In Vitro Expansion of Chondrocytes

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Summary

An alternative therapy for the repair of damaged articular cartilage resides in the tissue engineering approach. However, routine tissue culture methodologies can hardly cope with the scale of cell production required for the manufacture of engineered cartilage tissue products. Consequently, in vitro cell expansion has become an essential step in the process of tissue engineering of articular cartilage and the optimization of expansion protocols is a fundamental issue that needs to be addressed. In particular, both the finding of alternative sources of stem/progenitor cells and the development of feasible large scale cell expansion processes are mandatory requirements for a sufficient production of chondrocytes. In this review we delineate the progress that has been made to date and the challenges remaining for the successful production of the large number of articular chondrocytes that would be eventually required by the cartilage tissue engineering industry.

Keywords: tissue engineering; chondrocytes; chondroprogenitors; expansion; stem cells

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Introduction

Articular cartilage

Articular cartilage, also called hyaline cartilage, is the thin, smooth, glistening white tissue that covers the surface of all the diarthrodial joints in the human body. It has an incredibly low coefficient of friction which, coupled with its ability to bear very large compressive loads, makes it ideally suited for placement in joints, such as the knee and hip. The smoothness and thickness of the cartilage determines the load-bearing characteristics and mobility of the joints. It is a tissue only a few millimeters thick, but with excellent wear characteristics. Its mechanical and structural capacity depends on the integrity of its extracellular matrix. Extracellular components of collagen, proteoglycans, non-collagenous proteins and water provide the shear, compressive, and permeability characteristics of the articular cartilage (1-3). Chondrocytes sparsely distributed throughout the matrix of structural macromolecules work together with hydrated extracellular glycosaminoglycans to attract and then sequentially extrude water. This charged mechanical interaction permits cartilage to perform its mechanical functions without appreciable wear (1-3). The functions of articular cartilage include load transmission and distribution, smooth articulation, and aid in lubrication (1, 4). Load transmission and distribution is due to the ability of the structural matrix to deform, which leads to increased joint contact areas and distributed mechanical stresses (1). It also has the ability to respond to applied loads through fluid exudation and redistribution within the interstitial tissue.

Articular cartilage is not a homogeneous tissue. Instead, it has a very complex composition and architecture that permits it to achieve and maintain proper biomechanical function over the majority of a human lifespan. Water constitutes between 65-80% of the entire wet weight of articular cartilage (5) and is about 15% more concentrated at the surface than in the deeper zones (1). Chondrocytes are the only cells of articular cartilage, and they are responsible for the production of the extracellular matrix. Distributed throughout the matrix, chondrocytes compose less than 5% wet weight (1, 2). Collagen makes up about 15-22% of the wet weight and contains 90-95% type II collagen fibers with a small percentage of types IX and XI (1, 2). This is what provides the high tensile stiffness, strength and resiliency of the tissue. Proteoglycans constitute about 4-10% of the total wet weight and are a mix of large aggregating (50-85%) and large non-aggregating (10-40%) proteoglycans (1, 2). They are responsible for pressure elasticity and charged interactions with water (3, 6). Non-

collagenous proteins, elastins, integrins and other macromolecules of protein are responsible for the matrix organization and maintenance (2). The distribution and arrangement of these components, however, is not uniform. Instead, articular cartilage is divided into four zones: superficial, middle, deep, and calcified. The superficial zone is characterised by flattened chondrocytes, relatively low quantities of proteoglycans, and high quantities of collagen fibrils arranged parallel to the articular surface (7). The middle zone, in contrast, has round chondrocytes, the highest level of proteoglycans among the four zones, and a random arrangement of collagen (8). The deep zone is characterised by collagen fibrils that are perpendicular to the underlying bone, and columns of chondrocytes arrayed along the axis of fibril orientation (9). The calcified zone is partly mineralised, and acts as the transition between cartilage and the underlying subchondral bone (9). It is the composition and highly complicated interaction of these components that makes regeneration and replacement techniques of articular cartilages challenging.

Diseases and injuries of articular cartilage

Diseases of the joints affect over 500 million people worldwide (10). Articular cartilage lesions are generated during the course of many joint diseases, notably osteoarthritis, in conjunction with a large number of genetic or metabolic conditions, such as acromegaly, Paget's Disease, the Stickler-Syndrome and hemophilia (11), or as a result of trauma. Traumatic lesions may occur directly or indirectly in consequence of an intraarticular fracture, a high-intensity impact or following ligament injuries (11, 12). Some of the most common diseases related to articular cartilage lesions are arthritis, degenerative joint disease or osteoarthritis, rheumatoid arthritis and osteochondritis dissecans. Approximately 46 million people in the U.S. suffer from some form of arthritis, and that number is expected to rise to 60 million by the year 2020, which will constitute nearly 20% of the projected population (13). This disease causes joint pain, stiffness, and depending on its severity, it can turn simple, everyday activities into arduous tasks. Degenerative joint disease (DJD) represents over 40% of the arthritic patient population (13). It affects nearly 33 million people in the U.S. and accounts for an estimated 7.3 million physician visits per year (13). In simplest terms, the disease is born of cyclic wear-and-tear that results in the breakdown of cartilage at the ends of bones that in turn cause pain in the joint. The disease is commonly referred to as osteoarthritis (OA). The systemic condition, or rheumatoid arthritis, is a far less common autoimmune condition. Rheumatoid arthritis is not an inherited disease, although researchers believe that some people have genes that make them susceptible to the disease

(13). People with these genes will not automatically develop rheumatoid arthritis. Rheumatoid arthritis (RA) affects an estimated one to two percent of the world's population (10). Osteochondritis dissecans (OCD) is a disorder in which a fragment of cartilage and subchondral bone separates from an articular surface (11). The etiology is uncertain, although trauma and ischemia have been implicated. It has been estimated that 4% of all cases of osteoarthritis of the knee diagnosed in men were the direct result of OCD (10).

Articular cartilage injuries may occur as a result of either traumatic mechanical destruction or progressive mechanical degeneration. With mechanical destruction, a direct blow or other trauma may injure the articular cartilage. Occasionally, an articular cartilage fragment breaks loose from the underlying bone. This fragment, called a loose body, may float in the joint, interfering with normal joint motion. Progressive mechanical degeneration of the articular cartilage, commonly referred to as wear and tear, occurs with the progressive loss of the normal cartilage structure and function. This initial loss begins with cartilage softening and proceeds to actual fragmentation of the cartilage. A loss of articular knee cartilage continues, the underlying bone has no protection from the wear and tear of daily living and begins to break down, an event that may lead to OA. Causes of progressive mechanical degeneration of the articular knee cartilage include high-impact twisting injuries, joint instability and inadequate muscle strength or endurance (13).

