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FLOW CYTOMETRIC ANALYSIS OF HUMAN CHONDROCYTES CULTURED IN A NEW MEDIUM FOR AUTOLOGOUS THERAPIE AND TISSUE ENGINEERING CARTILAGE

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ABSTRACT

Autologous Cell Implantation (ACI) is a currently practiced cell-based therapy to repair cartilage defects. Several strategies have been explored to expand the number of chondrocytes *ex vivo*. However, these methods are unable to provide sufficient quantity of chondrocytes with unaltered phenotype. To maintain the original phenotype in monolayer culture and to expand cell proliferation, primary human chondrocytes isolated by enzymatic digestion were cultured in a DMEM medium supplemented with Ac-Gly-Gly-OH dipeptide The aim of our study was to investigate and compare by flow cytometry the viability and the cell proliferation of chondrocytes obtained by culture in medium containing the dipeptide with the cells cultured in a classical system. The results we obtained provide that proliferation and viability of chondrocytes cultured in presence of DMEM medium containing Ac-Gly-Gly-OH were higher and thus can be used in the culture of chondrocytes devoted to reconstructive clinical procedures.

KEYWORDS: chondrocytes, osteoartritic cartilage, flow cytometric analysis, viability test, cell proliferation, PKH-26, tissue engineering

INTRODUCTION

The estimated number of articular cartilage incidences worldwide is around 30 million cases of knee osteoarthritis and 1.2 million cases of focal defects (*U.S. Markets for Current and Emerging Orthopedic Biomaterials Products and Technologies*, 2002). About 11% of these defects are suitable for cartilage repair procedures (Aroen *et al.,* 2004).

Osteoarthritis is an age-related disorder generally affecting articular cartilage. In adult vertebrates, articular cartilage is devoid of nerves, blood vessels or lymphatics and contains only one cell type, the chondrocyte. These resident cells are highly specialized and solely responsible for the maintenance and turnover of extracellular matrix macromolecules including type II collagen, aggregating proteoglycans and non-collagenous proteins (Muir, 1995). The biochemical performance of cartilage depends on the biochemical and biophysical properties of extracellular matrix macromolecules and thus on the normal metabolic activity and homeostatic status of chondrocytes (delise *et al.*, 1999). The challenge is to produce cartilage tissue with suitable structure and properties *ex vivo,* which can be implanted into joints to provide a natural repair.

Developments of therapeutic strategies for cartilage repair have increasingly focused on the promising technology of cell therapy based on the use of autologous chondrocytes or of other cell types to regenerate articular cartilage *in situ.* The culturing of chondrocytes is currently an important issue, in particular within the framework of tissue regeneration in human and veterinary medicine. As a matter of fact, the autologous transplantation of cartilage cells into a diseased tissue is an advantageous method of treating diseases of the cartilage. Autologous Cell Implantation (ACI) is a currently practiced cell-based therapy to repair cartilage defects (Brittberg *et al.,* 1994). Several strategies have been explored to expand the number of chondrocytes *ex vivo*.

As a matter of fact, in order to avoid the use of an invasive method for total replacement of osteoarthritic joints, cartilage cells are taken from the patient and then cultured *in vitro* in a chondrocyte expansion medium, so as to be multiplied and finally re-implanted in the tissue. The portion of the joint affected by osteoarthritis is thus reconstructed and grafted onto the healthy portion. However, these methods are unable to provide sufficient quantity of chondrocytes with unaltered phenotype because chondro-

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cytes propagated in monolayer culture lose their original characteristics by assuming a fibroblastoid morphology and shift from production of collagen.

In this work, we present a method to maintain the original phenotype in monolayer culture and to expand cell proliferation of primary human chondrocytes isolated from osteoarthritic patients.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (DMEM) was obtained from Cambrex Bio Science (Verviers, Belgium), fetal calf serum, penicillin, streptomycin, amphotericin B and L-glutamine were from Gibco (Carlsbad, USA). Hyaluronidase, trypsin, collagenase from *Clostridium histoliticum*, the PKH-26 were from Sigma-Aldrich (St. Louis, MO, USA), Ac-Gly-Gly-OH by BACHEM (Villers-le-Bretonneux, France) and Calcein-AM supplied by Molecular Probes (Eugen, USA). The flow cytometer was a Becton-Dickinson FACScan apparatus (San Jose, CA, USA) with CellQuest Pro software for acquisition and analysis.

