HBSS, according to the technique described by Kruyt *et al.*[16] Sytox Green labeling was performed by incubating cells with 100 nM Sytox Green (Molecular Probes, Leiden, The Netherlands) in PBS for 20 min at room temperature. CM-DiI and Sytox Green labeled cells were analyzed by fluorescence microscopy (E800, Nikon, Tokyo, Japan). The viable and dead cells were expressed as a percentage of the total amount of labeled cells.

Cell seeding and culturing on scaffolds

CM-DiI-labeled chondrocytes or periosteal cells were seeded on PEGT/PBT scaffolds using a dynamic cell-seeding protocol: 1×10^6 cells in 40 μL culture medium were applied on top of a pre-wetted and dry-blotted scaffold in an Eppendorf vial. Scaffolds with cells were left for 6 h to allow the cells to attach, then 1 mL of culture medium was added and the constructs were placed on a roller-bar or rotating incubator for 3 days. For the cultured group, after these 3 days the chondrocyte-scaffold constructs were placed into 24-well plates and cultured for 2 weeks in culture medium.

Implantations

Five weeks after the first operation the animals were operated again. All cell-scaffold constructs to be implanted and controls were washed five times for 5 min with a sterile 0.9% NaCl solution at room temperature.

The knee was opened using a medial parapatellar incision. Holes of 4 mm diameter $\times$ 4 mm deep were created in the central, weight-bearing portion of the medial and lateral condyles of both knees using a low-speed drill (150 rpm) and constant cooling. The defects were irrigated and autogenic cell-scaffold constructs were press fit into the defects. Twelve groups were distinguished (Table 1). Implants were distributed in such a manner that duplicate ob-

Table 1. Groups in the Implantation Study

Group	*Cell type*	*Scaffold*	*Serum*	*Seeded or cultured*	*HYA*	*T = 0* (n)	*T = 5* (n)	*Nomenclature*
1a	No cells	CM					4	
1b	No cells	RP					4	
2	Chondrocytes	CM	FBS	S		6	6	CCFS
3	Chondrocytes	CM	AS	S		5	5	CCAS
4	Chondrocytes	CM	FBS	C			5	CCFC
5	Chondrocytes	CM	AS	C			6	CCAC
6	Chondrocytes	CM	AS	C	HYA	5	6	CCAC+H
7	Chondrocytes	RP	FBS	S		3	6	CRFS
8	Chondrocytes	RP	FBS	C			6	CRFC
9	Periosteal cells	CM	FBS	S			4	PCFS
10	Periosteal cells	CM	AS	S		3	5	PCAS
11	Periosteal cells	CM	AS	S	HYA		5	PCAS+H
12	Chondrocytes (dead)	CM	FBS	S			4	

Serum, the serum with which the culture medium was supplemented for 10%; FBS, fetal bovine serum; AS, autogenous serum; CM, compression-molded, salt-leached PEGT/PBT 300 55/45 scaffold; P, PEGT/PBT 300 55/45 scaffold produced by rapid prototyping; seeded (S), cells seeded onto the scaffolds by a dynamic procedure for 3 days prior to implantation; cultured (C), after seeding the scaffolds and cells were kept in culture for 2 weeks before implantation; HYA, 5 mL of 10 mg/mL hyaluronan i.a. immediately postoperatively.

servations were avoided as much as possible and that the implants could not influence each other. Animals in two groups received an injection of 1 mL (10 mg/mL) hyaluronan (HYA) (Ostenil, Chemedica, Munich, Germany) intra-articularly after closure of the wound.

