

Membrane-seeded autologous chondrocytes: cell viability and characterization at surgery

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Received: 8 April 2005 / Accepted: 24 January 2006 / Published online: 13 June 2006
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Abstract The implantation of chondrocytes, seeded on matrices such as hyaluronic acid or collagen membranes, is a method that is being widely used for the treatment of chondral defects. The aim of the present study was to evaluate the distribution, viability and phenotype expression of the cells seeded on a collagen membrane just at the time of the implantation. Twelve patients who were suffering from articular cartilage lesions were treated by the MACI[®] procedure. The residual part of each membrane was tested by colorimetric assay (MTT) and histochemical and ultrastructural analyses were carried out. In all of the samples a large number of viable cells, quite homogeneously distributed, was detected. The cells expressed the markers of the differentiated hyaline chondrocytes. These data reassure in that the MACI procedure provides a suitable engineered tissue for cartilage repair, in line with the clinical evidences emerging in the literature.

Keywords Chondrocytes · Articular cartilage · Cells · Cultured · Histochemistry · Tissue engineering

Introduction

Autologous chondrocyte implantation (ACI) is an established method for the treatment of chondral defects [6]. Some prospective studies have demonstrated subjective and objective improvement in joint function at 12 months after surgery [3, 13]. MRI, second-look surgery and biopsies have shown the formation of nearly normal cartilage in a good number of patients [13, 18, 19]. On the basis of these results, ACI may be considered one of the most effective treatments of articular cartilage lesions. However, this method is not devoid of some problems with regard to, for example, the wide surgical approach, the demanding technique, the presence of periosteal flap and the cell-holding in site [14, 16].

In order to overcome some of these hurdles, new tissue engineering techniques, widely used nowadays, have been developed using chondrocytes seeded on biological matrices such as hyaluronic acid [15] or collagen membranes [7] or atelocollagen gel [17]. Despite the wide diffusion of these methods, there are still some areas that would need better clarification. The presence of numerous, viable and well-differentiated cells at surgery is an essential requisite for the success of all these methods. It has been shown that autologous chondrocytes in suspension before implantation are able to maintain their differentiated phenotype and are capable of proliferating fairly well [9, 20]. On the other hand, little has been reported so far about cell characterization in membrane-seeded ACI techniques.

The aim of the present study was to evaluate the distribution, viability and phenotype expression of the cells seeded on a collagen membrane just at the moment of the implantation.

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Materials and methods

In 2003, 12 consecutive patients, 8 males and 4 females, mean age of 34 years, suffering from cartilage lesions of the knee (10 cases) and the ankle (2 cases), underwent a collagenic scaffold-based ACI procedure (MACI[®]-Verigen, D). Autologous chondrocytes were isolated at the Verigen laboratories from cartilage slices obtained from non-bearing areas of the patients' joints during a preliminary arthroscopic surgery. Cells were propagated in monolayer cultures in autologous serum for 2 or 3 weeks, according to the cellular growth rate, and were passaged on average 3 times to obtain at least 10×10^6 cells. Cells were then seeded on 20 cm² type I/III collagen membrane of porcine origin. The cell-seeded membranes were implanted in cartilage defects by means of either arthroscopic or mini-open surgery, using fibrin glue to ensure adhesion. At each implantation, the residual part of the membrane was collected and tested for cell viability, and histochemical and ultrastructural analyses were also performed.

Cell viability analysis

Cell viability was evaluated by MTT (dimethylthiazol-diphenyltetrazol bromide; thiazolil blue) colorimetric assay. MTT (Sigma, Italy) is a water soluble tetrazolium salt that yields a yellowish solution when prepared in medium lacking in phenol red (RTMI 1640, Sigma). Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by the active mitochondrial dehydrogenases of living cells. The MTT solution (5 mg MTT/ml medium) was added to three samples (1 cm²) of each membrane, being assayed to equal 1/10 of the original culture medium volume, and incubated for 3 h. The solution was then removed and acidic isopropanol (0.04–0.1 N HCl in absolute isopropanol) was added to solubilize the stain.

The results were evaluated by means of the spectrophotometric assay (570 nm), yielding absorbance as a function of viable cell number.