Isolation of chondrocytes

Osteoarthritic articular chondrocytes were isolated using Green*,* 1971 and Kuettner *et al.* 1982 protocols from patients with osteoarthritis undergoing arthroplasty under sterile techniques (CF 2 Hospital, Bucharest, Romania). All enzymatic solutions were prepared in Dulbecco's modified Eagle's medium (DMEM) supplemented with a mixture of antibiotics and antimycotics (penicillin 10 U/ml, streptomycin 10 mg/ml, amphotericin B 0.025 mg/ml), L-glutamine 0.002M and 10% of fetal calf serum. The fragments of cartilage were minced into small pieces and incubated with 0.1% of sheep teste hyaluronidase in DMEM medium for 20 min at 37˚C. The pieces were washed with PBS (Phosphate Saline Buffer ph 7.4, osmolality 320-330 mosmol kg^{-1}) and maintained in a trypsin solution (0.25 g/100 ml PBS buffer ph 7.4) for 60 min at 37˚C. The articular cartilage pieces were washed again with PSB buffer and incubated at 37˚C and 5% CO_2 overnight in 0.2 % collagenase from *Clostridium histolyticum* in DMEM medium with 10% fetal calf serum. Cells were then centrifuged for 15 min at 3000 rpm, washed with PBS buffer and then centrifuged for 15 min at 3000 rpm. The obtained pellet was divided into two equal parts, the one for classical monolayer culture and the others in a DMEM medium supplemented with Ac- Gly-Gly-OH dipeptide.

Monolayer Culture of Chondrocytes

The cells were seeded into 1.5 cm chambers (Nalge Nunc International, Naperville, I L, USA) which provide enough surface area to allow $4x10^4$ isolated chondrocytes to proliferate in DMEM medium without or with Ac- Gly-Gly-OH dipeptide at 200 μm final concentration. This medium was supplemented with a mixture of antibiotics and antimycotics (penicillin 10 U/ml, streptomycin 10 mg/ml, amphotericin B 0.025% mg/ml), with L-glutamine 0.002M and 10% of fetal calf serum. The cultures were maintained at 37^0 C in a humidified 5 % CO_2.

Flow cytometric analysis

Flow cytometric analyses were performed on a FACScan cytometer using CellQuest Pro software for acquisition and analysis. Cells in suspension in isotonic PBS buffer pH 7.4 were gated for the light scatter channels on linear gains, and the fluorescence channels were set on a logarithmic scale with a minimum of 10,000 cells analyzed in each condition.

Cell proliferation test using PKH-26

Prior to being cultured, the chondrocytes were irreversibly labelled with a vital fluorescent membrane intercalate derived from arcridine orange, the PKH-26, with a view to following cell proliferation via flow cytometry, which entails a decrease in the overall fluorescence of the cells.10^6 chondrocytes in suspension in 1 ml of the "diluent C" of the labelling kit are added to 1 ml of a 2 μM solution of PKH-26 in the same diluent. After 4 min of incubation at ambient temperature, the reaction is stopped by the addition of 2 ml of fetal calf serum and then, after incubating for one minute, 4 ml of complete DMEM medium are added. After centrifugation (5 min, 2000 g at 25° C), the resulting sediment is washed 3 times with 10 ml of complete DMEM medium. The cells are then cultured for 3 days under the described conditions, detached from the support, placed back in suspension in a PBS buffer at pH 7.4 and analyzed by flow cytometry in the logarithmic mode FL2.

Morphological changes assessment of chondrocytes by light scattered measurements.

Analysis of the scattered light by flow cytometry in the mode FSC/SSC provides informations about cell size and structure. In fact, intensity of light scattered in a forward direction (FSC) correlates with cell size while if is measured at a right angle to the laser beam (side scatter/SSC) it correlates with granularity, refractiveness and presence of intracellular structures that can reflect the light were associated with cell shrinkage.

Flow cytometric assay of cell viability using Calcein-AM

Cell viability assessment was studied according to the procedure of Bratosin *et al*., 2005 and applied to chondrocytes by Takács-Buia *et al.,* 2008. The membrane-permeable dye Calcein-AM was prepared as a stock solution of 10 mm in dimethylsulfoxide stored at -20°C and as a working solution of 100 μM in PBS buffer ph 7.4. Chondrocytes ($4 x10^5$ in 200 μl PBS buffer) were incubated with

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10 µl Calcein-AM working solution (final Calcein-AM concentration: 5 µM) for 45 min. at 37°C in the dark and then diluted in 0.5 ml of PBS buffer for immediate flow cytometric analysis of Calcein fluorescence retention in cells. Experiments were performed at least three times with three replicates each time.