Self repair capability of articular cartilage

In articular cartilage, the combination of the lack of blood supply and a few cells distributed widely amongst a dense extracellular matrix leads to a limited ability to heal (1, 11, 14, 15). The usual inflammatory response of hemorrhage, formation of fibrin clot, cellular production and migration of mesenchymal cells is absent (11). However, spontaneous repair of articular cartilage may eventually occur depending on the depth of the lesion. While surface defects that do not penetrate the subchondral bone have to rely on sparsely populated chondrocytes for matrix remodeling, deeper lesions may introduce a blood supply from the well-vascularised subchondral bone (16). With the blood come various types of stem cells and fibrocytes that modulate to fibrochondrocytes (17-19). In addition, large quantities of growth factors are also released from the bone (20-22), playing an important role in initiating the repair response. Nevertheless, these combination of cells and growth factors produce a relatively disorganised network of collagen fibres partially filling the defect with structurally

weak tissue (16). The heterogeneous composition and inferior biomechanical properties of spontaneously formed cartilage undoubtedly contribute to its functional incompetence and perishability (16). Apart from the depth and degree of damage, other factors such as age, traumatic or chronic condition, associated instability, and genetic predisposition are also factors affecting healing of cartilage (1, 2, 11). For example, age affects healing in part because in newborns, the multi-functioning mesenchymal stem cells needed for healing account for 1 in every 10,000 cells in bone marrow and reduces to 1 in 100,000 in teens, 1 in 400,000 by age 50 and 1 in 2 million in an 80 year old (16). Clearly, for a successful (reproducible and durable) repair result, a more homogeneous repair cell population, which is capable of producing hyaline-like cartilage, is required.

Table 1. Current therapeutic techniques for the treatment of articular cartilage defects

Methods	Techniques	Disadvantage
I-Lavage and arthroscopy		
Lavage	Irrigation of a joint with solutions of NaCl, Ringer or Ringer and lactate.	Only short term pain relief. Not solid evidences of any biological or repair activity being instigated.
II-Debridement		
Shaving and debridement of cartilage	Mechanical removal of diseased chondral tissue.	Instability of the joint. High rate of re-injury.
Thermal chondroplasty (laser and radiofrequency)	Alternative debridement by heat or radiofrequency energy.	and tissue necrosis by thermal damage.
II-Marrow Stimulation Techniques		
Abrasion chondroplasty	All these techniques involve surgical access to the subchondral bone until bleeding occurs inducing spontaneous repair responses.	Repair tissue becomes fibrocartilaginous (less durability and strength). Restriction of mobility.
Multiple drilling (Pridie drilling)		
Microfracture		
III-Transplantation		
Autologous grafting (periosteal/perichondrial/osteochondral plug transfer)	Replace lost cartilage with tissue grafts from own patient.	Poor fixation of graft to damage area. Inflammatory and joint mobility restriction. Donor site morbidity and availability.
Allogeneic grafting	Replace lost cartilage with tissue grafts from different individuals.	Immunological reaction and disease transmission. Limited supply of graft. Handling and storage of frozen tissue.
IV-Cell-based therapies		
Autologous Chondrocyte Transplantation	Removal of patient own cells by biopsy. *in vitro* cell culture and implantation into defect under a periosteal patch.	Expensive treatment. Two surgical procedures. Potential damage to surrounding cartilage. Restricted activity to allow cell integration. Long term degeneration.
Marrow Stromal Cells		Only animal models so far.

Currently available repair techniques

Articular cartilage injuries affect more than 1 million people each year in the U.S., with approximately 600,000 surgical procedures being performed annually for the treatment of these injuries (10). The limited ability of articular cartilage to self repair has led to a wide variety of treatment approaches for focal chondral defects with varying levels of success (16, 23). These techniques may be classified either as 1) debridement of loose or impinging chondral flaps; 2) stimulation of the intrinsic repair mechanism from subchondral bone; 3) transplants to fill the defect with autografts or allografts; 4) cell-based therapy to regenerate the chondrocytes and surrounding matrix; or 5) combinations of these techniques with growth factors or biologically active carriers to influence the repair process. Most of these techniques are briefly described in Table 1.

Tissue engineering approach

An alternative therapy for the repair of damaged articular cartilage resides in the tissue engineering approach. Tissue engineering is an interdisciplinary field that applies principles and methods of engineering and the life sciences toward the development of biological substitutes that restore, maintain and improve the function of damaged tissues and organs (24). Such tissue reconstitution process can be conducted either entirely *in vitro* or partially *in vitro* and then completed *in vivo*. In Figure 1 a schematic representation of this technology is depicted. In the process of articular cartilage tissue engineering, two main phases can be identified. A first phase where few isolated cells need to be expanded in order to provide sufficient cells and a second phase where a cartilage is engineered either inside the body (*in vivo*) or in a cell culture (*in vitro*) using an appropriate scaffold. Tissue engineering can potentially use cells taken either from the patient (autologous) or from a donor (allogeneic), and these cells may be mature cells (e.g., chondrocytes) or immature cells (e.g., mesenchymal stem cells). Scaffolding technologies plays a crucial role in this technology. Biocompatible scaffolds, the most common of which involve collagen or polymer (25), allow cells to be grown in systems such as rotating bioreactors (26, 27), and spinner flasks (28). Expanded cells are seeded onto three dimensional scaffolds to form cell-polymer constructs, which are cultured *in vitro* and then used either as implants or for *in vitro* research. All these systems are designed not only to maintain the proper culture medium and environmental conditions

