Engineered articular cartilage: influence of the scaffold on cell phenotype and proliferation

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Articular cartilage defects do not heal. Biodegradable scaffolds have been studied for cartilage engineering in order to implant autologous chondrocytes and help cartilage repair.

We tested some new collagen matrices differing in collagen type, origin, structure and methods of extraction and purification, and compared the behavior of human chondrocytes cultured on them.

Human chondrocytes were grown for three weeks on four different equine type I collagen matrices, one type I, III porcine collagen matrix and one porcine type II collagen matrix. After 21 days, samples were subjected to histochemical, immunohistochemical and histomorphometric analysis to study phenotype expression and cell adhesion. At 7, 14 and 21 days cell proliferation was studied by incorporation of [3H]-thymidine.

Our data evidence that the collagen type influences cell morphology, adhesion and growth; indeed, cellularity and rate of proliferation were significantly higher and cells were rounder on the collagen II matrix than on either of the collagen I matrices. Among the collagen I matrices, we observed a great variability in terms of cell adhesion and proliferation. The present study allowed us to identify one type I collagen matrix and one type II collagen matrix that could be usefully employed as a scaffold for chondrocyte transplantation.

Introduction

Cartilage repair is a complex problem. Full-thickness defects do not heal because of absence of vascularity and low chondrocyte proliferation capacity [1].

Among the methods proposed to treat these defects [2], autologous chondrocyte implantation has yielded promising results [3]. However, the use of suspended cells and a periosteal flap to hold the chondrocytes in place presents both theoretical and technical difficulties [4, 5].

To address these problems, cell-seeded matrices have been studied in cartilage engineering in order to facilitate *in vivo* implantation and promote cartilage repair [6]. A three-dimensional scaffold aids cell differentiation, allows for easier implantation and supports cell proliferation at the lesion site. Several natural and synthetic matrices have been studied *in vitro* and *in vivo*, among them fibrin gel [7, 8], hyaluronic acid [9], polyglycolic and polylactic acid [10], collagen gels, sponges and membranes [11–14], but results are not totally satisfactory.

In the attempt to identify ever improved scaffolds for cartilage engineering, we tested some new collagen matrices differing in collagen type, origin, structure and methods of extraction and purification, and compared the *in vitro* behavior of human chondrocytes cultured on them.

Materials and methods

Chondrocyte cultures

Normal human articular cartilage was obtained from notch plasty during anterior cruciate ligament reconstructions. Cartilage slices were digested overnight in Dulbecco's modified Eagle's medium (DMEM), 2% fetal bovine serum (FBS) (both from Gibco, Italy), 0.25% collagenase I-A and 0.01% DNAse I (both from Sigma, Italy). Cell viability was evaluated by Trypan blue stain. Cells were grown as monolayers in 75 cm^2 culture flasks (Corning Inc., NY) for two weeks, trypsinized (Sigma) and grown in 24-well plastic culture plates (Nunc A/S, Denmark) for three weeks on six different types of collagen membranes at an initial density of 1×10^5 cells/cm^2. The culture medium contained DMEM, 10% FBS (Gibco, Italy), 50 μg/ml ascorbic acid (Sigma), 1% penicillin-streptomycin and 1% fungizone (both from Gibco). Cultures were grown at

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37 °C in a 5% CO_2 atmosphere. The medium was replaced at 48 h intervals.

After 21 days, samples were subjected to histochemical and immunohistochemical analysis for the study of phenotype expression and to histomorphometric analysis to quantify cell adhesion to the membranes. At 7, 14 and 21 days cell proliferation was studied by incorporation of tritiated [3H]-thymidine.

Membranes

Five new membranes were used in this study: four of equine type I (I-A, I-B, I-C, I-D) collagen and one of porcine type II collagen (II). They were compared with a porcine type I–III collagen membrane available on the market (Chondro-Gide – CG) (Geistlick Biomaterials, Switzerland). The specific production methods of these matrices are proprietary information of Opocrin SpA (Corlo di Formigine, Modena, Italy).

The four type I collagen membranes were obtained from equine tendons, extracted by enzymatic and acid treatment, purified, dissolved in 0.3% acetic acid, vacuum-homogenized by specific agitator, dried and formed as films in dies contained in an electrostatic field, and, finally, γ-irradiated. The telopeptide, which is connected with severe immunogenic reaction, was removed; in particular, the phenylalanine-rich hydrophobic regions of the carboxyl terminal telopeptides of the two α-1 chains of collagen, which lie between the domains of the triple helix and the trivalent lysine-derived cross-links, were eliminated. In membrane I-A, the collagen fibers were treated with alkali for better purification; membrane I-B differed from I-A for the removal of acetic acid and successive lyophilization; in membrane I-C, collagen was not treated with alkali; in membrane I-D, collagen was not treated with alkali and was air-dried.

