Histol Histopathol (2011) 26: 1265-1278

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

OA cartilage derived chondrocytes encapsulated in poly(ethylene glycol) diacrylate (PEGDA) for the evaluation of cartilage restoration and apoptosis in an *in vitro* model

Giuseppe Musumeci[1], Carla Loreto[1], Maria Luisa Carnazza[1], Iossif Strehin[2] and Jennifer Elisseeff[2]

[1]Department of Bio-Medical Sciences, University of Catania, Italy and [2]Department of Biomedical Engineering, Johns Hopkins University, Baltimore, MD, USA

Summary. Osteoarthritis (OA) is characterized by cartilage attrition, subchondral bone remodeling, osteophyte formation and synovial inflammation. Perturbed homeostasis caused by inflammation, oxidative stress, mitochondrial dysfunction and proapoptotic/antiapoptotic dysregulation is known to impair chondrocyte survival in joint microenvironments and contribute to OA pathogenesis. However, the molecular mechanisms underlying the programmed cell death (apoptosis) of chondral cells are not yet well defined. The present study was conducted to evaluate apoptosis of chondrocytes from knee articular cartilage of patients with OA. The aim of this study was to investigate and compare the apoptosis through the expression of caspase-3 in tissue explants, in cells cultured in monolayer, and in cells encapsulated in a hydrogel (PEGDA) scaffold.

Chondrocytes were also studied following cell isolation and encapsulation in poly(ethylene glycol) diacrylate (PEGDA) hydrogels. Specifically, articular cartilage specimens were assessed by histology (Hematoxlyn and Eosin) and histochemistry (Safranin-O and Alcian Blue). The effector of apoptosis caspase-3 was studied through immunohistochemistry, immunocytochemistry and immunofluorescence. DNA strand breaks were evaluated in freshly isolated chondrocytes from human OA cartilage using the TUNEL assay, and changes in nuclear morphology of apoptotic cells were detected by staining with Hoechst 33258. The results showed an increased expression of caspase-3 in tissue explants, in pre-confluent cells and after four passages in culture, and a decreased expression of caspase-3 comparable to control cartilage in cells encapsulated in hydrogels (PEGDA) after 5 weeks in culture.

The freshly isolated chondrocytes were TUNEL positive. The chondrocytes after 5 weeks of culture in hydrogels (PEGDA) showed the formation of new hyaline cartilage with increased cell growth, cellular aggregations and extracellular matrix (ECM) production. This is of particular relevance to the use of OA cells and tissue engineering in the therapeutic approach to patients.

Key words: Hydrogel, Immunohistochemistry, TUNEL, Apoptosis, Osteoarthritis

Offprint requests to: Dr. G. Musumeci Ph.D Department of Bio-Medical Sciences, University of Catania, Via S. Sofia 87, 95123 Catania, Italy. e-mail: g.musumeci@unict.it

Introduction

Osteoarthritis (OA) is classically defined as a progressive degenerative rather than an inflammatory disease, and is characterized by deterioration of joints, including loss of articular cartilage and subchondral bone, as well as osteophyte formation (Lorenz and Richter, 2006; Pritzker et al., 2006). OA affects about 8 million people in the United Kingdom and nearly 27 million people in the United States. It is one of the most common joint diseases and is associated with a substantial negative impact on the patient's quality of life as well as on healthcare costs (Scott et al., 1999; Mapel et al., 2004; Rosemann et al., 2007). It also influences disability in the middle-age and older populations, especially in developing countries.

It is known that OA is the result of mechanical and biological processes that modify cartilage homeostasis

(Hashimoto et al., 2008). Under normal physiological conditions chondrocytes maintain equilibrium between the synthesis and degradation of ECM components, thus regulating the structural and functional integrity of cartilage (Pooler, 2001; Goldring and Goldring 2004; Aigner et al., 2006; Goldring, 2006). Cell death with features of apoptosis has been detected in OA cartilage, as well as in animal models of OA. This was associated with matrix degradation and calcification, which suggests a role of cell death/survival in OA pathogenesis. Inducers and mechanisms which lead to chondrocyte death in OA cartilage have not been conclusively identified (Kuhn et al., 2004).

Apoptosis represents a physiological form of cell death that results in removal of apoptotic bodies by phagocytic cells. The role of apoptosis is to remove harmful, damaged or unwanted cells without inducing an inflammatory response and without the release of cell contents as observed during necrotic cell death (Majno and Joris, 1995; White, 1996; Alenzi, 2005). Cell loss in aged or osteoarthritic cartilage was initially thought to occur by necrosis (Roy and Meachim, 1968). However, more recent reports suggest that cell death occurs primarily via apoptosis or apoptosis-like programmed cell death (Kim et al., 2000; D'lima et al., 2001; Rosemann et al., 2007; Loreto et al., 2009). In situ electron microscopy studies of normal and osteoarthritic human cartilage have demonstrated that apoptosis has also been positively correlated with the severity of cartilage destruction and matrix depletion in human specimens (Kim et al., 2000; Alenzi, 2005).

The principle obstacle to clinical transplantation today is the shortage of donor organs. For this reason, tissue engineering and regenerative medicine have become promising and important fields of research, which may offer new sources of tissues and organs for transplantation. The major goal of tissue engineering is to replace, repair or enhance the biological function of damaged tissue or organs. The repair of cartilage becomes of clinical interest when three dimensional cell and tissue reconstructive procedures are correctly matched with the appropriate supporting biomimetic materials.

Poly(ethylene glycol) (PEG) based hydrogels have attracted broad interest as a scaffold material for tissue engineering applications (Peppas, 1987; Jeon et al., 1991; Hubbell, 1995). Hydrogels encompass a class of scaffolds with many attractive features for cartilage tissue engineering (Elisseeff et al., 2000; Mauck et al., 2000; Masuda et al., 2003). They are made from water-soluble polymer chains that are cross-linked to form water-insoluble gel networks. Cells in the polymer solution can be encapsulated in the hydrogel during the cross-linking process. The resulting gel network can be designed to have high water content, enabling efficient transport of nutrient and waste products to and from cells. Polymer cross-linking and hydrogel formation can be achieved by various physical and chemical mechanisms (Gutowska et al., 2001). In the process of photopolymerization, a light-sensitive chemical species initiates covalent cross-linking of the polymer chains to form a hydrogel.

The present study was conducted to evaluate apoptosis of chondrocytes from knee articular cartilage of patients with OA. In our recent publication we investigated apoptosis intissue explants where caspase-3 was over-expressed (Musumeci et al., 2011). After this cited study, we questioned whether apoptosis decreased or increased in the cell monolayer and in cells encapsulated in a hydrogel (PEGDA) scaffold.

The aim of this study was to investigate and compare apoptosis through the expression of caspase-3 in tissue explanted in monolayer cells and in cells encapsulated in the hydrogel PEGDA scaffold.

Activation of the apoptotic process was investigated via immunohistochemical detection of caspase-3 "in vivo" on OA cartilage specimens and via immuno-cytochemical detection of caspase-3 "in vitro", followed by DNA strand breaks evaluation [using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL) method] and the study of the nucleic morphology of apoptotic cells, detected by staining with the DNA-binding fluorochrome Hoechst 33258, on freshly isolated OA condrocytes. Caspase-3 immunoexpression in chondrocytes encapsulated in PEGDA hydrogels was also evaluated using immunohistochemistry following 2, 3 and 5 weeks of culture.

The neocartilage synthesized by the PEG-DA encapsulated chondrocytes was studied through histology, as matrix production can be observed using Hematoxylin and Eosin staining, while more specific chemical stains such as Safranin-O and Alcian Blue were used to assess synthesis of ECM molecules, such as glycosaminoglycan (GAG). Immunofluorescence was used to detect and quantify Type I (fibrocartilage) and Type II (hyaline cartilage) collagens.

Materials and methods

Patients

Biopsies of articular cartilage were derived from human donors. Human articular knee cartilage specimens were obtained from 22 patients, 15 males and 7 females with end-stage OA requiring total joint arthroplasty; the mean age of the OA patients was 58 years (range 28-76 years) with a Mankin score of 3 to 7 (van der Sluijs et al., 1992) and the mean disease duration was 10 years. The patients were not smokers, occasionally taking NSAIDs (nonsteroidal anti-inflammatory drugs) in addition to the classical treatment of knee OA composed of hot pack, therapeutic ultrasound and terminal isometric exercises. The females did not take estrogen replacement therapy (ERT). The diagnosis was based on clinical and radiological criteria. Control samples were obtained from five patients, 3 males and 2 females, without any history of any form of

arthritis either at the time or for 5 years following an arthroscopy for unexplained knee pain performed at a sports medicine facility. The mean age of these patients was 51 years (range 46-57 years). These donors are referred to as normal donors (ND), (van der Sluijs et al.,1992).