for cell propagation and growth, but also to transport the living construct to the ultimate operating site.
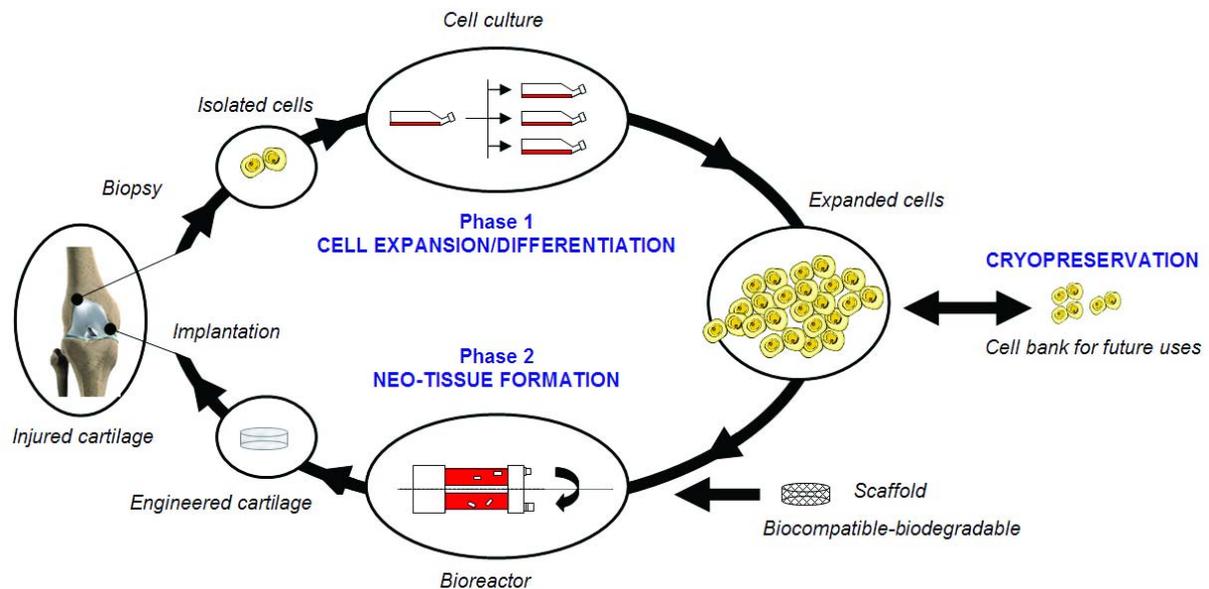
Fig. 1. Schematic representation of articular cartilage tissue engineering. Cells are harvested from the articular cartilage, expanded in tissue culture flasks and seeded on a biocompatible-biodegradable scaffold. The cell/scaffold constructs undergo a period of dynamic tissue culture in a bioreactor prior to implantation. This figure was adapted from reference (55) with permission.

Since 1991, when Vacanti *et al.* (1991) produced new hyaline cartilage from bovine chondrocytes on a polymer scaffold, tissue engineering of articular cartilage has notoriously evolved. A wide variety of scaffolds have been used to produce cartilage *in vitro*. Form and composition of scaffolds range from non-woven meshes and foams of alpha-hydroxypolyesters (29, 30), polyglactin (31) or hyaluronan alkyl esters (32) to photocrosslinked hydrogels (33) and sponges based on different types of collagen and glycosaminoglycans (34). Scaffolds filled with cells embedded in a fibrin or alginate gel have also been proposed (31, 35). The design of specific bioreactors have also improved the formation of cartilage tissues by providing an efficient and spatially uniform cell seeding and by allowing a better diffusion of nutrients to the cells into the scaffold. Cartilaginous tissue has been repeatedly engineered *in vitro* by using articular chondrocytes cultured on three-dimensional biodegradable scaffolds in tissue culture bioreactors. The success of the development of structurally suitable cartilaginous matrix *in vitro* has been found to depend on many culture conditions including cell seeding density and conditions (28, 36-38), the *in vitro* culture environment (28, 39, 40), bioreactor design (41) and the mechanical deformation and stimulation of engineered tissues under controlled conditions (42-47). The advances in all

Table 2. Selected current and emerging chondrocyte transplantation products. (Source: Medtech Insight, 2003)

Company	Product (s)	Description	Regulatory Status
Smith & Nephew	NeoCyte	Developed in collaboration with Advance Tissue Sciences, this product is designed to circumvent the need for total knee replacement	Preclinical trials. In 2003 both companies filed for IDE approval
	Stromal Tissue Culture System	Three-dimensional stromal tissue culture system	Development stage
Cell Transplantation Innovations/ProChon Biotech	BioCart	Designed for the propagation of human cartilage cells as a basis for autologous chondrocyte transplantation to treat articular damage in knees	Approved for commercial use in Israel
Co.don Tissue Engineering	Co.don chondrotransplant	Cultured autologous cartilage cell transplant for repairing focal cartilage defects in the knee	Clinical trials
Genzyme	Carticel	Autologous cultured chondrocytes	FDA approved in 1995
	Carticel II	Second-generation product	Phase II clinical trials
	Quick Tack	Tack for attaching periosteum. Osteoarthritis treatment	Phase II clinical trials
IsoTis	VivesCart	Second-generation tissue engineering cartilage developed in conjunction with Massachusetts Institute of Technology	Preclinical trials. FDA approval expected by 2005
Isto Technology	Istocyte Chondral Allograft	Three-dimensional articular cartilage graft formed using pediatric cadaveric donor chondrocytes	Preclinical trials
Verigen Transplantation Service International	CACI	One-step procedure that replaces excised damaged tissue with a purified resorbable porcine collagen membrane	CE-Mark approved
	MACI	Two-steps process that involves culturing autologous chondrocytes and inserting them into a collagen matrix which is then implanted into the defect site	Clinical trials
	MACI (A)	Alternative approach to the MACI procedure	Development stage

FDA = Food and Drug Administration. IDE = Investigational Device Exemption. CE = *Conformité Européene*. CACI = Collagen-covered Autologous Chondrocyte Implantation. MACI = Matrix-activated Autologous Chondrocyte Implantation. Advance Tissue Science' assets were acquired by Smith & Nephew in May 2003.

these areas have allowed the achievement of suitable (biochemical and histological) engineered constructs. Such engineered constructs showed both high fractions and homogeneous distributions of proteoglycan and collagen, with collagen type II representing the dominant fraction of total collagen (39).

Widespread discourse about the early experiments with tissue engineering has generated public demand and expectations that engineered tissues will be available before long. There are, however, critical hurdles that still need to be overcome. In the case of engineered cartilage, it would seem preferable to avoid harvesting of normal tissue and have a single operation for the implantation of the engineered tissue. In addition, consideration of the limited proliferative and regenerative capacity of adult chondrocytes and their potential dedifferentiation upon expansion leads to the goal of an alternate source of cells. Regarding the scaffolds, only limited materials are currently available and approved by the regulatory authorities (13), and innovative synthetic materials, such as polypeptides or novel biodegradable polymers, need to be further improved with regard to: i) nature of polymers favoring cell-substrate interactions and bioactive molecules release; ii) pore size and scaffold geometry (foam, mesh or gel) that affects the induction/maintenance of the chondrocyte phenotype (48).