For the fifth membrane, type II collagen was collected from porcine trachea, extracted by enzymatic and acid treatment, purified, dissolved in 50% HCl, formed as films in dies with an electrostatic charge, dried, and γ-irradiated.

Phenotype analysis

Membranes were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4 °C for 20 min and washed three times with phosphate-buffered saline (PBS). Histochemical analysis was performed by safranin-O and alcian blue stains. For immunohistochemistry, specimens were incubated with monoclonal anti-chondroitin sulfate (chondroitin-S) antibody (Sigma) at 1 : 200 dilution, and with polyclonal anti-S-100 protein antibody (Dako, Italy), diluted 1 : 3000, and anti-type I and II collagen antibody (Monosan, The Netherlands) diluted 1 : 150 and 1 : 100, respectively. Rabbit and mouse immunoglobulins at the same dilutions as the primary antibodies were used as controls. After three washes with 0.05 M Tris-HCl (pH 7.6), the reaction was visualized using the DAKO LSAB 2 system HRP kit. Stainings were viewed and photographed with a Leica Microscope (Leica Cambridge Ltd., UK).

Histomorphometric analysis

Computerized morphometric analysis was performed using the Leica Q500MC Image Analysis System (Leica Cambridge Ltd., UK), as described previously [15]. The analysis was performed on S-100-labeled slices and the area fraction (Aa %) occupied by the cells on the whole field was calculated. Nine fields were studied in each specimen. Data are expressed as mean ± SD of the mean. Statistical analysis was carried out using the ANOVA test. Significance was established at $p < 0.05$.

Proliferation analysis

At 7, 14 and 21 days of culture, [3H]-thymidine uptake was measured on four samples per group. Cells were incubated with 1 μCi/ml [3H]-thymidine (Sigma) at 37° for 6 h, then washed twice with cold PBS and treated with perchloric acid (PCA) at 4° for 10 min. The acid-insoluble fraction was digested with 0.5 M PCA at 80° for 20 min. Aliquots were counted in liquid scintillation (Packard, Italy) and results were expressed as disintegrations per minute (dpm). Statistical analysis was carried out using the ANOVA test. Significance was established at $p < 0.05$.

Results

Phenotype analysis

Membranes presented a dual appearance: there was a layer with tightly packed fibers and another, sparser layer on which cells were seeded.

On all membranes, cells appeared quite well differentiated, they stained metachromatically for safranin O and were labeled with anti-S-100 protein, type II collagen and chondroitin-S antibodies, albeit cells had a flattened morphology on some type I collagen matrices (Fig. 1(a)–(f)).

Matrix CG showed one or two layers of cells adhering to the membrane which slightly expressed the chondrocyte markers but had an elongated shape and exhibited patchy immunoreaction for type I collagen (Figs. 1(a), 2(a), (b)).

On membrane I-A, cells were arranged in few layers and were positive for S-100 protein, type II collagen and chondroitin-S (Figs. 1(b), 2(c)). Cell adhesion on membranes I-B was scarce (Fig. 1(c)). Persistence of cellular phantoms and inclusions was observed on membranes I-C (Figs. 1(d), 2(d)) and I-D (Figs. 1(e), 2(e)).

In membrane II, we observed round cells clearly staining for the chondrocyte markers that were arranged in several layers and penetrated the membrane (Figs. 1(f), 2(f), 3).

Histomorphometric analysis

The area fraction occupied by the cells on each group of membranes is illustrated in Fig. 4. Cellularity was richer on membrane II and, to a lesser extent, on membrane I-A.

Proliferation analysis

Cells grown on membrane II showed the highest proliferation rate. Proliferation was similar among