Explantation

Five days after implantation, the animals were euthanized with an overdose of pentobarbital. The cell-scaffold constructs were isolated from the osteochondral defects and cut in two equal parts: one part was prepared for GMA sectioning and histological examination, the other part was stained with Sytox Green as described above, embedded in OCT compound, and immediately frozen. Forty μm cryosections were cut, fixed in acetone for 10 min at -20°C, stained with DAPI (Sigma), mounted with an anti-fading reagent (Molecular Probes, Leiden, The Netherlands), and analyzed using fluorescence microscopy. At a magnification of 400 $\times$, five areas of each section were photographed using three filters: the Texas Red filter (excitation, 540–580 nm; dichroic mirror, 595) for CM-DiI label; the DAPI filter (excitation, 340–380 nm; dichroic mirror, 400 nm); and the FITC filter (excitation 465–495 nm; dichroic mirror, 505) for Sytox Green. The three photographs of each area were combined using Photoshop 6.0 software (Adobe) and analyzed. Analysis was done by two independent observers, who were blinded for the different groups. CM-DiI labeled cells without a nucleus (Fig.1A) or with a green (Sytox Green) nucleus (Fig. 1A) were regarded to be dead, and CM-DiI-labeled cells showing a blue (DAPI) nucleus (Fig. 1A and B) were regarded viable. The data of the two observers were combined and averaged.

Cell viability at implantation

In order to examine cell viability and seeding quality at implantation, control scaffolds with CM-DiI-labeled cells were incubated with Sytox Green at the day of the implantation, fixed, embedded, and analyzed as described above.

Statistics

The one-way ANOVA and the post-hoc Newman Keuls tests were used for statistical analysis. The significance level was $p < 0.05$.

Validation of methodology

The optimal labeling protocol was established by labeling of chondrocytes with 5, 10, 20, or 40 μM CM-DiI in Hank's balanced salt solution (HBSS) (Molecular Probes), according to the technique described by Kruyt *et al.*[16] To validate the Sytox Green staining as a means to assess cell death, vital and devitalized, Sytox Green-labeled cells were counted and compared to similar samples analyzed by the trypan blue exclusion assay.

Transfer of label in vitro *and* in vivo

Rabbit articular chondrocytes were cultured in a 6-well plate and a 0.4 μm filter (transwell PC, Costar) was placed above the cells. Permeability of the filter for

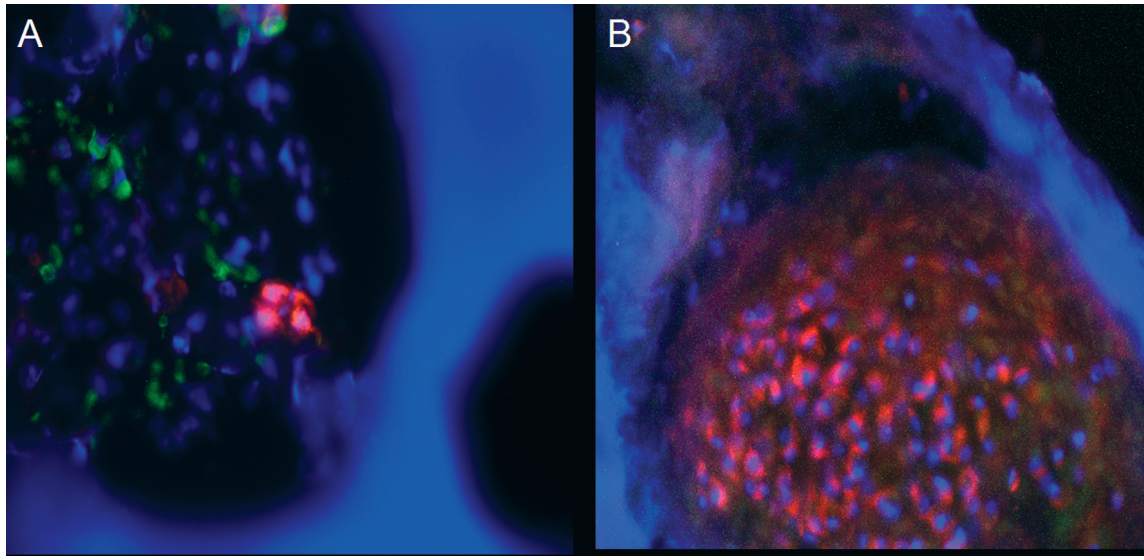

FIG. 1. Fluorescence micrographs of CM-DiI (red) stained chondrocytes with a blue (DAPI) nucleus are considered viable. (**A**) The green nuclei are of nonviable CM-DiI labeled (red cell, right upper corner) and non-CM-DiI labeled cells. CM-DiI labeled cells without blue nuclei are also regarded nonviable. (**B**) A CM-DiI labeled chondrocyte cluster. Notice chondrocytes with and without blue nucleus.