RESULTS AND DISCUSSION

Light scattering properties of chondrocytes cultured in monostrat in dmem medium without or with Ac- Gly-Gly-OH

The cell's ability to scatter light is expected to be altered during cell death, reflecting the morphological changes such as cell swelling or shrinkage, breakage of plasma membrane and, in the case of apoptosis, chromatin condensation, nuclear fragmentation and shedding of apoptotic bodies. Analysis of the scattered light by flow cytometry in the mode FSC/SSC provides information about cell size and structure.

During apoptosis, the decrease in forward light scatter (which a results from cell shrinkage) is not initially paralleled by a decrease in side scatter. A transient increase in right angle scatter can be seen during apoptosis in some cell systems. This may reflect an increased light reflectiveness by condensed chromatin and fragmented nuclei. However, in later stages of apoptosis, the intensity of light scattered at both, forward and right angle directions, decreases. Cell necrosis is associated with an initial increase and then rapid decrease in the cell's ability to scatter light simultaneously in the forward and right angle direction. This is a reflection of an initial cell swelling followed by plasma membrane rupture and leakage of the cell's constituents (Darzynkiewicz *et al.,* 1997).

Figure 1 shows the morphological changes of human osteoarthritic chondrocytes cultured in different ways which were associated with cell shrinkage (decreased forward scatter and characteristic features of apoptosis. After 5 days of chondrocytes culture (seeding) using a standard

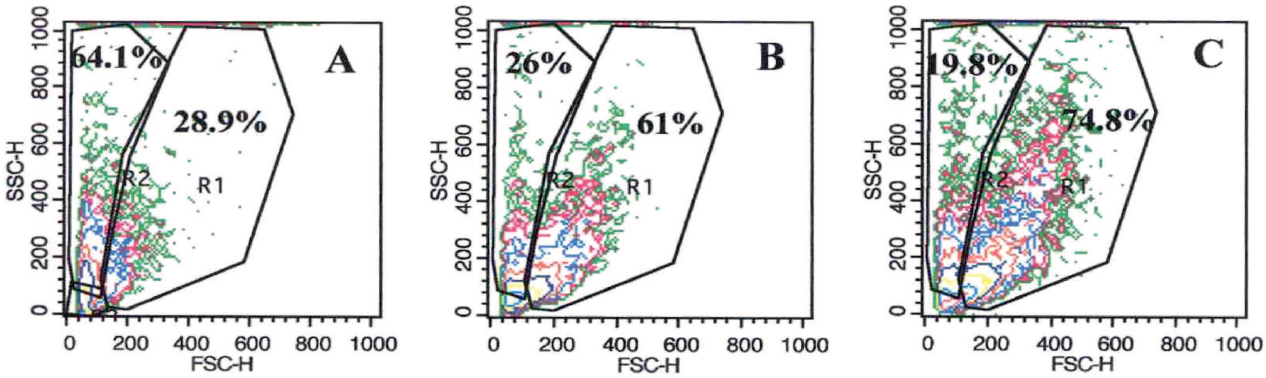

Fig. 1. Flow cytometric analysis of human osteoarthritic chondrocytes morphological changes after 5 days cultured in classical monolayer system in DMEM medium without (B) or with Ac-Gly-Gly-OH (C). (A):Osteoarthritic chondrocytes before seeding. Abscissae: forward scatter (cell size); ordinates: side scatter (cell density, granularity or refractiveness). R1: viable chondrocytes; R2: apoptotic chondrocytes; R3: cellular remainders of chondrocytes. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.

protocol (Fig.1 B), we noticed the separation of the same regions as before (A), with a percentage of viable cells almost double (61%). However, when we grew the chondrocytes in DMEM medium with Ac-Gly-Gly-OH, about 75% of them remained in the region (R1) of the dot-plot, described as viable region. Consequently, we can conclude that the culture system in DMEM medium with Ac-Gly-Gly-OH, allows chondrocyte division, while normal cell morphology is retained in a more homogeneous population.

Proliferation rate of the chondrocytes. Concentration Kinetics of dipeptide.

In order to carry out this test, 76,000 cells were cultured for five days under the culture conditions presented below, in DMEM medium with Ac- Gly-Gly-OH dipeptide at a final concentration between 0 and 500 µM.