Finally, there remain difficulties in the incorporation of neo-cartilage with adjacent healthy tissue (49). There is incomplete understanding of the relationship between cartilage and vascular response to wounding. Engineered cartilage needs to attach to the implantation site without evoking an angiogenic response. Enhancement of cartilage antiangiogenic activity may be needed, but these metabolic processes are not yet fully understood (49).

At the moment, cell-based therapies involving the implantation of cells are the centre of most of the innovations emerging in the field of articular cartilage repair (Table 2). However, some three-dimensional tissue engineering products can be currently found in their development stages (e.g., Stromal Tissue Culture System, Smith&Nephew; Table 2). Success in tissue engineering technology would obviate the need for tissue transplantation, and if the appropriate precursor cell pools could be obtained from embryonic, fetal or adult allogeneic sources, then the numerous problems associated with the use of donor tissue would be avoided.

Challenge of cell expansion in cartilage tissue engineering

One of the challenges that tissue engineers will have to address in the near future is the development of feasible large scale cell expansion processes. The estimated number of articular cartilage incidences worldwide is around 30 million cases of knee osteoarthritis and 1.2 million cases of focal defects every year (50). If tissue engineering products are to be used in the future for the treatment of all these incidences, it is crucial to estimate the scale of cell production needed for all these repair procedures, essential question that is rarely approached in reported tissue engineering studies. Such estimation has been presented in Table 3. As a guideline for this estimation, it has been presumed a typical size of focal defect in articular cartilage of 4 mm thick and 40 mm in diameter, while for osteoarthritis defects, two implants (opposing joint surfaces) of 4 mm x 40 mm would be required. In the literature, few systematic studies have addressed the effects of the initial cell density on cartilage formation. It is known that polylactide-polyglycolide scaffolds seeded with a density of less than 10 million cells per ml will typically result in little, if any cartilaginous material (36, 51).

Table 3. Estimation of cell necessities for tissue engineering treatments

Indication	Incidence of surgical procedures Worldwide		
Focal defects in Articular Cartilage	1.2×10^6		
Osteoarthritis (OA of the knee)	3×10^7		
Tissue engineering treatment		**Number of implants**	**Cells required** [a]
Focal defects in Articular Cartilage			
Typical human defect size (4mm x 40mm) [b]		1	3.2×10^8
10% of focal defect incidences [c]		1.2×10^5	3.9×10^{13}
Osteoarthritis			
Typical human OA (2 x 4mm x 40mm) [b]		2	6.4×10^8
10% of OA defect incidences [c]		3×10^6	1.9×10^{15}

[a] 64 million cells/cm^3
[b] Typical sizes provided by Smith&Nephew Research Centre, York, UK
[c] Assuming 10% of incidences are appropriate for treatment

As the cell seeding density increases, the integrity of the cartilaginous product appears enhanced and distribution of the cartilaginous material more homogeneous. Of course, not all of the cells in initial seeding solutions attach to scaffolds. Whether the attached cells represent a subpopulation that exhibits some preferential property has not been addressed. However, as a means to get to an end product, seeding at high cell density seems to be

desirable. One study that systematically tested for the effect of cell seeding density on cartilage formation revealed that seeding scaffolds at a density ranging from 20 to 100 million cells per ml resulted in formation of clinically appropriate cartilage when implanted subcutaneously into nude mice; lower seeding density (e.g., 10 million cells per ml) exhibited reduced cartilaginous formation when assessed by wet weight and thickness (36). Consequently, in the estimations presented in Table 3, a seeding density of 64 million cells/ml has been considered as appropriate. This value of seeding density would result in cell demands of 320 and 640 million cells for the treatment of one single focal defect and osteoarthritis defect respectively. Although not all the registered incidences may be suitable for tissue engineering treatments, a recent study have shown that approximately 10% of all knee arthroscopies showed cartilage defects that may be appropriate for cartilage repair procedures (52). Therefore, considering just 10% of all the surgical procedure incidences, the total demand of cells that the production of tissue engineering products would have to face are 3.84×10^{13} and 1.92×10^{15} cells for focal defects and osteoarthritis defects respectively (Table 3). The question is: can routine tissue culturing methodologies cope with this scale of cell production? If we think about autologous applications and assume the possibility of harvesting 2 million articular chondrocytes from a donor's biopsy (53), single treatments of our presumed cartilage defects would require a 160 fold (focal defect) and 320 fold (osteoarthritis) multiplication of the donor cells, values that seems to be achievable with standard culture methodologies (53). However, the future of cartilage tissue engineering may not be in the use of adult autologous chondrocytes applications. The limitations of adult chondrocytes to maintain their phenotype expression and differentiation ability after extensive expansion *in vitro* has led to the investigation of the potential use of pluripotent stem cells and progenitor cells as a source for tissue engineering. In addition, autologous applications include the need for two surgical procedures (biopsy followed by implantation), and it has associated an expensive cost (16). Although success in tissue engineering would eventually obviate the need for tissue transplantation, only if the appropriate precursor cell pools are obtained from embryonic, fetal or adult allogeneic sources, then the numerous problems associated with the use of donor tissue would be avoided. It would be especially in these potential allogeneic applications where the expansion of the cell population *in vitro* becomes an essential step in the development of feasible large scale process of tissue engineering of articular cartilage. Both optimization of the culture conditions and the validation of a cryopreservation procedure to form cell banks are fundamental challenges that need to be addressed (54, 55).