Isolation of chondrocytes and culture conditions

Our lab usually works with human and bovine cells, which we isolate and expand. We have found that it is best to use primary or passage zero chondrocytes when possible. To isolate the chondrocytes from OA and non OA articular cartilage, the cartilage pieces were incubated in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Grand Island, NY, USA) containing 0.2% collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 5% fetal bovine serum (GIBCO) for 14-16 h at 37°C and 5% CO_2. The resulting cell suspension was then filtered through 70 μm nylon filters (Cell Strainer; Falcon, Franklin Lakes, NJ, USA) and washed three times with phosphate buffered saline (PBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin. The number and size distribution of the isolated cells were then determined with a Z2 Coulter Counter and Size Analyzer (Beckman Coulter, Inc., Palo Alto, CA, USA). After isolation, the chondrocytes were plated onto separate 10 cm tissue culture dishes at a density of 10.000 cells/cm^2. Cells were incubated at 37°C and 5% CO_2 in chondrocyte medium composed of DMEM containing 10% fetal bovine serum, 0.4 mM proline, 50 μg/ml ascorbic acid, 10 mM HEPES, 0.1 mM non-essential amino acid, and 100 U/ml penicillin and 100 μg/ml streptomycin. The culture medium was changed twice weekly. The cells were observed with an Axioplan Zeiss light microscope (Germany) and photographed with a digital camera (Canon, Japan).

Immunocytochemistry

Immunostaining for caspase-3 was performed on control and OA cell cultures in pre-confluent cells and after four passages in monolayer. Cells were fixed with 4% phosphate-buffered paraformaldehyde for 20 min at room temperature; quenching was performed with a solution of 2% hydrogen peroxide (H_2O_2) and 10% methanol in phosphate-buffer saline (PBS), for 1 min. To permeabilize the cells, fresh 0.3% Triton X-100 in PBS was used for 5 minutes. The cells were then treated with 5% bovine serum albumin (BSA) in PBS for 1 hour at room temperature. BSA is a blocking agent used to prevent non-specific binding of the antibody. After blocking, the cells were incubated overnight with a primary mouse monoclonal anti-caspase-3 (1:200 dilution, ABcam plc, MA, UK) antibody. A fluoresceine isothiocyanate (FITC) labeled anti-mouse antibody (1:100, Santa Cruz Biotechnology) was used as a secondary antibody. Coverslips containing the cells were washed and mounted in PBS/glycerol (50:50) and placed on glass microscope slides. In all instances negative controls without primary antibody were performed. Coverslips were analyzed using a Nikon Eclipse TE200 fluorescence microscope and photographed with a digital camera (Canon, Japan) and positive cells were counted over the entire coverslip. Staining intensity was assigned a semi quantitative score 0 = no reactivity, 1 = weak reactivity, 2 = moderate reactivity, and 3 = strong reactivity (Loreto et al., 2009). Four independent observers (two anatomical morphologists and two histologists) assessed the immunocytochemical reactions, histological findings and histochemical results; in the few cases where their evaluations diverged, an agreement was reached after discussion.

In situ detection of apoptotic cells (TUNEL)

Once primary chondrocytes isolated from OA and non OA cartilage reached 80-90% confluency, apoptosis was demonstrated by DNA fragmentation and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL, in situ cell death detection kit, fluoroscein; Roche Applied Science, Mannheim, Germany). The same assay was used to quantify apoptosis of articular chondrocytes in cartilage explant cultures according to the manufacturer's protocol.

Positive controls were incubated for 10 minutes at room temperature with 1500 U/ml DNAse1 in 50 mM Tris pH 7.5, 10 mM $MgCl_2$, and 1 mg/ml BSA. Negative controls were incubated with label solution only (without terminal transferase). Apoptotic cells were counted under a fluorescence microscope (Nikon Eclipse TE200). Photographs were taken with a digital camera (Canon, Japan).

Hoechst 33258 staining

Changes in nuclear morphology were detected by staining with the DNA-binding fluorochrome Hoechst 33258 (Polysciences, Inc. Warrington, PA, USA).

Cell Encapsulation in PEGDA hydrogels

Cells were encapsulated in PEGDA hydrogels and cultured for 2, 3 and 5 weeks as described previously (Terraciano et al., 2007), (Figs. 1E,F). Briefly, chondrocytes (passage 3 or 4) were suspended in 10% (w/v) PEGDA (SunBio, Orinda, CA, USA) solution in sterile PBS with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA). The photoinitiator Irgacure 2959 (Ciba Specialty Chemicals, Tarrytown, NY, USA) was added at 0.05% (w/v) final concentration. The photoinitiation and encapsulation processes have been previously determined to be biocompatible. Chondrocytes were resuspended in 95 μl of polymer solution at a concentration of 20x10^6 cells per milliliter. The cell-polymer solution was added to a cylindrical silicone-tubing mold with an internal

diameter of 4.75 mm and a construct height of 5 mm. The tubing was attached to a glass microscope slide using silicon lubricant. Prepolymer (macromer) was exposed to UV light (365 nm) for 5 minutes to induce gelation. Constructs were removed from the molds and incubated at 37°C, and 5% CO_2 in chondrocyte medium. The culture medium was changed twice weekly. The scaffolds were photographed with a digital camera (Canon, Japan).

Histology

Cells in monolayer

After four passages the cells were harvested for histological studies. They were washed in PBS, fixed for 30 min in 2% paraformaldehyde at 4°C, rewashed in PBS and stained with Hematoxylin for 6 min, then washed in H_2O and counterstained with Eosin for 1 min. Coverslips containing the cells were washed and mounted in PBS/glycerol (50:50) and placed on glass microscope slides.

Cells in PEGDA hydrogel

After 2, 3 and 5 weeks of culture, constructs were harvested for histological studies. The hydrogels were fixed overnight in 2% paraformaldehyde at 4°C,then washed in PBS, specimens were dehydrated in graded ethanol solutions, cleared in xylene and paraffin-embedded, according to standard histological technique. Sections 4-5 μm in thickness were mounted on sialane-coated slides and stored at room temperature. Slides were dewaxed in xylene, hydrated using graded ethanol solutions and H_2O, and stained for routine histologic and morphological evaluation by Hematoxylin and Eosin (H&E). The sections were observed with an Axioplan Zeiss light microscope (Germany) and photographed with a digital camera (Canon, Japan).

Immunohistochemistry

For immunohistochemical analysis, sections were incubated for 30 min in 0.3% H_2O_2/methanol to quench endogenous peroxidase activity, and rinsed for 20 min with phosphate-buffered saline (PBS; Bio-Optica, Italy). High-temperature antigen unmasking was conducted in a microwave oven. A monoclonal rabbit antibody at 1:100 dilution was used for caspase-3 localization (Cell Signaling Technology, Inc., MA, USA). After an overnight incubation in a humidified chamber at 4°C, sections were incubated with the secondary antibody; detection was performed with the Streptavidin-biotin method using 3,3'-diaminobenzidine (DAB, Dako, Denmark) as the chromogen (LSAB 2 System-HRP, Dako, Denmark). Sections were counterstained with hematoxylin, observed with an Axioplan Zeiss light microscope (Germany) and photographed with a digital camera (Canon, Japan). Positive controls consisted of tissue specimens with known antigenic positivity. Negative controls were incubated without the primary antibody. Staining intensity was assigned a semi quantitative score: 0 = no reactivity, 1 = weak reactivity, 2 = moderate reactivity, and 3 = strong reactivity (Loreto et al., 2009). Four independent observers (two anatomical morphologists and two histologists) assessed the immunohistochemical reactions, histological findings and histochemical results; in the few cases where their evaluations diverged, an agreement was reached after discussion.

Histochemistry

After 2, 3 and 5 weeks of culture, constructs were harvested for histochemistry studies. The hydrogels were fixed overnight in 2% paraformaldehyde at 4°C. After an overnight washed in PBS, specimens were dehydrated in graded ethanol solutions, cleared in xylene and paraffin-embedded, according to standard histological technique. Sections 4-5 μm in thickness were obtained and mounted on sialane-coated slides and stored at room temperature. Slides were dewaxed in xylene, hydrated using graded ethanol solutions and H_2O, and stained for histochemistry by Safranin-O and by Alcian Blue (Bio-Optica, Italy) for GAG detection. The sections were observed with an Axioplan Zeiss light microscope (Germany) and photographed with a digital camera (Canon, Japan).

Immunofluorescence

For immunohistochemical analysis, sections were incubated for 30 min in 0.3% H_2O_2/methanol to quench endogenous peroxidase activity then rinsed for 20 min with phosphate-buffered saline (PBS; Bio-Optica, Italy). High-temperature antigen unmasking was conducted in a microwave oven. A mouse polyclonal anti-collagen Type II (Research Diagnostics Inc., Flanders, NJ, USA) antibody was used at 1:100 working dilution, and a rabbit anti-mouse collagen Type I (Research Diagnostics Inc., Flanders, NJ, USA) antibody was used at 1:50 working dilution. After overnight incubation in a humidified chamber at 4°C, sections were incubated with the Alexa Fluor-conjugated secondary antibody (Cell Signaling Technology, Inc., MA, USA), for one hour at room temperature, mounted in PBS/glycerol (50:50) and observed with a Nikon Eclipse TE200 fluorescence microscope and photographed with a digital camera (Canon, Japan). Positive controls consisted of cartilage from lung or fetus. Negative controls were incubated without the primary antibody. Staining intensity was assigned a semi quantitative score: 0 = no reactivity, 1 = weak reactivity, 2 = moderate reactivity, and 3 = strong reactivity (Loreto et al., 2009). Four independent observers (two anatomical morphologists and two histologists) assessed the immunohistochemical reactions, histological findings and histochemical results; in the few cases where their evaluations

diverged, an agreement was reached after discussion.