The chondrocyte cultures carried out with a Ac-Gly-Gly-OH dipeptide concentration of 200 µM provided proliferation rates 3 to 5 times higher than the proliferation rate observed after culturing in a medium not supplemented with Ac-Gly-Gly-OH dipeptide (Fig. 2).

Cell proliferation test using PKH-26

The cells initially labelled with the PKH-26 have high fluorescence intensity (curve A). This intensity decreases over the course of the cell division since the PKH-26 is then distributed between the cells derived from the divi-

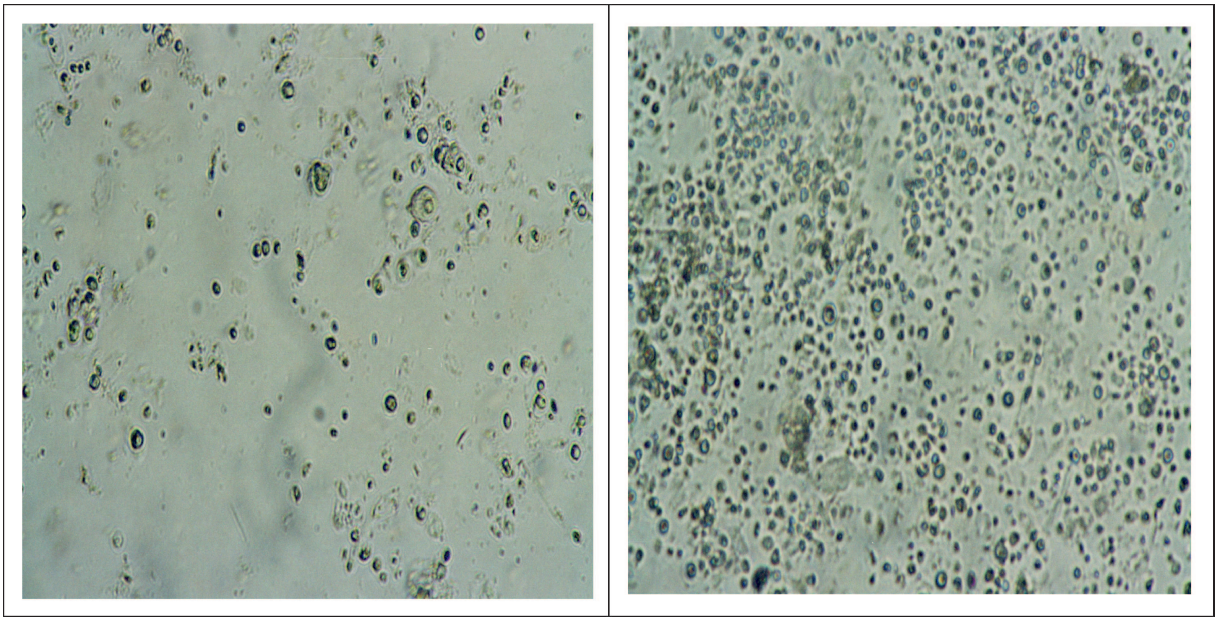

Fig. 2. Cell-seeding density analyses by comparative microscopic analyses of human osteoarthritic chondrocytes after 5 days of culture cultured in classical monolayer system in DMEM medium without (A) or with Ac-Gly-Gly-OH (B). It can easily be seen the massive cell multiplication