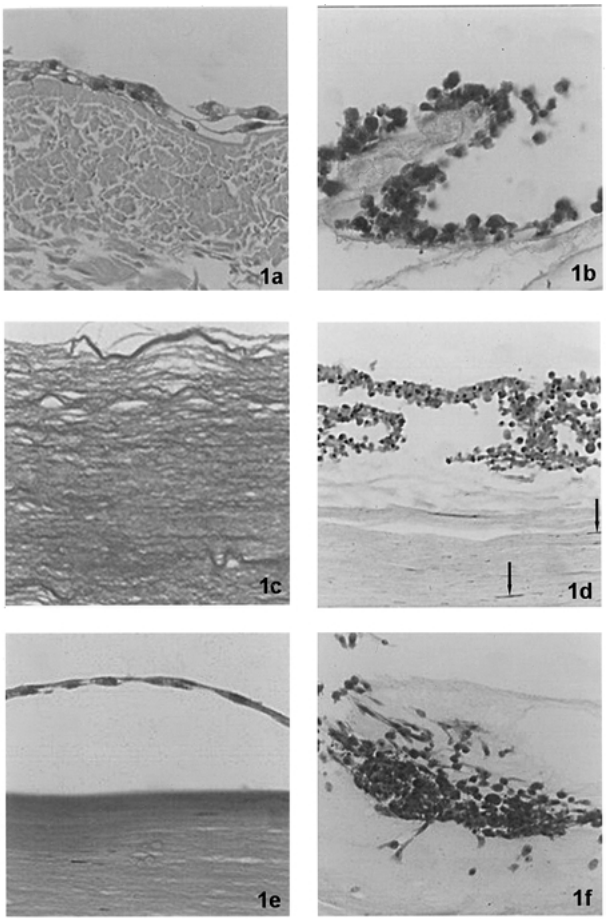

Figure 1 Histochemical analysis (safranin O stain) of human mature chondrocytes cultured on different collagen matrices. (a) Type I, III collagen membrane (CG): cells shows a flattened morphology (× 200); (b) Type I collagen membrane (I-A): numerous round cells express a positive metachromatic reaction (× 400); (c) Type I collagen membrane (I-B): there is scarce or even null cell adhesion on the membrane (× 200); (d) Type I collagen membrane (I-C): cellular phantoms and inclusions are evident within the membrane (arrows) (× 100); (e) Type I collagen membrane (I-C): some inclusions are observed within the membrane (× 200); (f) II collagen membrane (II): cells are arranged in several layers and exhibit a rounded morphology. It can be observed a metachromatic stain (× 200).

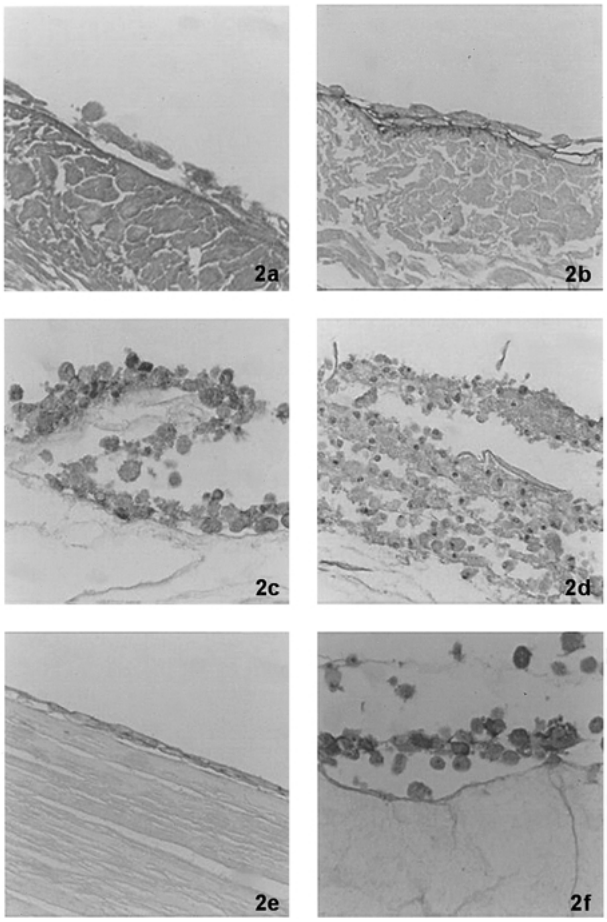

Figure 2 Human mature chondrocytes cultured on different collagen matrices. (a) Type I, III collagen membrane (CG): few layers of cells adhering to the matrix show patchy immunostain for type I collagen (× 400); (b) Type I, III collagen membrane (CG): few layers of cells adhering to the matrix slightly immunostain for chondroitin-S (× 200); (c) Type I collagen membrane (I-A): multilayer of round cells shows positive immunoreaction for chondroitin-S (× 400); (d) Type I collagen membrane (I-C): multilayer of cells slightly labeled with anti-chondroitin-S antibodies (× 200); (e) Type I collagen membrane (I-D): cells show a scarce expression of chondroitin-S (× 100); (f) Type II collagen membrane (II): all cells have a rounded morphology and immunostain for chondroitin S (× 400).

membranes I-A, I-C, I-D and CG. Membrane I-B exhibited the lowest levels of thymidine uptake (Fig. 5).