CM-DiI was tested by applying a 40 μM CM-DiI solution on top of the filter. The same model was used to monitor transfer of label from labeled to unlabeled chondrocytes. Vital or devitalized CM-DiI-labeled rabbit articular chondrocytes were placed on top of the filter. After time periods of up to 10 days, the cells at the bottom of the well were examined for transfer of CM-DiI using fluorescence microscopy.

For the *in vivo* experiment, CM-DiI-labeled chondrocytes were seeded on CM scaffolds. Cells in some of the constructs were devitalized by two cycles of freezing and thawing. The constructs were implanted subcutaneously in nude mice. After 5 and 10 days, the mice were sacrificed and the scaffolds were harvested with the surrounding fibrous tissue. To assess *in vivo* label transfer, consecutive sections were examined using light (thionine staining) and fluorescence microscopy, respectively.

Ability of Sytox Green to penetrate tissue

The ability of the Sytox Green dye to penetrate 4 mm diameter $\times$ 4 mm scaffolds in which tissue has been grown was evaluated by incubating 4 mm diameter $\times$ 4 mm devitalized pieces of bovine articular cartilage with the Sytox Green solution according to the protocol described. Frozen sections were examined using fluorescence microscopy.

RESULTS

CM-DiI labeling

For both cell types, the optimal CM-DiI concentration appeared to be 40 μM. The amount of labeled cells increased with the concentration of CM-DiI. At 5 μM CM-DiI, only 2.5% of the cells were labeled. Using a concentration of 40 μM CM-DiI, 68% of the periosteal cells and 43% of the chondrocytes were labeled. At this concentration, the CM-DiI label slightly reduced viability for both cells types; before labeling 91% of the cells were viable, and after the label procedure about 76% of the cells were viable (both cell types). The viability did not decrease further in time during the period of evaluation. At higher concentrations, the label caused a reduction of viability below 50%.

Sytox Green labeling of dead cells

When Sytox Green labeling was compared with trypan blue exclusion to assess the viability of cultured cells, the viability rates were the same (data not shown). The dye appeared to be able to diffuse to the center of 4 $\times$ 4 mm devitalized cartilage biopsies (data not shown). We concluded that Sytox Green stained devitalized cells only and was able to penetrate cartilaginous tissues.

Testing and transfer of the label in vitro

The CM-DiI label freely diffused through the 0.4 μm filters and did label the underlying chondrocytes. When labeled cells were placed on top of the filter, no evidence for transfer of label was found within 6 days. First after 7 days, transfer of label from devitalized chondrocytes to the living chondrocytes was observed. Transfer of label from living chondrocytes to the underlying chondrocytes was first observed after 11 days. The label did not have an effect on the seeding of the cells in the scaffold; cells

were distributed homogenous throughout the scaffold (data not shown).

The fluorescence of both CM-DiI and Sytox Green was not affected by the processing of labeled cells/tissue for cryosectioning. Embedding in GMA did not affect the CM-DiI fluorescence; however the fluorescence of Sytox Green could no longer be detected.

In vivo *label transfer in a nude mouse model*

The implanted chondrocyte clusters could easily be distinguished from infiltrating mouse cells (Fig. 2A and B). No transfer of label was detected during the first 5 days after implantation of either vital or devitalized implants (Fig. 2A). At day 7, labeled mouse cells could be detected next to devitalized implants (Fig. 2B). Transfer of label from vital implants was not detected during the 10 days of this experiment.