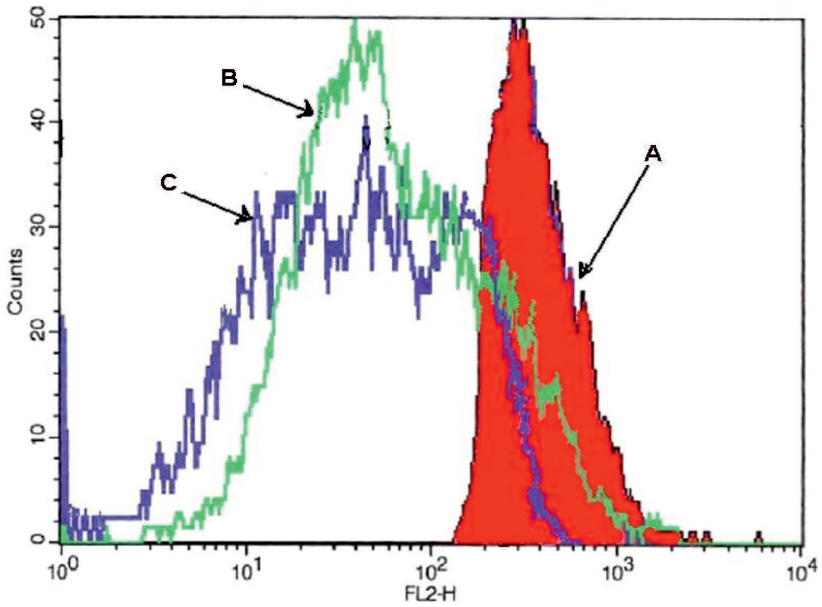

Fig. 3. Comparative flow cytometric histogram analysis of human osteoarthritic chondrocytes labelled with the PKH-26. A: Osteoarthritic chondrocytes before seeding; B: after 5 days of culture in classical monolayer system in DMEM medium without (B) or with Ac-Gly-Gly-OH (C). Abscissae: log scale red fluorescence intensity of PKH-26 (FL2). Ordinates: relative cell number. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.

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sion. It is clearly apparent that the intensity of the fluorescence of the chondrocytes after culturing (curves B and C) is lower than the fluorescence of the cells prior to culturing. This confirms the fact that cell divisions have indeed occurred and that the proliferation was effective.

The comparison between curves B and C likewise enables it to be established that the fluorescence is further decreased in the case of a population of chondrocytes cultured in the DMEM medium described above, which is supplemented with Ac-Gly-Gly-OH. This illustrates clearly that the cell divisions were more numerous in this case than during the culturing of chondrocytes in a DMEM medium not supplemented with Ac-Gly-Gly-OH.

Flow cytometric assay of chondrocytes viability using calcein-AM

The viability of the chondrocytes and the toxicity of the peptide were studied using the method developed by Bratosin *et al.* in 2005 and based on measuring the cellular esterase activity by means of Calcein. Calcein-AM is a non-fluorescent acetic ester of fluorescein which passively passes through the membranes of the viable cells and is transformed by the cytosolic esterases into fluorescent calcein, which provides an intense green signal at 530 nm and which is retained only by the cells having an intact plasma membrane. The disappearance of Calcein thus indicates both the decrease in the characteristic esterase activity of the senescent cells, undergoing apoptosis or subjected to the action of toxic substances, and the leakage of this compound from the cells due to the permeabilisation of the membrane thereof. These two complementary mechanisms make Calcein-AM an excellent test of cell viability and cytotoxicity.

The results obtained are presented in Figure 4. The intensity of the fluorescence on a logarithmic scale is plotted on the x-axis and the relative number of cells is plotted on the y-axis. Curve A, produced at time zero of the culture, shows that the suspension of chondrocytes taken from the osteoarthritic cartilage contains a significant proportion (75%) of dead cells. Curve B shows the number of viable chondrocytes after culturing in a DMEM medium such as the one described above, without Ac-Gly-Gly-OH. Curve C shows the number of viable chondrocytes after culturing in a DMEM medium identical to that of curve B, in the presence of 200 µm of Ac-Gly-Gly-OH. Curves B and C are superimposable, thereby demonstrating that the Ac-Gly-Gly-OH added to the culture medium is not toxic to the chondrocytes and does not reduce the viability there of. This non-toxicity makes it possible to anticipate the use of Ac-Gly-Gly-OH *in vivo*, for stimulating the proliferation of the chondrocytes, and more generally the differentiated cells of the chondrogenic lineage.

DISCUSIONS AND CONCLUSIONS

One of the limitations for applying cell-based regenerative medicine techniques to organ replacement is the difficulty of growing specific cell types in large quantities.