Statistical analysis

Each experiment was repeated 3 times for each of the 22 patients with OA and for each of the 5 control patients. Data analysis was performed using the SPSS-PC software (SPSS Inc., USA). Data was tested for normality with the Kolmogorov-Smirnov test. All variables were normally distributed. Comparisons between two means were tested with the Student's t test. A value of $P<0.05$ was considered statistically significant. Ten fields from randomly selected slides were observed under a fluorescence and a light microscope. Each field was photographed with a digital camera. On each photomicrograph three observers unaware as to the type of sample, identified and counted the number of total cells, as well as the number of these cells exhibiting a positive reaction. The proportion of positive cells was calculated for each photomicrograph and a mean value was obtained for each sample. The results were expressed as a percentage.

Cohen's kappa was applied to measure the agreement between the three observers and averaged over all three to evaluate overall agreement using the following grading: 0-0.2 (slight), 0.21-0.40 (fair), 0.41-0.60 (moderate), 0.61-0.80 (substantial), and 0.81-1.0 (almost perfect).

Results

Isolation of chondrocytes and culture conditions

The differences in morphology between freshly isolated OA chondrocytes and normal chondrocytes cultured from two days to one-week was monitored by light microscopy. The chondrocytes isolated from OA cartilage showed a fibroblastic-like morphology (Fig. 1A,B) while the chondrocytes from control cartilage showed normal chondrocyte morphology (Fig. 1C,D).

Immunocytochemistry

Immunocytochemistry results through fluorescence microscopy showed an activation of apoptosis detected by labeling caspase-3 in pre-confluent cells and in cells after the fourth passage from OA cartilage and control cartilage. Strong (Grade 3) caspase-3 immunoreaction was observed in injured cartilage, in pre-confluent cells, suggesting the activation of programmed cell death (Fig. 2A). The controls (normal cells) exhibited weak (Grade 1) caspase-3 immunoreaction in pre-confluent cells, with only a few cells fluorescing (Fig. 2B). Strong (Grade 3) caspase-3 immunoreaction was observed in injured cartilage in cells after the fourth passage, suggesting the activation of programmed cell death (Fig. 3A). The controls (normal cells) exhibited weak (Grade 1) caspase-3 immunoreaction in cells after the fourth passage, with only a few cells fluorescing (Fig. 3B). No immunoreaction was observed in the negative control treated with 1% BSA without the primary antibodies (Figs. 2C, 3C). The percentage of caspase-3-positive cells was significantly greater ($P<0.05$) in OA compared with control chondrocytes. Interobserver agreement, measured using the Kappa coefficient, was 0.82 (almost perfect).

In situ detection of apoptotic cells (TUNEL)

Marked differences between OA and normal chondrocytes were observed following analysis of freshly isolated cells by fluorescence microscopy (Nikon Eclipse TE200). Chondrocytes from OA cells showed a large proportion of apoptotic TUNEL-positive cells (Fig. 4A) while chondrocytes from normal donors did not show any cytoplasmic signs of apoptotic cell death (Fig. 4B). The OA chondrocytes also exhibited morphological evidence of apoptosis, clear cytoplasmic and cell surface blebs, altered nuclear shape, apoptotic bodies and a parallel loss of nuclear volume. Some apoptotic nuclei showed no evidence of chromatin condensation, indicating that bleb formation is an early event during apoptosis. The percentage of TUNEL-positive cells was significantly greater ($P<0.05$) in OA compared with control chondrocytes. Interobserver agreement, measured using the Kappa coefficient, was 0.88 (almost perfect).

Hoechst 33258 staining

Normal cell nuclei had a homogeneous pattern of staining with Hoechst 33258 (Fig. 4C). In contrast, nuclei of OA cells were smaller and brighter than normal cells, and nuclear fragmentation and condensation were evident (Fig. 4D,E). These findings suggest that the OA chondrocytes demonstrated changes consistent with apoptosis.

Immunohistochemistry

Light microscopy observation showed different patterns of immunopositive cells in the two sets of specimens. Activation of apoptosis was investigated via immunohistochemistry by detection of caspase-3, in tissue explants "in vivo", and in chondrocytes encapsulated in hydrogels after 2, 3 and 5 weeks of culture. Strong (Grade 4) caspase-3 immunoreaction was shown in injured cartilage, suggesting the activation of programmed cell death "in vivo" OA chondrocytes (Fig 5A). Moderate/weak (Grade 2) caspase-3 immunoreaction was seen in OA chondrocytes encapsulated in PEGDA hydrogels after 2 and 3 weeks of culture, suggesting the activation of programmed cell death (Fig. 6 A,D). Weak (Grade 1) caspase-3 immunoreaction was observed in OA chondrocytes encapsulated in PEGDA hydrogels after 5 weeks of culture, suggesting the inhibition of programmed cell death (Fig. 6G). The control group exhibited weak (grade 1) caspase-3

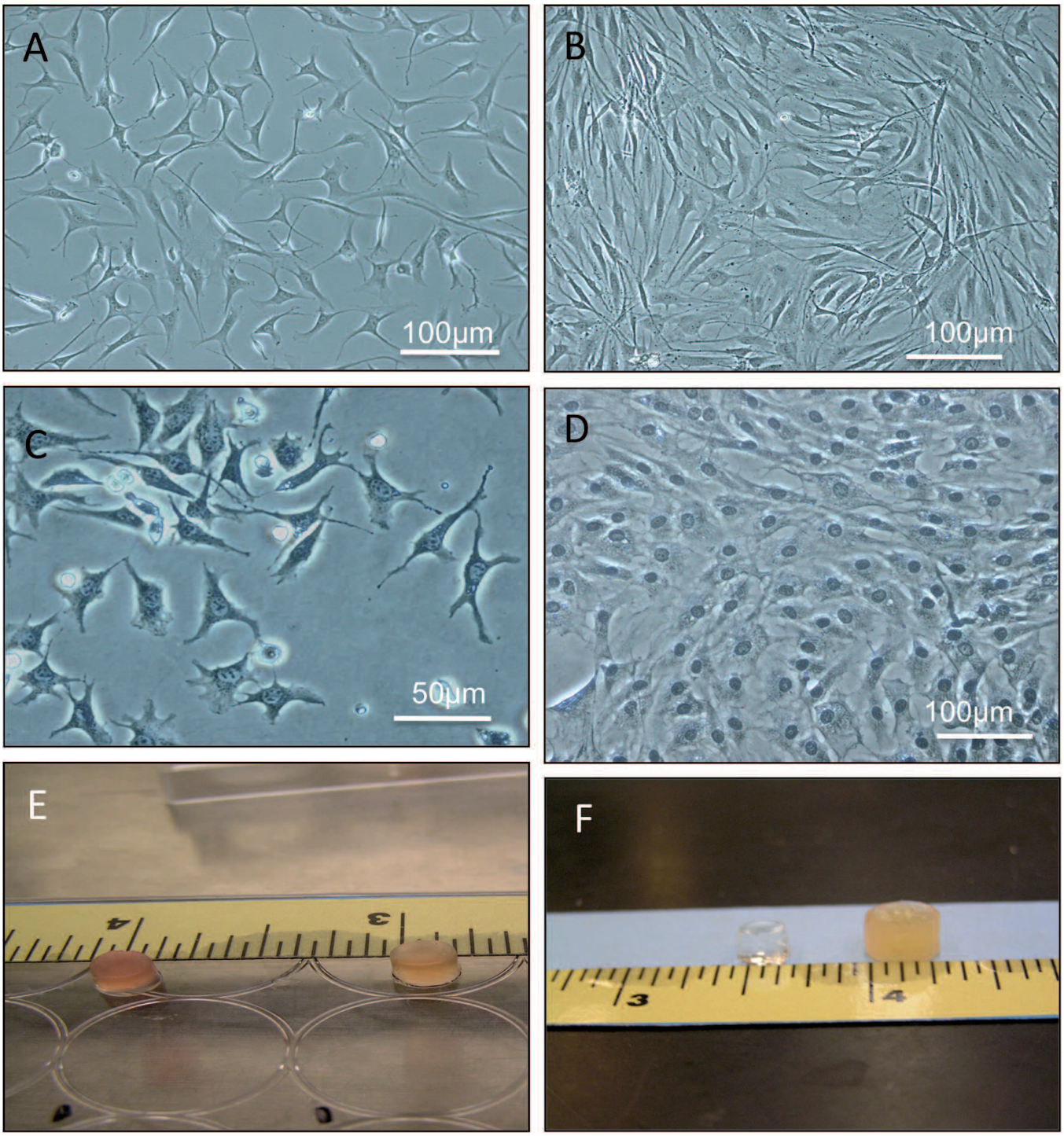


Fig. 1. A. OA chondrocytes after 2 days of culture with fibroblast-like morphology. **B**. OA chondrocytes after 1 week of culture with fibroblastic-like morphology. **C.** Normal chondrocytes after 2 days of culture with typical chondrocyte morphology. **D.** Normal chondrocytes after 1 week of culture with typical chondrocyte morphology. **E.** PEGDA scaffold after 3 weeks of cell culture. **F.** PEGDA scaffold after 5 weeks of cell culture. Notice the difference in size between the hydrogel scaffold at day one of encapsulation and after 5 weeks. The size doubled over the 5 weeks period. This suggests that there is cellular growth in the hydrogel. Scale bars: A, B, D, 100 μm; C, 50 μm.

immunoreaction (Fig. 5B). No immunoreaction was observed in the negative control treated with PBS without the primary antibodies (Fig. 5C). The percentage of caspase-3-positive cells was significantly greater ($P<0.05$) in OA compared with control chondrocytes in tissue explants ("in vivo"). The percentage of caspase-3-positive cells was significantly greater ($P<0.05$) in OA compared with control chondrocytes after 2 and 3 weeks encapsulated in PEGDA hydrogels but was not significantly greater ($P<0.05$) after 5 weeks of encapsulation in PEGDA hydrogels. Interobserver agreement, measured using the Kappa coefficient, was 0.84 (almost perfect).