Cell viability in PEGT/PBT scaffolds in osteochondral defects

The auto-fluorescence of the scaffold did not substantially affect the evaluation. In devitalized implants, a survival rate of $3.9 \pm 3.1\%$ was found. At $t = 0$ days, the viability of cells in all chondrocyte groups was approximately 90% independent of pre-culturing or scaffold type. The viability of the periosteal cells cultured in media with AS was 79% at the time of implantation.

At explantation ($t = 5$ days), all groups with chondrocytes, with the exception of one (with added hyaluronan), showed no significant decrease in cell viability compared to $t = 0$ (Fig. 3A). However, a significant decrease in cell viability was observed for the periosteal cells: 78.7 ± 8.8 at $t = 0$ and $19.8 \pm 11.9\%$ at $t = 5$ ($p < 0.001$).

When both cell types were expanded in media with AS, seeded on to CM scaffolds, and implanted (Fig. 3B), $95.3 \pm 2.2\%$ of the chondrocytes were viable after 5

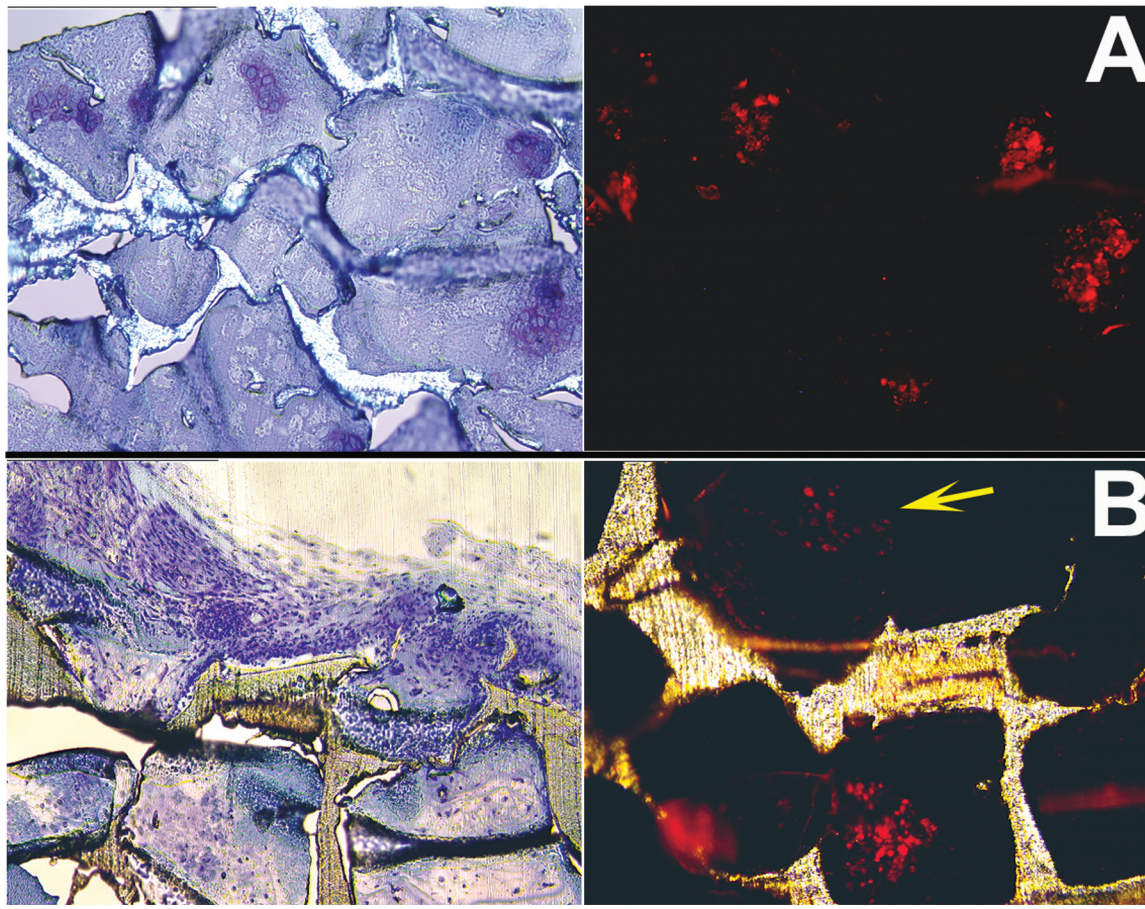

FIG. 2. Consecutive GMA sections of compression molded PEGT/PBT polymer scaffolds seeded with CM-DiI labeled rabbit chondrocytes and implanted subcutaneously in a nude mouse. Notice correspondence between the fluorescent CM-DiI labeled chondrocytes clusters (left) and with the thionine-stained chondrocytes (right). (**A**) Five days postimplantation of viable rabbit chondrocytes, no label transfer was detected. (**B**) Implantation of devitalized rabbit chondrocytes; after seven days some evidence for label transfer was detected..