Histochemical and immunohistochemical analysis

The samples were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C and then embedded in paraffin. Specimens were stained with safranin-O. For the immunohistochemistry, non-specific binding was blocked with 3% normal goat serum in a phosphate-buffered saline (PBS), pH 7.4, for 30 min at room temperature; slides were then incubated overnight with primary antibodies at 4°C. Sections were incubated with polyclonal antibodies

anti S-100 protein (Dako, Italy), a cytoplasmatic marker of chondrocyte phenotype, diluted at 1:3,000, anti-collagen type I (Monosan, The Netherlands) and II (Calbiochem-Oncogene, CA, USA) at 1:150, and monoclonal antibodies anti chondroitin sulphate (chondroitin-S) (Sigma) at 1:200. Rabbit and mouse immunoglobulins, at the same dilutions as the primary antibodies, were used as controls. After three washes with Tris-HCl (0.05 M, pH 7.6), revelation of the reactions was accomplished by DAKO LSAB + kit, HRP. Stainings were viewed and photographed with a Leica Microscope (Leica Cambridge Ltd., UK).

Ultrastructural analysis

For scanning electron microscopy (SEM), the membranes were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), post-fixed in 1% osmium tetroxide, dehydrated in increasing ethanol concentrations and then CPD-dried. They were mounted on stubs and gold-sputtered. Specimens were observed with a Philips 505 microscope.

Results

Cell viability analysis

The presence of numerous viable cells was observed in all samples, with quite a homogeneous stain distribution, even if some of the areas revealed a greater concentration of converted dye (Fig. 1).

Considerable variability was found in mean cell numbers between the samples from different patients, in relation to the different amounts of cells obtained from cartilage slices and seeded on the membrane.

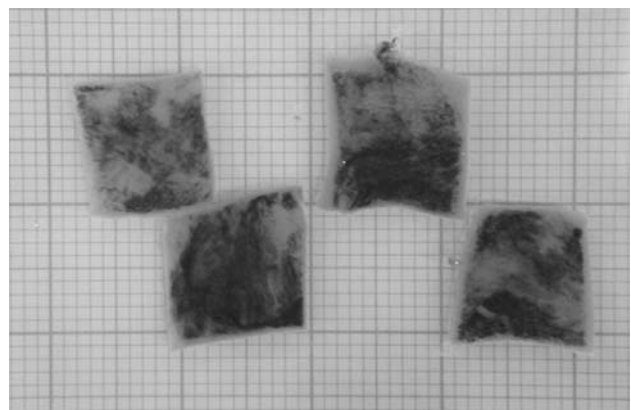


Fig. 1 Macroscopic view of human chondrocytes seeded on type I, III collagen membrane after MTT incubation. The converted blue dye shows the presence of numerous viable cells with quite a homogeneous stain distribution

Highest values were observed in samples from the younger patients (Fig. 2, Table 1).

Histochemical and immunohistochemical analysis

In all of the samples, chondrocytes that were grown on the membrane were arranged in multi-layered sheets, and sometimes invaded deeper into the matrix. The cells appeared to be quite well differentiated, although some had a flattened morphology. They slightly stained metachromatically for safranin O (Fig. 3), and clearly immunoreacted with anti-S-100 protein (Fig. 4), type II collagen (Fig. 5) and chondroitin-S antibodies. Only a few cells stained for type I collagen.

Ultrastructural analysis

The membranes presented a dual appearance: there was a smooth side with tightly packed fibres and a rougher side, a sparse layer, to which the cells were adhered. Numerous cells firmly adhered to the membrane and showed a round shaped morphology and a rough surface (Fig. 6).

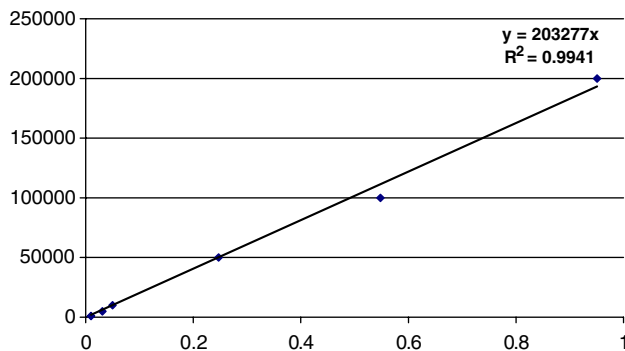


Fig. 2 The standard curve obtained from each membrane by MTT colorimetric assay (see also Table 1)

Table 1 The table shows the mean viable cell number/cm² obtained from each membrane by MTT colorimetric assay

Patients' characteristics and the number of cells initially seeded on each membrane are also reported (see also Fig. 2)