Histology

Cells in monolayer

The H&E staining was used to determine cell vitality in chondrocytes after four passages of culture from the control group (Fig. 3 D) and from OA cartilage (Fig. 3E). The chondrocytes from the control cartilage did not show any signs of cellular suffering, demonstrated by intense staining, while the chondrocytes from OA cartilage showed clear signs of cellular suffering, demonstrated by a slight staining.

Cells in PEGDA hydrogel

Results from histology using hematoxylin and eosin staining demonstrated the absence of structural alterations, comparable to control cartilage, in the OA chondrocytes encapsulated in PEGDA hydrogels after 2, 3 and 5-weeks of culture (Fig. 6B,E,H). The chondrocytes after 5 weeks of culture showed an increase in proliferation, cellular aggregations in nests (typical structures of the hyaline cartilage) and an increase in ECM production (Fig. 6H).

Histochemistry

Specific chemical stains, such as Safranin-O and Alcian Blue, were used to assess synthesis of GAG containing proteoglycans. Histochemistry results demonstrated a fair reduction in proteoglycans synthesis by OA chondrocytes encapsulated in the scaffolds following 5 weeks of culture, when compared to control chondrocytes (Fig. 7A-D).

Immunofluorescence

Immunofluorescence results showed a clear distinction between the synthesis of Types I and Type II collagen, indicative of fibrocartilage and hyaline cartilage formation respectively. The control and OA groups both exhibited moderate (Grade 2) collagen Type I and strong (Grade 3) collagen Type II immunoreaction (Fig 8A,B,D,E). No immunoreaction was observed in the negative control treated with PBS without the primary antibodies. The percentage of Collagen Type I-

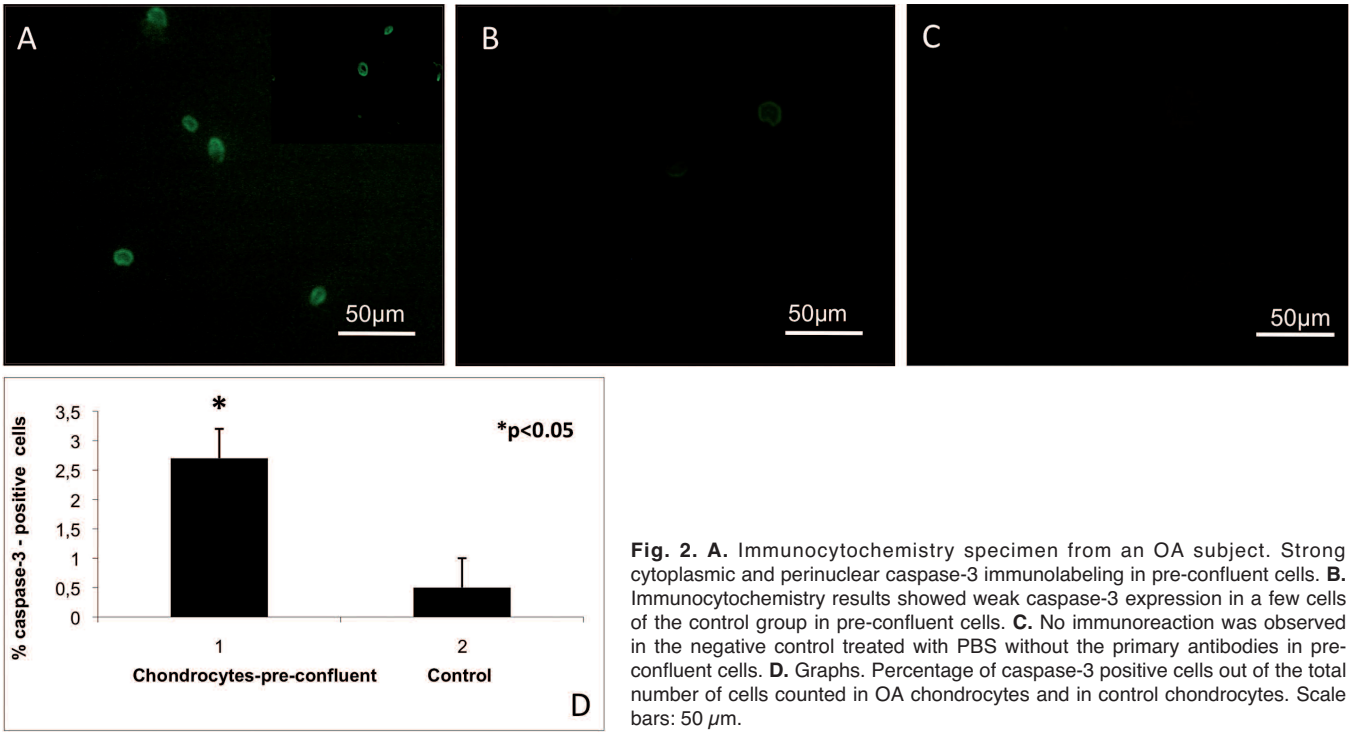


Fig. 2. A. Immunocytochemistry specimen from an OA subject. Strong cytoplasmic and perinuclear caspase-3 immunolabeling in pre-confluent cells. **B.** Immunocytochemistry results showed weak caspase-3 expression in a few cells of the control group in pre-confluent cells. **C.** No immunoreaction was observed in the negative control treated with PBS without the primary antibodies in pre-confluent cells. **D.** Graphs. Percentage of caspase-3 positive cells out of the total number of cells counted in OA chondrocytes and in control chondrocytes. Scale bars: 50 μm.

positive cells and Collagen Type II-positive cells was not significantly greater ($P<0.05$) in OA chondrocytes after 5 weeks encapsulated in PEGDA hydrogels compared with control chondrocytes. Interobserver agreement, measured with the Kappa coefficient, was 0.86 (almost perfect).

Discussion

Articular cartilage is a remarkably complex tissue, one that is difficult to repair or re-engineer. Due to its poor capacity for repair, injuries to cartilage tissue are irreversible unless something is done to fix it. Many studies have examined the occurrence of apoptosis in OA tissue and its implications for articular surface degeneration in both humans and experimental models (Hough and Webber, 1990; Hashimoto et al., 1999; Kim et al., 2000). In this study we have evaluated the apoptosis process of chondrocytes from knee articular cartilage, from patients with OA, both "in vivo", on paraffin sections and "in vitro" after isolation of these osteoarthritic chondrocytes. This evaluation was also demonstrated with chondrocytes after their encapsulation in a PEGDA hydrogel.