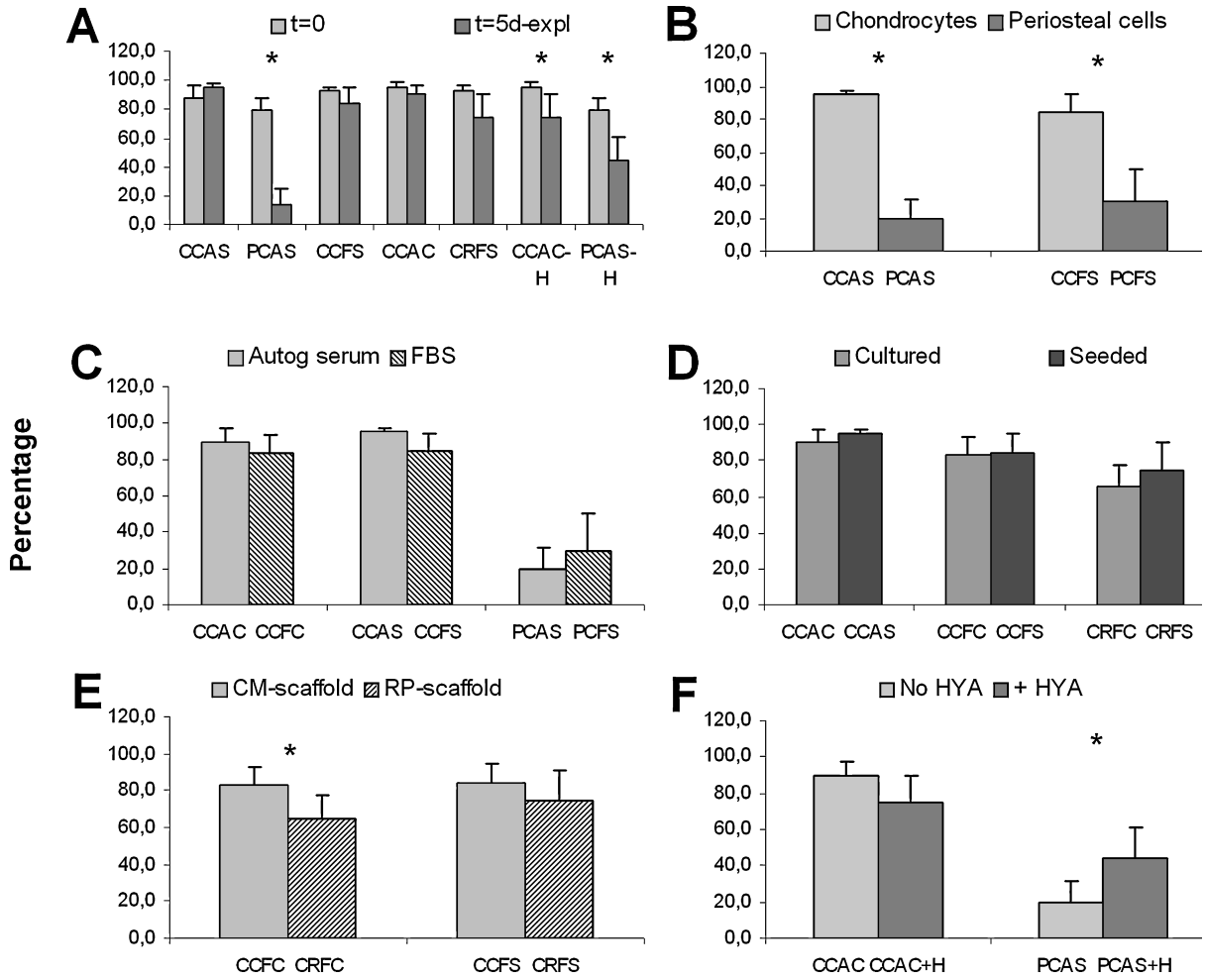

FIG. 3. Viability of chondrocytes and periosteal cells after implantation into osteochondral defects. **(A)** Viability at explantation ($t = 5$ days) compared to that at implantation ($t = 0$). **(B)** Difference in viability between chondrocytes and periosteal cells. **(C)** Effect of FBS or AS supplemented to culture medium. **(D)** Effect of present extracellular matrix. **(E)** Effect of scaffold pore interconnectivity. **(F)** Effect of hyaluronan. Codes for the groups are as follows: First letter indicates cell type: C, chondrocytes; P, periosteal cells. Second letter indicates type of scaffold: C, CM scaffold; R, RP scaffold. Third letter indicates type of serum in culture medium: F, FBS; A, AS. Fourth letter indicates seeded (S) or cultured (C) cells in scaffold. 0, analyzed at implantation (t = 0); H, hyaluronan added (see Table 1). *Significant difference at $p < 0.05$.

days, but only 19.8 ± 11.9% of the periosteal cells were ($p < 0.0001$).

Chondrocytes expanded in medium supplemented with AS showed a slightly, but not significantly, increased viability compared to those expanded in medium containing FBS. In contrast, periosteal cells tend to show increased viability after being expanded in culture medium with FBS compared to those being expanded in culture medium with AS (29.9 ± 19.9 and 19.8 ± 11.9%, respectively, not significant) (Fig. 3C).