Dedifferentiation and redifferentiation of chondrocytes

Dedifferentiation

In the process of cartilage tissue engineering, it is important to ensure that the expanded cell population retains its phenotypic function. However, a major problem of mammalian articular chondrocytes in culture is the phenomenon of dedifferentiation (56). As a result of this process of dedifferentiation, chondrocytes isolated from their tissue-specific extracellular matrix fail to produce cartilage matrix after extensive expansion in monolayer culture. After suspension in growth medium chondrocytes adhere at the bottom of cell culture plastic wares (monolayer cultures) where they can grow and proliferate to form a confluent cell layer. However, the cells lose their morphology as well as their biochemical and functional properties. Such dedifferentiated cells behave completely differently compared to the cells in their original tissue environment. After a few days in monolayer culture, they begin to change their appearance to fibroblast-like morphology (57). The typical formation of chondrons and pericellular matrix is not observed in monolayer culture. Biochemical investigations reveal a switch of collagen synthesis. Thus, instead of the cartilage-like collagen type II, cultured chondrocytes mainly synthesize collagen type I and III which are absent from normal cartilage (58). Also the dedifferentiated chondrocytes present gradual shift from the synthesis of large aggregating proteoglycans (aggrecan) to low molecular weight proteoglycans (versican) and a decrease of alkaline phosphatase (AP) activity (57, 59, 60). This phenomenon has been particularly studied in culture. Dedifferentiation occurs when chondrocytes released from their extracellular matrix are cultured under conditions that promote a flattened cell morphology, such as at low cell density in monolayer, and it is gradually manifested as soon as at passage 1 (61). Although the mechanisms involved the dedifferentiation process are not fully understood, it is believed to be mediated by the formation of actin stress fibers which occurs when the cells spread on an adhesion-permitting substrate (59, 62).

This phenomenon of cell dedifferentiation is probably the most important limitation on the expansion potential of adult articular chondrocytes in tissue engineering applications, where extensive expansion would be eventually necessary for the development of feasible large scale operations. Moderate seeding densities and frequent subcultivations usually optimize growth of most cell types. However, if that approach is used for chondrocytes, there is a

progressive, and often irreversible loss of function (49, 60). In addition, chondrocytes derived from articular cartilage biopsies have only a limited proliferative potential. Not only they dedifferentiate upon repeated passaging (60), but also the number of cell divisions chondrocytes undergo *in vitro* decreases with age (63). It has been known for many decades that chondrogenesis can be enhanced if chondrocytes are seeded at high density (64), suspended in solution (65), isolated chondrons (66), or culture as pellets (56). However, conditions that favor maintenance of phenotype are usually not those that favor increases in numbers (67). As a result, there may be limitations in the numbers of suitable cells that can be grown *in vitro* for subsequent repair of cartilage defects.

Redifferentiation

Dedifferentiated chondrocytes appears to be very similar to fibroblasts (68). However, in contrast to fibroblasts they can still redifferentiate to normal chondrocytes. Expanded chondrocytes in monolayer culture are probably still determined by a cell memory system to form cartilage in a favorable environment. Although the mechanisms involved in restoration of the differentiated phenotype have not been fully elucidated yet, the dedifferentiation process is believed to be mediated by the formation of actin stress fibers which occurs when the cells spread on an adhesion-permitting substrate (59, 62). It is also well documented that dedifferentiation can be prevented or reversed (redifferentiation) by culturing chondrocytes under conditions that inhibit cell flattening, preventing the formation of stress fibers (69-73). Some of these culture conditions are:

High cell density cultures

The beneficial effect of cell density on the phenotype stability of articular chondrocytes was early investigated in monolayer cultures (61). Briefly, after three weeks in culture, chondrocytes grown at high density expressed predominantly large proteoglycans that aggregated with hyaluronic acid, whereas in low-density cultures a smaller, non-aggregating form was present. Additionally, both high and low density were expressing type I collagen, although the high-density cells also had an extensive extracellular matrix of type II collagen. These early observations supported the conclusion that high seeding density stabilises the chondrocyte phenotype to a greater extent than low seeding density. They also suggested that

enhanced dedifferentiation at low density may be due to cell spreading, rather than to selective proliferation of a phenotypically unstable subpopulation of cells.

Pellet cultures

Articular chondrocytes has also been maintained in high-density aggregated cultures (74-77). This system has been reported to support cell proliferation while maintaining the chondrocyte phenotype (78, 79), although the strong interactions cell-cell generated during this kind of culture make the harvesting of viable chondrocytes difficult to achieve.

Hydrogel cultures

Dedifferentiated chondrocytes can reexpress phenotypic markers of the articular chondrocyte when they are cultured in three-dimensional hydrogels such as agarose, collagen and alginate (80). Dedifferentiated chondrocytes propagated as a suspension culture in agarose gels re-expressed a differentiated phenotype where both collagen and proteoglycan returned to rates exhibited by primary chondrocytes (60). According to these authors, approximately 80% of the cells survived the transition from the flattened morphology of anchorage-dependent culture to the spherical morphology of anchorage-independent culture and then deposited characteristic proteoglycan matrix domains. The rates of proteoglycan and collagen synthesis returned to those of primary chondrocytes, demonstrating a complete return to the differentiated phenotype. Type I collagen, presumably because of its availability in large quantities relative to other collagen types, has also been utilized as a gel substance for culture of chondrocytes. Collagen gels have been demonstrated to support chondrocyte proliferation (81-84), although data addressing the ability of collagen gels to support the synthesis of cartilaginous material *in vitro* is minimal. Matsusaki *et al*. (1998) reported that even under the use of basic fibroblast growth factor (bFGF) for stimulation of proliferation, chondrocytes in collagen gel culture were able to maintain the differentiated phenotype for 4 weeks, although other authors reported that depending on the type of collagen gel used, the percentage of cells that maintained a chondrocyte-like morphology after 1 week in culture varied form 40 to 90% (81). Therefore, the effect of collagen gels on chondrocyte phenotype and the capacity of redifferentiation remain an issue. Finally, it has been also reported that articular chondrocytes remain metabolically active when cultured in alginate beads (85-87). Although there seems to be an initial cell loss after transfer to the alginate beads, the remained cells are able to proliferate and maintain their typical chondrocyte phenotype (88). The potential of this system to redifferentiate the chondrocytes has also been investigated by cultivating them in

alginate beads after monolayer expansion. This resulted in the reexpression of two main markers of differentiated chondrocytes (aggrecan and type II collagen) although several weeks were necessary for total suppression of type I and III collagen synthesis (indicators of a modulated phenotype) and to reach a steady state of cell proliferation and synthesis of fully differentiated proteoglycans (89, 90). Additionally, the extent of proliferation and redifferentiation were seemed to be dependent on the formation of clonal populations of chondrocytes and correlated inversely with the initial cell seeding density (91).