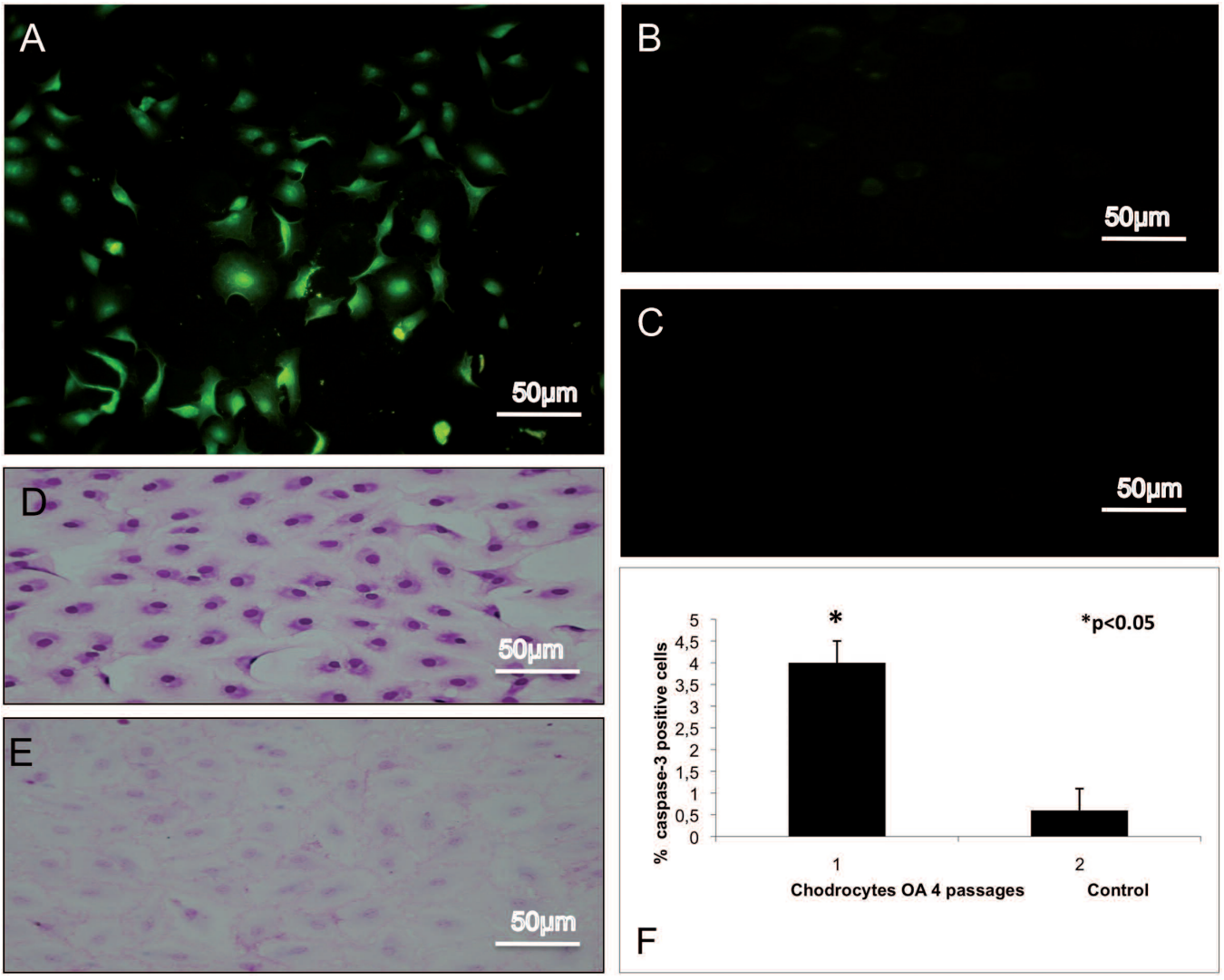


Fig. 3. A. Immunocytochemistry specimen from OA subject. Strong cytoplasmic and perinuclear caspase-3 immunolabeling in chondrocytes at fourth passage of culture. **B.** Immunocytochemistry results showed weak caspase-3 expression in a few cells of the control group in chondrocytes at fourth passage of culture. **C.** No immunoreaction was observed in the negative control treated with PBS without the primary antibodies in cells after four passages of culture. **D.** H&E staining in control chondrocytes at fourth passage of culture. **E.** H&E staining in OA chondrocytes at fourth passage of culture. **F.** Graphs. Percentage of caspase-3 positive cells out of the total number of cells counted in OA chondrocytes and in control chondrocytes. Scale bars: 50 μm.

The field of tissue engineering focuses on the restoration, maintenance, or improvement of tissue function through the development of biological substitutes (Vacanti, 1988; Langer and Vacanti, 1993; Vacanti, 2006). The physical and biological properties of the hydrogel scaffold play an important role in the development of engineering tissues. The field focuses on the use of three components: the scaffold, which is composed of different biomaterials and varying mechanics, the cell type, which can vary from embryonic stem cells to adult cells, and soluble signaling molecules that stimulate cells to proliferate, differentiate and synthesize extracellular matrix. An ideal scaffold has to be both mechanically functional in a specific environment and interact with cells to efficiently induce new tissue development, characterized by an ECM that closely resembles native tissue (Lutolf and Hubbell, 2005). Scaffolds are thus designed to perform a number of biological functions, including stimulation of cells to synthesize ECM, proliferate, migrate, prevent apoptosis and/or differentiate. They can be composed of natural or synthetic materials or a combination of both. Scaffolds composed of biological polymers (natural scaffolds) can communicate or interact with cells to induce desired biological responses. Characterizing the extracellular matrix produced by cells in the scaffolds is critical for evaluating the success of a tissue engineering system.

The present study was conducted to evaluate

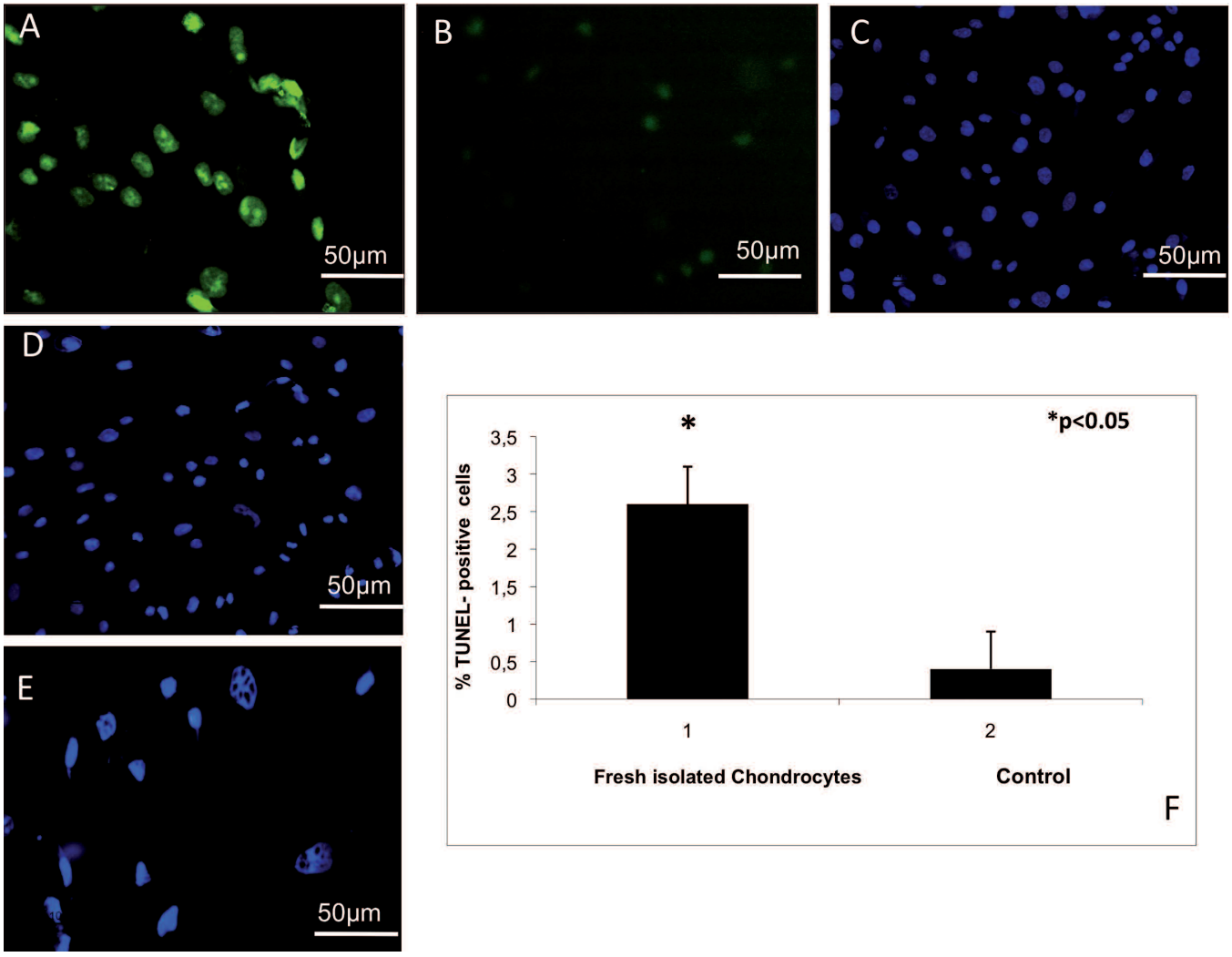


Fig. 4. A. TUNEL-positive chondrocytes from a subject with OA cartilage. In OA chondrocytes there is an increase in nuclear density, a change in nuclear shape and alterations in chromatin with cytoplasmic blebs. Some apoptotic nuclei showed no evidence of chromatin condensation, indicating that bleb formation is an early event during apoptosis. **B.** TUNEL-positive chondrocytes from a subject with normal cartilage. **C.** Hoechst 33258 staining of freshly isolated chondrocytes from control subject. **D, E.** Hoechst 33258 staining of freshly isolated chondrocytes from OA subject have nuclear morphology changes indicative of apoptotic chondrocytes. Smaller and brighter nuclei than that of normal cells. **F.** Graph. Percentage of TUNEL-positive cells out of the total number of cells counted in OA chondrocytes and in control chondrocytes. Scale bars: 50 μm.

apoptosis of chondrocytes from knee articular cartilage of patients with OA. We investigated by light microscopy the different morphologies between OA pre-confluent chondrocytes and normal pre-confluent chondrocytes, cultured from two days to one-week. The chondrocytes from OA cartilage showed a fibroblast-like morphology, while the chondrocytes from control cartilage showed typical chondrocyte morphology. Moreover, the cell vitality in chondrocytes after four passages of culture from a normal control group and from OA cartilage has been assessed by H&E. The chondrocytes from control cartilage did not show any signs of cellular suffering, demonstrated by intense staining, while the chondrocytes from OA cartilage showed clear signs of cellular suffering, demonstrated by slight staining.