There were no differences in survival of chondrocytes between any groups when chondrocytes were implanted after being seeded or implanted after an additional culture period of 2 weeks (Fig. 3D).

The CM scaffolds yielded more viable chondrocytes than did the RP scaffolds: for seeded cells 84.6 ± 10.2% and 74.2 ± 16.4%, not significant; for cultured cells 83.3 ± 10% and 65.1 ± 12%, respectively, not significant (Fig. 3E).

When HYA was administered, the number of periosteal cells surviving the implantation increased (44.2 ± 16.6 compared to 19.8 ± 11.9% ($p < 0.05$) (Fig. 3F). This effect from HYA was not observed for chondrocytes.

Histology of osteochondral defects

In general, the explanted scaffolds contained fibrin and areas with apoptotic cells as can be expected during the

wound healing process (Fig. 4). Scaffolds with seeded chondrocytes from groups that were supplemented with hyaluronan contained more clusters of cells with a cartilage-like appearance compared to those to which no HYA was added (Fig. 4A).

DISCUSSION

In this study the viability of autogenic chondrocytes and periosteum-derived progenitor cells in tissue-engineered cartilage constructs was evaluated following implantation into osteochondral defects in rabbit knees for 5 days. This short-term follow-up was selected to study cell viability upon transfer from cell culture conditions to an osteochondral defect wound bed. Only viable cells can be expected to contribute to tissue repair.