Case	Joint	Age	Sex	Seeded cells	Viable cells/cm ² (mean ± SD)
1	Knee	54	M	19 × 10 ⁶	(7.52 ± 2.79) × 10 ³
2	Knee	22	F	17 × 10 ⁶	(12.94 ± 2.54) × 10 ³
3	Knee	50	F	15.4 × 10 ⁶	(17.82 ± 6.25) × 10 ³
4	Knee	23	M	20.3 × 10 ⁶	(100.28 ± 22.14) × 10 ³
5	Knee	29	F	20.4 × 10 ⁶	(110.07 ± 39.18) × 10 ³
6	Knee	43	M	18.7 × 10 ⁶	(5.34 ± 3.25) × 10 ³
7	Knee	48	M	17.5 × 10 ⁶	(4.74 ± 2.37) × 10 ³
8	Ankle	42	F	18.2 × 10 ⁶	(33.13 ± 25.92) × 10 ³
9	Knee	19	M	21.6 × 10 ⁶	(99.54 ± 25) × 10 ³
10	Knee	35	M	20 × 10 ⁶	(103.81 ± 37.26) × 10 ³
11	Ankle	39	M	19 × 10 ⁶	(79.48 ± 62.57) × 10 ³
12	Knee	13	M	20.1 × 10 ⁶	(87.88 ± 55.17) × 10 ³

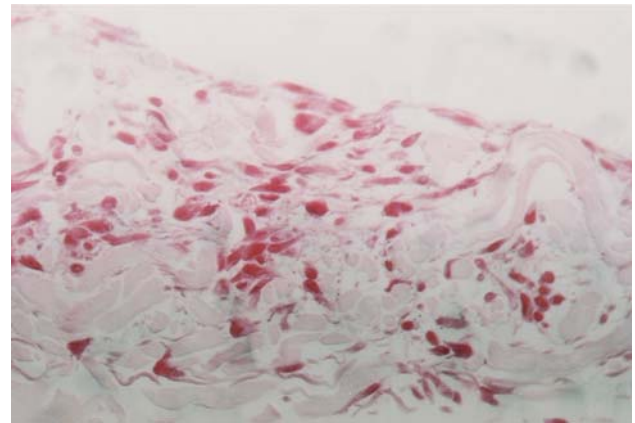


Fig. 3 Human chondrocytes seeded on type I, III collagen membrane before the implantation. Numerous cells are located on the membrane and deeper in the matrix forming a multi-layer engineered tissue. (Safranin O stain, ×200)

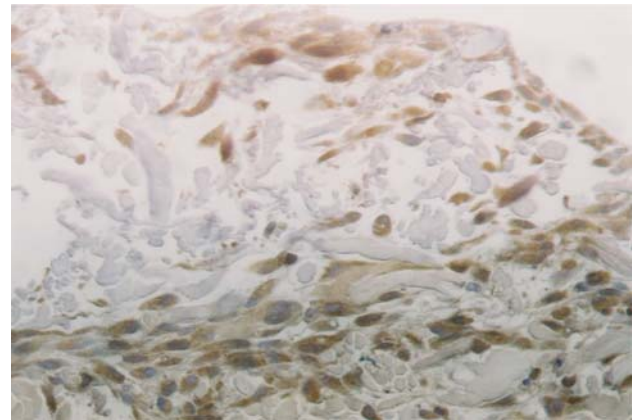


Fig. 4 Human chondrocytes seeded on type I, III collagen membrane. A marked cytoplasmic immunoreaction for S-100 protein is evident (×400)

Discussion

Cell phenotype and proliferation analysis should be an essential step in the evaluation of all tissue-engineered

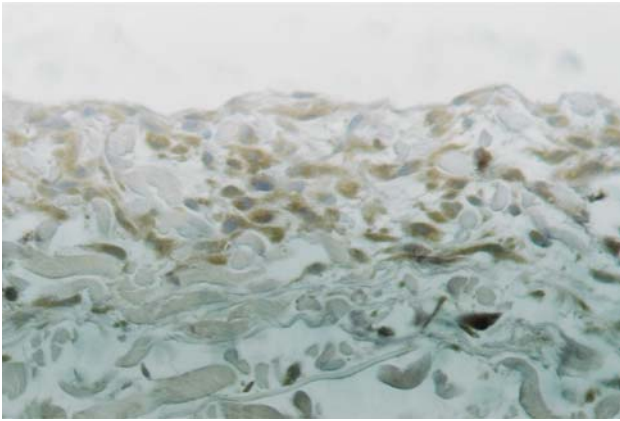


Fig. 5 Human chondrocytes seeded on type I, III collagen membrane. Cells express a positive immunoreaction for type II collagen, while the membrane remains unstained ($\times 400$)