Apoptosis through the expression of caspase-3 was over-expressed in tissue explants as confirmed by our recent publication (Musumeci et al., 2011). Compared to our recent publication, we investigated apoptosis through the expression of caspase-3 in monolayer cells and in cells encapsulated in a PEGDA hydrogel scaffold. The results show an increase of expression of caspase-3 in pre-confluent cells and in cells after four passages of culture in monolayer. However the cells after 5 weeks of culture in a PEGDA hydrogel demonstrated a decrease of caspase-3 immuno-expression comparable to control cartilage.

Freshly isolated chondrocytes from human OA cartilage exhibited morphological evidence of apoptosis, clear cytoplasmic, cell-surface blebs, altered nuclear shape, apoptotic bodies and a parallel loss of nuclear volume. Some apoptotic nuclei showed no evidence of chromatin condensation, indicating that bleb formation is an early event during apoptosis. Chondrocytes from normal donors did not show any cytoplasmic signs of apoptotic cell death. Changes in nucleic morphology, such as smaller and brighter nuclei and nuclear fragmentation and condensation, were evident in OA chondrocytes when compared to normal cells. These

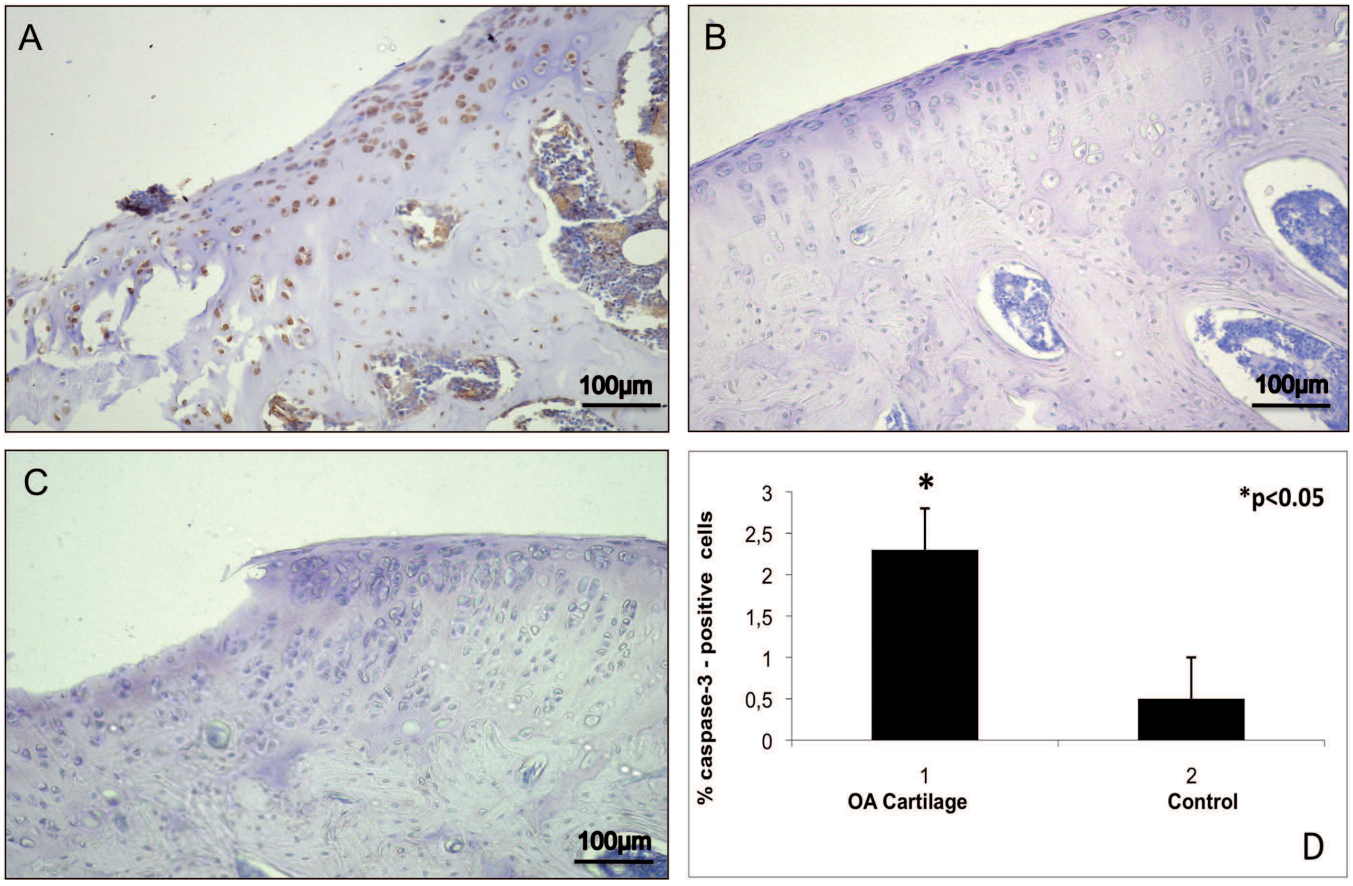


Fig. 5. A. Caspase-3 immunohistochemistry specimen from an OA subject (tissue explanted). Strong perinuclear caspase-3 immunolabeling of chondrocytes. **B.** Caspase-3 immunohistochemistry specimen from a control subject (tissue explanted). Weak/absence caspase-3 immunolabeling of chondrocytes. **C.** No immunoreaction was observed in the OA cartilage (tissue explanted) treated with PBS without the primary antibodies. **D.** Graphs. Percentage of caspase-3 positive cells out of the total number of cells counted in OA cartilage and control cartilage. Scale bars: 100 μm.

findings suggest that the OA chondrocytes demonstrated differences in predisposition toward apoptosis. Data recently published (Musumeci et al., 2011).

Studies demonstrated that OA chondrocytes showed a strong caspase-3 immunoexpression when compared to controls, "in vivo", in tissue explants (as previously reported Musumeci et al., 2011) in pre-confluent cells and in the cells after four passages of culture in monolayer. The OA chondrocytes after 3 weeks of encapsulation in a PEGDA hydrogel showed a moderate caspase-3 immunoexpression, but after 5 weeks, these cells demonstrated a decrease of caspase-3 immunoexpression comparable to control cartilage. This reduction in enzyme, which correlates with a decrease in apoptosis, could be interpreted as an enhancement of chondrocyte survival and proliferation. Thus, the 3D culture in PEGDA hydrogel could make the cells more resistant to apoptosis, probably because cells find the microenvironment conditions to be similar to the "in vivo" tissue.

The new cartilage and cell morphology in the hydrogel constructs was studied through histology using Hematoxylin and Eosin staining. The results demonstrated the absence of structural alterations of OA chondrocytes encapsulated in the hydrogels after 2, 3 and 5 weeks of culture. In particular, after 5 weeks the chondrocytes showed an increase in cellular growth, cellular aggregations in nests (typical structures of the hyaline cartilage) and an increase in ECM production.

More specific chemical stains, such as Safranin-O and Alcian Blue, were used to assess synthesis of ECM molecules like GAG containing proteoglycans. These results confirmed that OA chondrocytes after 5 weeks of culture in PEGDA hydrogels produced new cartilage and new ECM comparable to the control cartilage, as shown by the presence of proteoglycans.