In general, a major fraction, if not all, of autogenic chondrocytes survived the transfer into the osteochondral wound bed. In contrast, only a minor fraction of progenitor cells retained their viability. Our findings indicate that cell survival in tissue-engineered constructs is mainly influenced by cell type. In contrast to periosteal cells, articular chondrocytes originate from a nonvascular tissue dominated by mechanical forces and low oxygen concentrations. Accordingly, chondrocytes may be less affected by deprivation of oxygen and nutrients or the presence of inflammatory mediators, even after being expanded in monolayer culture, than are progenitor cells from periosteum.

Periosteal cells were not stimulated to differentiate into the chondrogenic lineage before implantation. Differentiated periosteal cells may show an improved viability compared to undifferentiated cells. In a recent study, Ball *et al.* demonstrated increased viability of differentiated, allogeneic perichondrium cells upon implantation compared to that of nondifferentiated cells.[35]

Periosteum consists of two layers. The major cell types are fibroblasts from the fibrous layer and progenitor cells from the cambium layer. Next to these, the tissue also contains small amounts of vascular smooth muscle cells and endothelial cells. The cell isolate is therefore heterogeneous. By using culture medium in which L-valine is substituted with D-valine, we attempted to inhibit fibroblast growth. However, our data indicate that we were unable to fully eliminate these cells (unpublished data). We therefore cannot exclude that viability data for the periosteum group reflect in fact the selective survival of a subpopulation of a heterogenous cell isolate. In addition, it remains to be elucidated, whether the low viability of periosteal cells upon implantation reported here also applies for progenitor cells from other origins, such as bone marrow or adipose tissue.

There is little evidence in the literature about the fate of implanted cells in tissue-engineered constructs. To our knowledge, we report here for the first time a detailed analysis on cell viability in the initial osteochondral repair phase. Quintavalla *et al.* studied fluorescently labeled mesenchymal stem cells after implantation in osteochondral defects in goats. Although some of the labeled cells were present 2 weeks postimplantation, cell viability was not assessed.[36] Mierisch *et al.* and Dell'Accio *et al.* studied the contribution of transplanted chondrocytes in a rabbit and goat model, respectively, of autologous chondrocyte transplantation.[37,38] Mierisch *et al.* found the labeled cells were not incorporated in the repair tissue, and therefore the authors doubt whether the implanted cells contribute to the repair process. Dell'Accio and co-workers showed fluorescently labeled, transplanted chondrocytes to be present up to 14 weeks

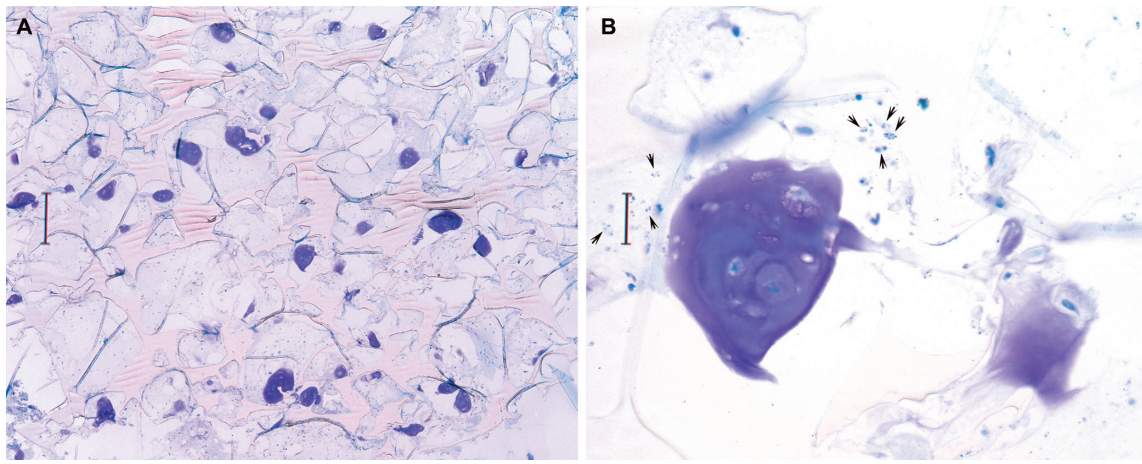

FIG. 4. Photographs of a histological section of an explanted cell-scaffold construct that contained chondrocytes, cultured for 2 weeks within the 3-D CM scaffold. Immediately after implantation, hyaluronan was injected in the knee joint. **(A)** Overview showing cartilaginous "islands" throughout the scaffold. Bar, ~260 μm. **(B)** Detail showing apoptotic cells (arrows). Bar, ~32 μm.