Three-dimensional cell carriers

As with chondrocytes cultures in hydrogels, culturing chondrocytes on appropriated carriers that allow a three-dimensional distribution of the cell and hence inhibit cell flattening can prevent and/or reverse dedifferentiation. This seems to be the case of microcarrier cultures. For instance, the use of collagens type I microcarriers (cellagen) has been reported to support both chondrocyte proliferation and phenotype expression (92). Articular chondrocytes propagated in serial passages (5 passages) as monolayer and re-seeded at passage 6 in cellagen microcarriers had no detectable staining for collagen type I and stained intensely for collagen type II. More recently, macroporous gelatin microcarriers (CultiSpher-G) have been also found to be effective matrices for human nasal chondrocyte (93) and bovine chondroprogenitor cells (94) expansion, while maintaining the ability of chondrocyte differentiation. These studies demonstrated that the microcarrier suspension culture system supports growth and enhances expression of the differentiated phenotype. Attachment to a constrained surface and the fluid shear forces on the microcarriers during suspension culture may have helped chondrocytes to reacquire their rounded shape and produce cartilage matrix components. Although the exact mechanism by which chondrocyte redifferentiation is induced through microcarrier expansion has not yet been elucidated, this technique shows promise for cartilage tissue engineering approaches. The maintenance of the chondrocyte phenotype has been also reported when cultured in three-dimensional biodegradable polymer scaffolds to regenerated cartilaginous tissues *in vitro* (95). In this system, the surface chemistry and geometry of the polymer scaffold, (e.g., polyglycolic acid (PGA) mesh) with fiber diameter comparable to that of a chondrocyte, minimize the focal points for cell adhesion and thus prevent cell flattening (28, 95). This ability to support the growth of human chondrocytes and to maintain their original phenotype has been also reported using a hyaluronic-acid-based biodegradable polymer (96). Human chondrocytes, expanded in monolayer cultures for three to four passages and seeded on this material were able to express

and produce collagen type II and aggrecan and downregulate the production of collagen type I.

Growth Factors

Growth factors can in principle modulate chondrocyte proliferation and differentiation (97). It has been reported that chondrocytes expanded in monolayer for two passages in the presence of fibroblast growth factor-2 (FGF-2) dedifferentiate, but fully maintained their potential for redifferentiation in response to environmental changes (98) and also modulated the subsequent responsiveness of the cells to bone morphogenetic protein-2 (BMP-2). The use of sequential exposure of bovine calf articular chondrocytes to FGF-2 during monolayer expansion and to BMP-2 during three-dimensional culture on PGA scaffolds improved the engineering cartilage tissue (99). In addition, it has been demonstrated the chondrogenesis of human adult articular chondrocytes can be enhanced if they are expanded in the presence of a combination of factors and differentiated with factors belonging to the same superfamily of BMPs (100). Concretely, it has been reported that chondrocytes expanded in monolayer in the presence of FGF-2/TGF-β displayed a higher proliferation rate and more dedifferentiation, but also higher capacity to redifferentiate in response to supplementation of serum free medium with TGF-β and dexamethasone during three-dimensional cultures. Similar results have been recently reported by culturing bovine chondroprogenitor cells in the presence of TGF-β1 (55). These results evidence that growth factors during chondrocyte expansion not only influence cell proliferation and differentiation, but also the cell potential to redifferentiate and respond to regulatory molecules upon transfer into a three-dimensional environment.

The issue of phenotype expression and differentiation is of vital importance on the success of cartilage tissue engineering applications. All the approaches described above could potentially improve the redifferentiation capacity of autologous chondrocytes isolated from a small biopsy and expanded *in vitro*. Nevertheless, unless the expansion is performed in three-dimensional carriers soon after isolation, it has not yet been demonstrated that extensively expanded chondrocytes can be induced by exogenous factors to regenerate cartilaginous tissue at a rate and to an extent comparable to freshly isolated chondrocytes. Moreover, there are also evidences suggesting that it is increasingly difficult to redifferentiate them in three-dimensional cultures after extensive monolayer expansion (82). The problem resides in the

fact that is precisely extensive expansion what it would be necessary for the development of feasible tissue engineering products in the near future. Whether from allogeneic sources, or from some kind of autologous progenitor cells, the use of alternative sources of cell seems to be mandatory for the success of tissue engineering therapies.

Alternative source of cells

Establishing a reliable source of cells is a principal priority for tissue engineers (101). Cells used in tissue engineering may be drawn from a variety of sources, including primary tissues and cell lines. Primary tissues may be xenogeneic (from different species), allogeneic (from different members of the same species), syngeneic (from a genetically identically individual) or autologous (from the same individual).

Xenogeneic and/or allogeneic

Although animal cells are a possibility, ensuring that they are safe remains a concern, as does the high likelihood of their rejection by the immune system (102). For those reasons, human cells are favored. Currently, the use of allogeneic cells in the setting of cell-polymer constructs is still limited by the need for host immunosuppression. However, with the advent of techniques to render cells immunologically "transparent", the use of banked xeno/allogeneic cells may become a clinical reality (101). The creation of cells that could be used as universal donors might for instance be achieved by using molecules that mask the histocompatibility proteins on the cell surface that normally identify the donor cells as non-self (103-105). This type of approach is being explored to make pig cells acceptable for transplantation to patients with Parkinson's disease (105). In principle, such universal donor cells would not be expected to be rejected by the recipient; they could be generated for various types of cells from many different tissues and kept growing in culture until needed. Nevertheless, it is not yet clear how universal donor cells will perform in large-scale clinical trials (101).

Stem and progenitor cells

Because of the difficulties exposed before for the different cell sources, the majority of tissue engineering advances to date have employed primary autologous cells (106). In the case of articular cartilage, the limitations of adult chondrocytes to maintain their phenotype expression and differentiation ability after extensive expansion *in vitro* has led to the

investigation of the potential use of pluripotent stem cells and progenitor cells as a source for tissue engineering. The recent identification and isolation of human embryonic stem cells offers one approach to the problem. Cells derived from human embryonic blastocysts can be proliferated through multiple generations and made to differentiate into the appropriate cell type (107). Recent studies on embryonic stem cells involved in the creation of cartilage has shown encouraging results (108, 109), suggesting that in the future these cells may become a potential tool for repairing articular cartilage defects. However, researchers are still a long way from being able to manipulate embryonic stem cells in culture to produce fully differentiated cells that can be used clinically (102). In addition, the use of embryonic stem cells poses major ethical problems that need to be addressed.