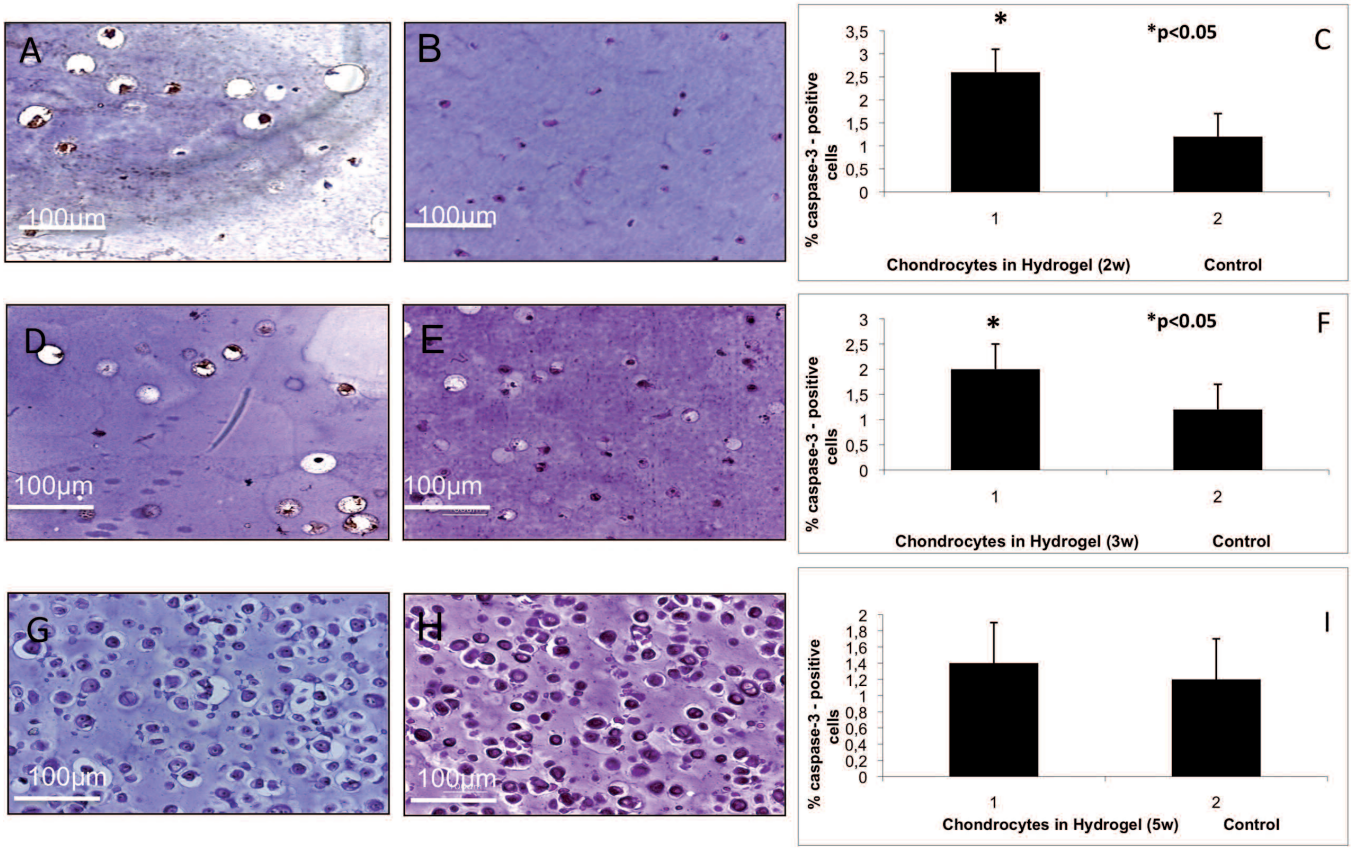
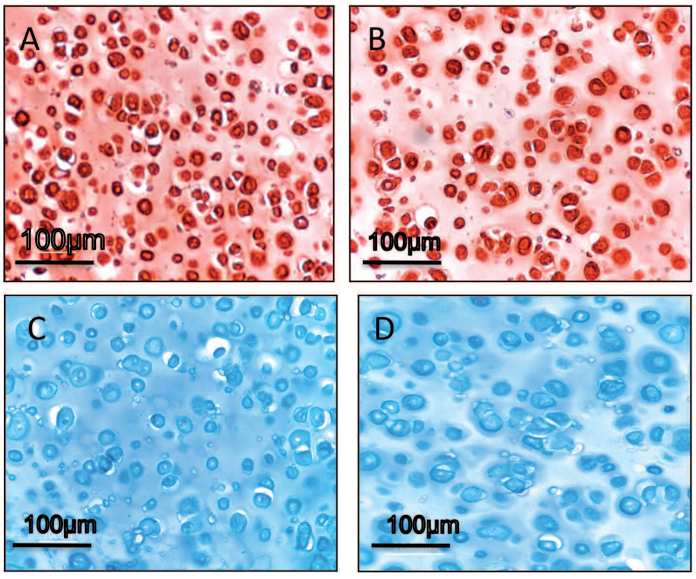


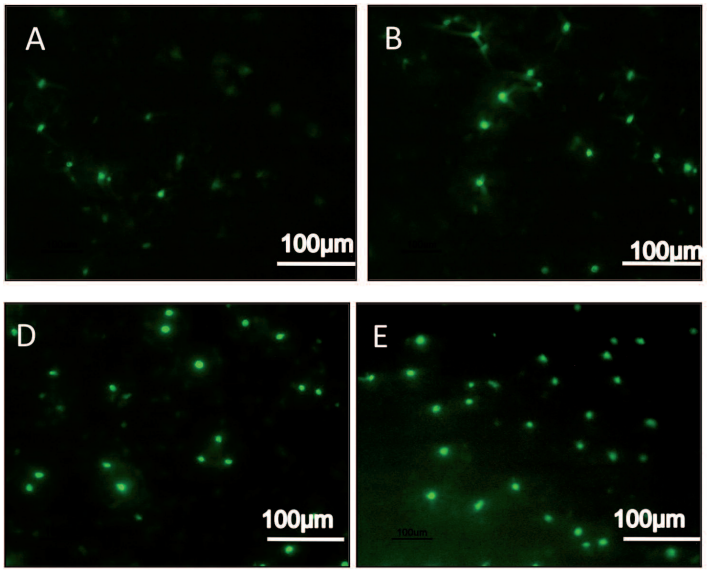
Fig. 6. A, D. Caspase-3 immunohistochemistry specimen from OA chondrocytes after 2 and 3 weeks of encapsulation in PEGDA hydrogels. Moderate/weak perinuclear caspase-3 immunolabeling in chondrocytes. **G.** Caspase-3 immunostained specimen from OA chondrocytes after 5 weeks of encapsulation in PEGDA hydrogels. Weak caspase-3 immunolabeling in the chondrocytes comparable to control chondrocytes. **B, E.** OA chondrocytes following 2 and 3 weeks of culture in PEGDA hydrogel constructs. H&E staining. Histology results demonstrated the absence of structural alterations comparable to control chondrocytes. **H.** OA chondrocytes after 5 weeks of encapsulation. H&E staining. Histology results demonstrate normal chondrocytes without structural alterations, comparable to control chondrocytes. Chondrocytes showed an increase of cellular growth, cellular aggregations in nests (typical structures of the hyaline cartilage) and an increase in ECM production. **C, F, I.** Graphs. Percentage of caspase-3 positive cells out of the total number of cells counted in OA chondrocytes and in control chondrocytes after 2 weeks **(C)**, after 3 weeks **(F)**, after 5 weeks **(I)** in PEGDA hydrogels. Scale bars: 100 μm.

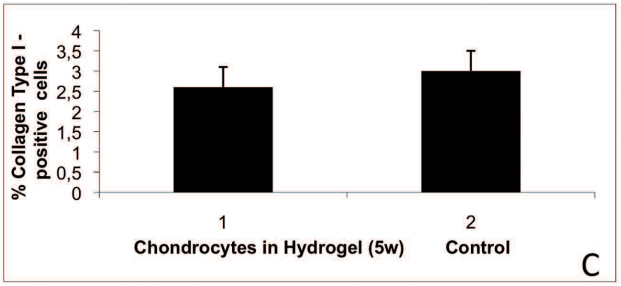


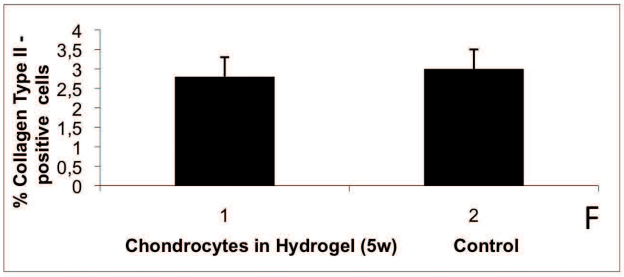
E	Safranin-O	Alcian Blue Stain
Control	4	4
Chondrocytes after 5 weeks in Hydrogel	2	2

Legend:
Stain intensity were assessed by light microscopy. Intensity of staining (IS) was graded on a scale of 0-4, according to the following assessment: 0 = no detectable staining, 1 = weak staining, 2 = moderate staining, 3 =strong staining, 4 = very strong staining

Fig. 7. A. Safranin-O staining in control chondrocytes showed an absence of proteoglycan reduction. **B.** Safranin-O staining in OA chondrocytes encapsulated in PEGDA hydrogel after 5-weeks of culture showed fair proteoglycan reduction. **C.** Staining with Alcian Blue showed in control chondrocytes, absence of proteoglycan reduction. **D.** Staining with Alcian Blue showed in OA chondrocytes, encapsulated in PEGDA hydrogel after 5 weeks of culture showed fair proteoglycan reduction. **E.** Table. Histochemical grading in PEGDA hydrogel after 5 weeks. Scale bars: 100 μm.







G	Collagen Type I	Collagen Type II
Control	Grade 2	Grade 3
OA cartilage	Grade 2	Grade 3

Legend:
Grade 0 = 0-0.2 (slight);
Grade 1 = 0.21-0.40 (fair);
Grade 2 = 0.41-0.60 (moderate);
Grade 3 = 0.61-0.80 (substantial);
Grade 4 = 0.81-1.0 (almost perfect).

Fig. 8. A. Moderate collagen Type I immunoreaction was shown in PEGDA hydrogels after 5 weeks of culture, suggesting the presence of fibrous cartilage in the chondrocytes. **B.** Moderate collagen Type I immunoreaction was shown in control chondrocytes. **D.** Strong collagen Type II immunoreaction was shown in PEGDA hydrogels after 5 weeks of culture, suggesting the presence of hyaline cartilage in the chondrocytes. **E.** Strong collagen Type II immunoreaction was shown in control chondrocytes. **C, F.** Graphs. Percentage of Collagen Type I and Type II positive cells out of the total number of cells counted in OA chondrocytes and in control chondrocytes after 5 weeks in PEGDA hydrogel. **G.** Table. Immunohistochemical grading in PEGDA hydrogel after 5 weeks. Scale bars: 100 μm.