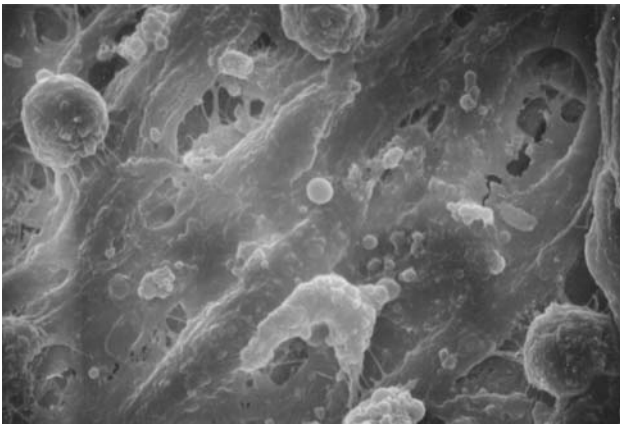


Fig. 6 Scanning electron micrograph of human chondrocytes seeded on type I, III collagen membrane. Numerous cells (*arrows*) firmly adhere to the membrane and show a round shaped morphology and a rough surface ($\times 7,500$)

products because the implantation of dedifferentiated or not-proliferating cells would not justify the therapeutic employment of these biotechnologies.

The chondrocyte culture may undergo a dedifferentiation process, consisting in a fibroblast-like morphology, a reduction of the type II collagen and aggregating proteoglycans synthesis, and an increase of type I collagen expression [1, 4]. Chondrocyte dedifferentiation is common in monolayer cultures, while a long phenotype maintenance is supported by the presence of a tri-dimensional matrix [1, 4, 11]. The chemical and ultrastructural characteristics of the matrix may have great influence on the behaviour of chondrocytes in culture. A Type I collagen membrane appears to be a matrix endowed with such properties which make that ideal and useful for cartilage tissue engineering [8, 10].

MACI is a method for the treatment of articular cartilage defects, that employs autologous chondrocytes expanded in monolayer culture, suspended, and then seeded on type I–III collagen membrane. In a previous study [9], we had observed that autologous chondrocytes in suspension before implantation were of “good cartilaginous quality” and that they possessed an excellent proliferation capacity. Zheng et al. [20] confirmed that cells in ACI procedure maintained chondrocyte phenotype and showed a low apoptotic rate. To our knowledge, there is no previous reference to the analysis of the phenotype and the proliferation activity of human chondrocytes before MACI implantation. Data about cell number and viability, but not about cell phenotype and distribution are provided by Verigen’s laboratory for each implant. It should, however, be remembered that it is quite possible that the presence of a membrane like the one found in the MACI technique could interfere with cell behaviour and modify phenotype expression.

The present study has demonstrated that the autologous chondrocytes that were analysed adhered to the membrane and had quite a homogenous distribution. These cells were viable in all the cases analysed, as shown by the spectrophotometric assay. The differences observed among patients in the number of cells seeded on the membrane and those found to be viable are probably due to several factors. In the first place, the amount of tissue collected from patients and sent to the laboratory for culture was variable. The cellularity of cartilage tissue and the proliferation capacity of chondrocytes in identical culture conditions are also quite variable between subjects, and this variability cannot merely be explained with age, but is more likely to stem from a number of characteristics that define tissue “quality” [5, 12].

We did not investigate whether during monolayer culture chondrocytes underwent dedifferentiation, however, other authors [20] demonstrated that cells grown in monolayer with the same culture conditions maintained a differentiated phenotype. Moreover, we felt it was important to establish the phenotypical expression of chondrocytes seeded on the I/III collagen membrane, since those are the cells that are then implanted, independently of what has taken place in the previous steps of the procedure. The positive immunostaining for S-100 protein, chondroitin-S, type II, but not type I collagen, confirms that cells maintained the characteristics of differentiated hyaline chondrocytes at the moment they were implanted.

These data reassure that the MACI procedure can indeed provide a suitable engineered tissue for cartilage repair, in line with the mid-term clinical results

described in the literature so far [2, 7]. Nevertheless, clinical and histological results of this method to attain stable clinical recovery and a hyaline-like cartilaginous scar are auspicated to substantiate these evidences in the long term.

Acknowledgments The authors wish to thank Miss Sandra Manzotti of the Laboratory of the Department of Orthopaedics, Polytechnic University of Marche, Italy, for the preparation of the histological sections.

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