A more immediate goal would be to isolate adult stem cell or progenitor cells present in a differentiated tissue. Such progenitors have taken some of the steps toward becoming specialized, but because they are not yet fully differentiated they stay flexible enough to replenish several different cell types. Also, in comparison to the embryonic stem cells, the use of adult stem cells is generally well accepted by the society. Their progeny includes both new stem cells and committed progenitors with a more restricted differentiation potential. These progenitors in turn give rise to differentiated cell types (110). After transplantation some of these cells engraft tissues other than those of their own origin and respond to alternate environmental cues by displaying the phenotypes of such host tissues (111).

This approach has included mesenchymal stem cells, which are capable of differentiating into bone, cartilage, tendon and muscle (112-114). Bone marrow mesenchymal stem cells or stromal cells (BMSCs) are responsible for the maintenance of bone turnover through life and can be regarded as a mesenchymal progenitor/precursor cell population derived from adult stem cells. Cultured BMSCs can be stimulated to differentiate into bone, cartilage, muscle, marrow stroma, tendon, fat and a variety of other connective tissues (115, 116). The harvest of a limited bone marrow sample is an easy and relatively safe procedure. Large numbers of BMSCs can be obtained in culture, making it possible to engineer transplantable constructs composed of these cells in appropriate scaffolds (48). The properties of BMSCs are deeply influenced by the microenviromental conditions. Culture conditions for BMSC remain essentially the same as the ones originally described by Friedenstein *et al*. (1966). However, culture conditions that allow expansion without loss of differentiation potential are difficult to establish for most adult stem cells (48) and to obtain a large number of chondroprogenitors,

the effects of several growth factors on proliferation and differentiation of BMSC are being investigated (112, 117-119). Although cells potentially useful for connective tissue engineering can be isolated from a variety of tissue, BMSC remain, at the moment, the most interesting and widely accepted cell system to be used for both preclinical and clinical studies of cartilage regeneration and repair (48). Whereas a cartilage biopsy from the joint to obtain differentiated chondrocytes for cell therapy represents and additional injury to the cartilage surface, possibly detrimental to the surrounding healthy articular cartilage, the use of BMSCs avoids this problem altogether. Furthermore, BMSC can be transduced with various viral vectors and are, thus, interesting potential candidates for somatic gene therapy in local or systemic pathologies (120). A critical issue for the future, from a tissue engineering standpoint, is to learn how to control the permanent differentiation of stem cell populations into the desired cell types. There are also a number of technical hurdles such as the need for pure stem cell preparations (i.e., those without other cells such as fibroblast mixed in), methods to reduce cell adhesion during culture, and processes to increase the production of the large numbers of cells needed to create tissue (101).

Alternatively, cells with chondro-osteoprogenitor features have been isolate from several tissues, including periosteum, spleen, thymus, skeletal muscle, adipose tissue, skin, retina and articular cartilage surface (48, 121-127). However, for several reasons such as hardly accessible tissue source, low cell frequency and limited information the use of the majority of these progenitors in tissue engineering has not been always straightforward (48).

One of alternative population of chondroprogenitor cells have been recently identified from the superficial zones of articular cartilages (121). The articular cartilage is not a homogeneous tissue, with biochemical and morphological variations existing from the surface zone to the deeper calcified layer. Consequently, the differentiation and proliferation events occurring during the development of articular cartilage must be strictly controlled both temporally and spatially in order for the distinct zonal architecture of the tissue to be established. Various studies have shown that the surface zone of articular cartilage is centrally involved in the regulation of tissue development and growth. Not only does the surface of articular cartilage play a major role in the morphogenesis of the diarthrodial joint via differential matrix synthesis (128), but also the expression of many growth factors and their receptors at the articular surface (129, 130) suggest that this region represents an important signalling centre. In fact, it has been shown *in vivo* that the surface zone of the

articular cartilage is responsible for the appositional growth of articular cartilage (130). For such a mechanism to occur, a population of stem/progenitor cells must reside within the articular cartilage to provide transit amplifying progeny for growth. In that way, new progeny are generated at the articular surface, expand within the transitional zone and progress to terminal differentiation in the lower zone (130). Recently a number of studies have corroborated this hypothesis by the identification and partially characterization of a population of cells isolated from the superficial zone of the articular cartilage (55, 121, 131-133). These characteristics have included a prolonged cell cycle time at the articular surface (130), differential adhesion to fibronectin, differential integrin and Notch family expression and an ability to form large numbers of colonies *in vitro* from a low seeding (131, 132), properties that are common to known progenitor cell populations of other tissues (134).

Additionally, this population of cells exhibits a significant degree of plasticity in its differentiation pathways (121), another requisite of any stem cell population (135). The engraftment of bovine surface zone-derived cells in an embryonic chick tracking system resulted in the formation of a variety of connective tissue types including bone, tendon and perimysium (121). Moreover, the chondrogenic ability of this cell population has also been proved *in vitro*. Using a specific medium similar to that reported for the induction of chondrogenesis with mesenchymal stem cells, Martin *et al*. showed that this population of superficial zone cells were able to synthesise hyaline cartilage matrix when cultured in three-dimensional pellets (55). Further studies have also demonstrated the high expansion potential of these chondroprogenitor cells in comparison to normal bovine chondrocytes (133). The cells retained the ability to synthesise a cartilage-like hyaline matrix rich in glycosaminoglycans and collagen type II even after 11 passages which equated to 25 population doublings. On the other hand, normal chondrocytes isolated from bovine articular cartilages rapidly dedifferentiated in monolayer and had completely lost the ability to redifferentiate in pellet culture after 8 passages (or 13 population doublings), although this loss of redifferentiation potential could had occurred at an earlier stage of expansion as suggested by other authors (136, 137). The enhanced potential of these articular cartilage progenitor cells to retain the ability to form cartilage after extensive expansion in culture could constitute a major step forward for cartilage repair as it may enable the generation of large cell banks for use in future allogeneic tissue engineering applications. Investigations are currently focusing on employing these cells in cartilage repair strategies. It seems that the undifferentiated nature of these progenitor cells could be used to reproduce the structural and

hence biochemical properties of normal articular cartilage and thus integrate more fully into articular cartilage lesions. On this regard, it have been recently shown in a bovine model that a transplanted chondroprogenitor enriched population performs better at filling a partial depth lesion in a short period of time than a typical population of chondrocytes taken from the full thickness of articular cartilages (138).

Large scale culture systems for expansion of chondrocytes

Currently available culture systems for cell expansion are described bellow. The advantages and disadvantages of these systems are summarized in Table 4.