Discussion

In vitro chondrocyte phenotype and metabolism are modulated by numerous factors such as cells shape and density [16], method (anchorage-dependent vs. anchorage-independent) and time of culture [17], and medium composition [18]. Matrix characteristics exert a major influence on the behavior of chondrocyte cultures. Articular cartilage regeneration using engineered-tissue implants can be obtained only if the seeded cells achieve a high proliferation rate and produce a hyaline cartilage matrix. These metabolic parameters are influenced by the biochemical composition of the scaffold, so elucidation of specific cell-matrix interactions is important to define the optimal biochemical composition of a cell-delivery vehicle for cartilage repair [13]. An ideal scaffold should be non-toxic and non-immunogenic; it should have a biochemical composition similar to hyaline cartilage extracellular matrix, i.e. type II collagen, proteoglycans, hyaluronic acid; it should enhance cell adhesion and proliferation, and the maintenance of the chondral phenotype; finally it should be easy to handle and implant in articular defects.

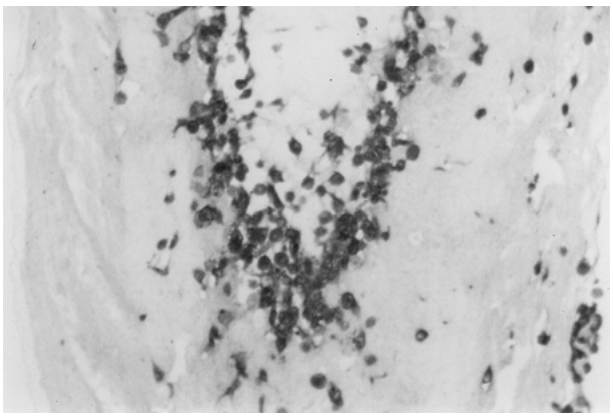

Figure 3 Human mature chondrocytes cultured on type II collagen membrane (II). Numerous cells, adhering and penetrating the membrane, are clearly labeled with anti-S-100 protein antibodies (× 200).

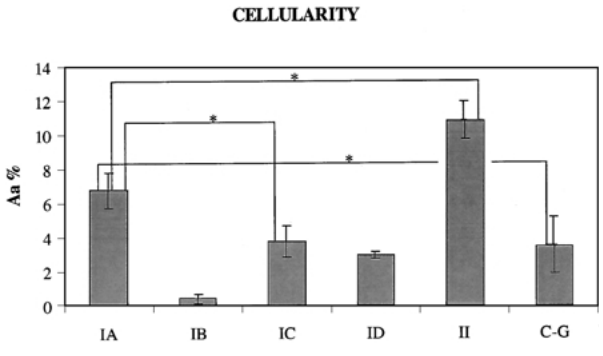

Figure 4 Histomorphometric analysis of the area fraction (mean Aa% $\pm$ SD) occupied by cells on each collagen membrane after 21 days in culture. Cellularity was richer on matrix II (type II collagen) and, to a lesser extent, on matrix I-A (type I collagen). *$p < 0.05$.

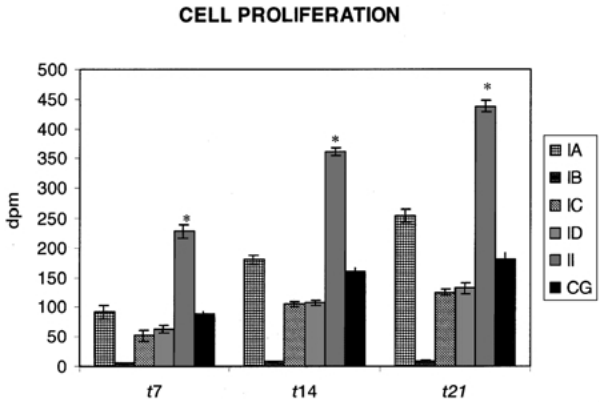

Figure 5 [3H]-thymidine incorporation by cells grown on each collagen matrix at 7, 14 and 21 days of culture. *$p < 0.05$.

We compared the effects on human chondrocyte behavior of various collagen matrices, different in source, method of extraction, purification and synthesis. Our data evidenced that the collagen type influences cell morphology, adhesion and growth; indeed, cellularity and rate of proliferation were significantly higher and cells were rounder on the collagen II matrix than on either of the collagen I matrices. Among the latter, we observed a great variability in terms of cell adhesion and proliferation; in particular, membranes I-B did not allow cells to adhere and grow. As regards biomaterial purity, inclusions were found in membranes I-C and I-D that do not allow to exclude the risk of an immune reaction in the host.

The present study of six membranes allowed us to identify one type I collagen membrane (I-A) and one type II collagen membrane (II) that could be usefully employed as a scaffold for chondrocyte transplantation. We are currently testing their behavior in articular defects *in vivo*.

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