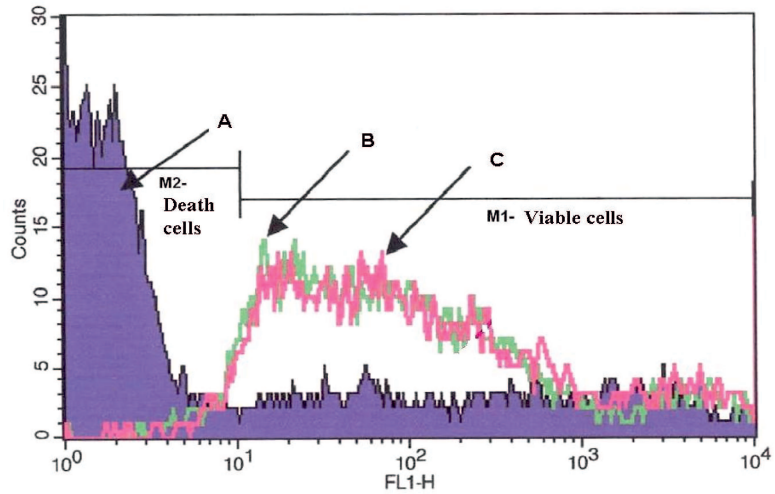

Fig. 4. Comparative flow cytometric histogram analysis of human osteoarthritic chondrocytes viability by cell esterase activity measurement using Calcein-AM.

A: Osteoarthritic chondrocytes before seeding; B: after 5 days of culture in classical monolayer system in DMEM medium without (B) or with Ac-Gly-Gly-OH (C). Abscissae: log scale green fluorescence intensity of Calcein (FL1). Ordinates: relative cell number. M1: region of fluorescent cells with intact membranes (living cells) and M2: region of nonfluorescent cells with damaged cell membranes (dead cells). Numbering refers to the cell percentage of each population. Number of counted cells: 10,000. Results presented are from one representative experiment of three performed.

Even when some organs, such as the liver, have a high regenerative capacity *in vivo*, cell growth and expansion *in vitro* may be complex. In the last 10 years major advances have been achieved on the expansion of a variety of primary human cells, with specific techniques that make the use of autologous cells feasible for clinical application. Numerous techniques based on autologous chondrocytes implantation (ACI) have been developed for the treatment of articular cartilage injury. Autologous chondrocytes implantation was first published by Brittberg *et al.* in 1994 and this technique has quickly becoming a successful and viable alternative treatment in orthopaedic surgery. Consequently, the risk of disease transfer and immunogenic response is minimized Nevertheless, the amount of cells that can be isolated from an articular cartilage biopsy is not sufficient to seed clinically relevant scaffold sizes (4 to 5 cm2). In ACI, high amount of cultured chondrocytes (approx. 12.0×10^6 cells cm^{-3}) are needed for implantation. Expansion of chondrocytes typically results in dedifferentiation: cells lose their spherical shape and acquire a fibroblastlike appearance (Von der Mark *et al.,* 1977; Watt, 1988). The major obstacle is to generate sufficient amount of healthy cells in short time and to preserve the chondrogenic properties of the cultured cell after serial passages (LeBaron & Athanasiou, 2000).

To increase the number of chondrocytes before implantation more rapidly, various techniques have been used. Longer duration of monolayer culture *in vitro*, repeated chondrocyte passaging, addition of growth factors to the culture medium and combinations of these methods are most commonly used (Marijnissen *et al*., 2000; Arevalo-Silva *et al.,* 2000; Martin *et al.,* 2001; Buia-Takacs *et al.,* 2010). All these techniques are useful to increase the number of cells before implantation, but all have limitations. A longer duration in monolayer culture causes the cell phenotype to change; the chondrocytes become more elongated and fibroblast-like and start to secrete collagen type I (normal chondrocytes secrete collagen type II). It has also been shown that fresh cells generate optimal quality neocartilage but that cell grown for longer than approximately 6 weeks *in vitro* demonstrate suboptimal tendencies. Similarly, the neocartilage quality is adversely affected by increasing the number of cell passages; beyond the third passage the utility of this technique declines.

The issue of phenotype expression and differentiation has led to the investigation of the potential use of pluripotent stem cells as a source for tissue engineering. These have included mesenchymal stem cells, which are capable of differentiating into bone, cartilage, tendon, and muscle (Caplan, 1990). Also cells with chondro-osteoprogenitor features havebeen isolate from several tissues, including periosteum, bone marrow, spleen, thymus, skeletal muscle, adipose tissue, skin, and retina (Mizuno & Glowacki, 1996; Levy *et al.,* 2001; Huang, *et al.,* 2002**;** Zuk *et al.,* 2001). However, for several reasons such as hardly accessible tissue source, low cell frequency, and limited information the use of these progenitors in tissue engineering has not been always straightforward (Cancedda *et al.,* 2003).

The results we obtained provide that proliferation and viability of chondrocytes cultured in presence of DMEM medium containing Ac-Gly-Gly-OH were higher and thus can be used in the culture of chondrocytes devoted to autologous chondrocytes implantation and reconstructive clinical procedures.

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