Imunofluorescence was used in order to quantify the secretion of collagen type I and II, as Type I collagen is characteristic of fibrous cartilage while Type II collagen is indicative of hyaline cartilage formation. These findings confirmed that OA chondrocytes after 5 weeks of 3D culture produce hyaline cartilage comparable to the control cartilage *in vivo*, as demonstrated by the increased production of collagen Type II with respect to collagen Type I.

In this study, we clearly demonstrated that OA chondrocytes grown in PEGDA hydrogel were able to redifferentiate into their primitive phenotype and expressed collagen Type I and II. This data has been also strengthened by other authors who have shown the growth of chondrocytes onto another kind of scaffold: the Hyaff®-11 membrane, which seems to erase the differences between the cells derived from normal and OA cartilages (Cavallo et al., 2009). Moreover, we found a decrease in apoptosis in cells encapsulated in the biomaterials, which resulted in regulation of the cellular balance between proliferation and cell death. The possibility of applying autologous cell transplantation (ACT) in conjunction with scaffold materials for repairing cartilage lesions in patients with OA could be of great interest to at least reduce the progression of the disease. This study is in agreement with the one conducted by Stoop et al., which demonstrated that chondrocytes from macroscopically intact cartilage of OA patients can be expanded in vitro in a quality suitable for scaffold-augmented ACT, although higher initial cell densities were needed to ensure sufficient cartilage formation (Stoop et al., 2007). Another research group focused their study on gene expression profiling and indicated that chondrocytes from OA donors showed a less differentiated state in ML (monolayer) compared with ND (normal donor) chondrocytes (Dehne et al., 2009). During 3D culture in scaffolds, the differences in gene expression between OA and ND chondrocytes were diminished (Dehne et al., 2009). Photo polymerizing materials could be injected into the body in liquid form and solidified in situ on exposure to a specific light source while maintaining considerable control over the form and shape of the implant.

Acknowledgements. This study was supported by grants provided by Medicine and Surgery Faculty of University of Catania, Italy and the National Institutes of Health (JHE, JHU)

Conflict of Interest. The authors have no conflict of interest.

References

Aigner T., Soeder S. and Haag J. (2006). Il-1beta and bmps--interactive players of cartilage matrix degradation and regeneration. Eur. Cell. Mater. 12, 49-56.

Alenzi F.Q. (2005). Apoptosis and diseases: Regulation and clinical relevance. Saudi. Med. J. 26, 1679-1690.

Cavallo C., Desando G., Facchini A. and Grigolo B. (2009). Chondrocytes from patients with osteoarthritis express typical extracellular matrix molecules once grown onto a three-dimensional hyaluronan-based scaffold. J. Biomed. Mater. Res. A 93, 86-95.

Dehne T., Karlsson C., Ringe J., Sittinger M. and Lindahl A. (2009). Chondrogenic differentiation potential of osteoarthritic chondrocytes and their possible use in matrix-associated autologous chondrocyte transplantation. Arthritis Res. Ther. 11(5):R133.

D'lima D.D., Hashimoto S., Chen P.C., Lotz M.K. and Colwell C.W. Jr (2001). Prevention of chondrocyte apoptosis. J. Bone. Joint. Surg. Am. 83, 25-26.

Elisseeff J., Mcintosh W., Anseth K., Riley S., Ragan P. and Langer R. (2000). Photoencapsulation of chondrocytes in poly(ethylene oxide)-based semi-interpenetrating networks. J. Biomed. Mater. Res. 51, 164-171.

Goldring M.B. (2006). Update on the biology of the chondrocyte and new approaches to treating cartilage diseases. Best. Pract. Res. Clin. Rheumatol. 20, 1003-1025.

Goldring S.R. and Goldring M.B. (2004). The role of cytokines in cartilage matrix degeneration in osteoarthritis. Clin. Orthop. Relat. Res. 427, 27-36.

Gutowska A., Jeong B. and Jasionowski M. (2001). Injectable gels for tissue engineering. Anat. Rec. 263, 342-349.

Hashimoto S., Takahashi K., Ochs R.L., Coutts R.D., Amiel D. and Lotz M. (1999). Nitric oxide production and apoptosis in cells of the meniscus during experimental osteoarthritis. Arthritis. Rheum. 42, 2123-2131.

Hashimoto M., Nakasa T., Hikata T. and Asahara H. (2008). Molecular network of cartilage homeostasis and osteoarthritis. Med. Res. Rev. 28, 464-481.

Hough A.J. Jr and Webber R.J. (1990). Pathology of the meniscus. Clin. Orthop. Relat. Res. 252, 32-40.

Hubbell J.A. (1995). Biomaterials in tissue engineering. Biotechnology. New York. pp 565-576.

Jeon S.I., Lee J.H,. Andrade J.D. and De Gennes P.G. (1991). Protein--surface interactions in the presence of polyethylene oxide: I. Simplified theory. J. Colloid Interface Sci. 142, 149-158.

Kim H.A., Lee Y.J., Seong S.C., Choe K.W. and Song Y.W. (2000). Apoptotic chondrocyte death in human osteoarthritis. J. Rheumatol. 27, 455-462.

Kuhn K., D'lima D.D., Hashimoto S. and Lotz M. (2004). Cell death in cartilage. Osteoarthritis Cartilage 12, 1-16.

Langer R. and Vacanti J.P. (2006). Tissue engineering. Science 260, 920-926.

Lorenz H. and Richter W. (2006) Osteoarthritis: Cellular and molecular changes in degenerating cartilage. Prog. Histochem. Cytochem. 40, 135-163.

Loreto C., Musumeci G. and Leonardi R. (2009). Chondrocyte-like apoptosis in temporomandibular joint disc internal derangement as a repair-limiting mechanism. An in vivo study. Histol. Histopathol. 24, 293-298.

Lutolf M.P. and Hubbell J.A. (2005). Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat. Biotechnol. 23, 47-55.

Majno G. and Joris I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. Am. J. Pathol. 146, 3-15.

Mapel D.W., Shainline M., Paez K. and Gunter M. (2004). Hospital, pharmacy, and outpatient costs for osteoarthritis and chronic back pain. J. Rheumatol. 31, 573-583.

Masuda K., Sah R.L., Hejna M.J. and Thonar E.J. (2003). A novel two-

step method for the formation of tissue-engineered cartilage by mature bovine chondrocytes: The alginate-recovered-chondrocyte (arc) method. J. Orthop. Res. 21, 139-148.

Mauck R.L., Soltz M.A., Wang C.C., Wong D.D., Chao P.H., Valhmu W.B., Hung C.T. and Ateshian G.A. (2000). Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. J. Biomech. Eng. 122, 252-260.

Musumeci G., Loreto C., Carnazza M.L. and Martinez G. (2011). Characterization of apoptosis in articular cartilage derived from the knee joints of patients with osteoarthritis.Knee. Surg. Sports. Traumatol. Arthrosc. 19, 307-313.

Peppas, N.A. (1987). Hydrogels in medicine and pharmacy. CRC Press. Boca Raton. pp 171.

Pooler A.R. (2001). Cartilage in health and disease. In: Arthritis and allied conditions: A textbook of rheumatology. Koopman W. (ed). Lippincott Williams & Wilkins. Philadelphia. pp 226-284.

Pritzker K.P., Gay S., Jimenez S.A., Ostergaard K., Pelletier J.P., Revell P.A., Salter D. and Van Den Berg W.B. (2006). Osteoarthritis cartilage histopathology: Grading and staging. Osteoarthritis Cartilage 14, 13-29.

Rosemann T., Laux G. and Szecsenyi J. (2007). Osteoarthritis: Quality of life, comorbidities, medication and health service utilization assessed in a large sample of primary care patients. J. Orthop. Surg. Res. 2, 12.

Roy S. and Meachim G. (1968). Chondrocyte ultrastructure in adult human articular cartilage. Ann. Rheum. Dis. 27, 544-558.

Scott J.C., Lethbridge-Cejku M. and Hochberg M.C. (1999). Epidemiology and economic consequences of osteoarthritis. In: Osteoarthritis. Clinical and experimental aspects. Pelletier J.P., Martel-Pelletier J. and Henrotin Y. (eds). Springer. Berlin. pp 20-52.

Stoop R., Albrecht D., Gaissmaier C., Fritz J., Felka T., Rudert M. and Aicher W.K. (2007). Comparison of marker gene expression in chondrocytes from patients receiving autologous chondrocyte transplantation versus osteoarthritis patients. Arthritis Res. Ther. 9, R60

Terraciano V., Hwang N., Moroni L., Park H.B., Zhang Z., Mizrahi J., Seliktar D. and Elisseeff J. (2007). Differential response of adult and embryonic mesenchymal progenitor cells to mechanical compression in hydrogels. Stem Cells 25, 2730-2738.

Vacanti J.P. (1988). Beyond transplantation. Third annual samuel jason mixter lecture. Arch. Surg. 123, 545-549.

Vacanti C.A. (2006). The history of tissue engineering. J. Cell. Mol. Med. 10, 569-576.

White E. (1996). Life, death, and the pursuit of apoptosis. Genes. Dev. 10, 1-15.

van der Sluijs J.A., Geesink R.G,, van der Linden A.J., Bulstra S.K., Kuyer R. and Drukker J. (1992). The reliability of the Mankin score for osteoarthritis. J. Orthop. Res. 1992 10, 58-61.

Accepted March 1, 2011