in defects in which the periosteal flap covering the defect was not delaminated. It was also shown that labeled cells contributed to tissue repair, suggesting that these cells are still vital after 14 weeks. The authors showed the absence of transfer of the label to other cells in a nude mouse model for 15 days. However, transfer of the label at 14 weeks cannot be excluded. Neither Mierisch *et al.* nor Dell'Accio *et al.* assessed cell viability specifically and directly.

In the present study, a number of experimental conditions were tested to validate the labeling procedure and establish the period in which transfer of label from one cell to another is unlikely. It was concluded that CM-DiI at a concentration of 40 μM is a suitable label for *in vivo* tracing of chondrocytes and periosteal cells without the risk of transfer of label to endogenous cells for at least 5 days postimplantation. These data are in accordance with earlier data described by Kruyt *et al.*[16] The decrease in cell viability on labeling with CM-DiI from 91 to 76% was comparable to data described by Andrade *et al.* and was proven to be due to manipulation of the samples rather than by a direct effect of the CM-DiI fluorochrome.[25]

CM-DiI derivatives have been previously used to label various cell types, including bone marrow cells, lymphocytes, hepatocytes, and neural tube cells.[25–27,39] In no instance has an altered cell function or dye cytotoxicity been reported. Analyses of the samples at $t = 0$ of labeled and seeded, but not implanted, cells did not reveal any cytotoxic effects either (internal control). Therefore, we state that the label and analysis method employed in this study are valid to establish the short-term survival of cells in implanted cell-scaffold constructs.

Our interest was primarily in cell viability in the short term. Therefore, we did not test the effect of labeling of periosteal cells on their ability to differentiate into the chondrogenic lineage and of chondrocytes to redifferentiate.

Our data did not demonstrate any significant differences in chondrocyte viability between cells cultured in media with FBS and AS. We conclude that FBS does not reduce cell viability via an immunological reaction (as assessed 5 days postimplantation).

In the present study, the amount of extracellular matrix formed by chondrocytes[40] prior to implantation did not significantly influence chondrocyte survival. Surprisingly, we did not find differences in chondrocyte viability after seeding in CM or PR scaffolds, except in the presence of extracellular matrix. Apparently, nutrient supply and diffusion toward cells within scaffolds do not depend on scaffold interconnectivity, but on the effect of extracellular matrix in fully interconnected scaffolds.

HYA increased the number of periosteal cells surviving the implantation. Periosteal cells are known to display MSC characteristics[41,42] and to express the receptor CD44 for HYA.[43] HYA may protect periosteal cells from cell death through its CD44 receptor.[21,44] Therefore, the addition of HYA, in combination with the induction of chondrogenic differentiation of progenitor cells prior to implantation, may increase cell viability for bone and cartilage tissue-engineering purposes and requires further research. An increase of chondrocyte viability after administration of HYA was not observed. This was probably due to the high percentage of chondrocytes already surviving the implantation without the addition of HYA, thus masking a potential chondro-protective effect of HYA.

In conclusion, upon implantation in osteochondral defects autogenic chondrocytes survived transfer from culture medium into an osteochondral wound bed, whereas most periosteal cells did not. This study indicates the importance of considering initial cell viability upon implantation of a tissue-engineered construct.

ACKNOWLEDGMENTS

This study was supported by a grant from Senter (BTS 00021). The help of May Bost, Petra Dijkstra, and Monique de Jong from the Central Animal Facility of Maastricht University is gratefully acknowledged.

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