Table 4. Selected culture systems for anchorage-dependent cells

Culture System	Advantages	Disadvantages
T-Flask	Simple Well characterized	Difficult to scale-up Labour intensive No continuous control of culture parameters No perfusion of culture medium
Cell Factories	Large scale (relative to T-flasks)	Still labour intensive in large scale No continuous control of culture parameters No perfusion of culture medium
Roller Bottle	Large surface area to volume ratio (relative to T-flask)	Still labour intensive in large scale No continuous control of culture parameters No perfusion of culture medium
Cell-Cube^{TM}	Large surface area to volume ratio (relative to T-flask) Perfusion of culture medium	Expensive Still labour intensive in large scale No continuous control of culture parameters
Pellet cultures	Simple Perfusion of culture medium Culture parameters can be continuously controlled	Cell harvesting Reduced proliferation rates
Hydrogel cultures	Perfusion of culture medium Culture parameters can be continuously controlled	Mass transfer limitation may occur Reduced proliferation rates
Microcarrier cultures	Greatest surface area to volume ratio Perfusion of culture medium Culture parameters can be continuously controlled Well documented technology	Cell exposed to shear forces Some cell types show reduced growth compared to monolayer culture

Monolayer culture

Expansion of anchorage-dependent cells, including primary chondrocytes or chondroprogenitor cells, largely rely on simple monolayer culture flasks (i.e., T Flasks, Petri dishes or tissue culture well plates) to date. However, if large scale operations are to be considered, alternatives to these traditional tissue culture flasks are mandatory. There are currently available several alternatives of large scale culture vessels and systems from different manufacturers. One of these alternatives is based on the roller bottle technology. The concept of growing cells as rotating cultures was originally developed at Johns Hopkins University (139) as a mean of growing larger quantities of anchorage-dependent cells. Since then, much larger glass roller bottles have been in common use for growing large numbers of cells, especially for viral vaccine production (140, 141). Besides providing larger surface areas for growth, this culture technique may also have two additional advantages over traditional static monolayer cultures: first, its gentle agitation prevents gradients from forming within the medium that may adversely affect growth; second, cells spend most of their time covered by only a thin layer of medium allowing superior gas exchange. The RollerCell 40 (CELLON S.A., Luxembourg) is a self-contained, automated roller bottle processing system , and its use has been recently reported for large scale production of retroviral vectors (142). Up to ten RollerCell 40 units can be linked together in series with a single CPU (RollerCell Max; CELLON S.A., Luxembourg), providing a surface area of 3.5×10^5 cm^2 (equivalent to 200 roller bottles of 1750 cm^2). An alternative to roller bottles is the Corning CellCube System (Corning Incorporated, USA), an integrated modular bioreactor system that has been successfully used in a variety of large scale culture applications (142-146). This system allows continuous perfusion of fresh media and can be scaled up to 3.4×10^5 cm^2 of growth surface using the same control package. Finally, if the choice of static systems is still preferred, multitray battery systems would be recommended. This system is designed for large scale operations, and its use has been reported in a variety of culture processes, including the production of human fibroblast interferon (147, 148). Two commercially available multitray systems are the Corning CellSTACK (Corning Incorporated, USA) and the Nunclon^{TM} Δ Cell Factory (Nunc, Denmark). Both products allow assembling up to 40 trays, providing a surface area of 2.5×10^4 cm^2.

Despite the improvements introduced by the use of large scale operation units, the main problem with monolayer systems reside in their low ratios of surface to volume. A flat

monolayer configuration is the most straightforward way of providing the surface for the cells to attach, but not necessarily the most efficient in term of scalability. Due to this limitation, optimization of the culture conditions for the expansion of the cell population in monolayer constitutes an essential step in the process of tissue engineering of articular cartilage. Melero-Martin *et al*. have recently illustrated the importance of culture optimization using chondroprogenitor cells in monolayer (149). The analysis of both seeding density and passage length was revealed crucial to establish optimal culture conditions for monolayer expansion. The authors reported that the determination of the optimal seeding density and the corresponding passage length for cell expansion in a serial passaging operation was a compromise between growth kinetics and process time (149). Additional considerations concerning the running cost of the process were introduced: although the optimal passage length gave the desired expansion factor in a minimum process time, the selection of an alternative value of passage length was shown to reduce the cost of the expansion process in more than 60%. Nevertheless, if production is to be significantly increased, the number of culture units has to be remarkably increased making the process time consuming and laborious. The result is that the process may not be cost effective. Although the large scale alternative systems impose a notorious improvement when compared to simple T-Flasks, their inherent limitations in term of scalability could finally compromise the suitability of these systems in very large scale operation.

Three-dimensional expansion methods

Three-dimensional systems could potentially provide the improved ratio of surface to volume necessary to cope with the scale of cell expansion require for allogeneic tissue engineering applications.

Pellet culture

Since most primary anchorage-dependent cells in suspension are known to form aggregates, these aggregates could be grown in suspension cultures that promote the multiplication of the cells in the aggregates. For example, articular chondrocytes has been maintained in high-density aggregated cultures (74-77) that support cell proliferation while maintaining the chondrocyte phenotype (78, 79). It has been also reported for that an appropriate mixing regime could promote controlled cell aggregation in suspension by maintaining aggregate diameters in a relatively narrow range of 11-32 μm (which corresponded to 1-16 cells) (28).

However the strong interactions cell-cell generated during this kind of culture make the harvesting of viable chondrocytes difficult to achieve.

Encapsulation inside hydrogel beads

Cells can be trapped inside hydrogel beads such as agarose, collagen and alginate and then introduced into bioreactors and culture in suspension. Since these kind of three-dimensional culture systems are known to prevent/restore articular chondrocyte differentiation, the number of reported studies using these methodology is extensive (60, 80-88, 150). One of the most studied is the alginate system. During alginate culture chondrocytes divide to form cell clusters and also synthesize cartilage-specific matrix components which form a halo around the cells (151). While cell number increase during initial culture, a plateau is reached at a later time indicating an inhibition of cell proliferation (86). Consequently, with this kind of culture techniques the recovery of viable cells for passage is necessary. Individual cells can be recovered by dissolving the alginate beads, using calcium chelating agents followed by enzymatic digestion to remove elaborated matrix, allowing repeat passage to be performed. Although there are little doubts about the chondrocytic phenotype enhancement produced by this kind of cultures, the expansion potential achievable with repeat passage in alginate beads it is less clear. While early studies have indicated that the rate of chondrocyte proliferation is greater in monolayer than these three-dimensional culture systems (152), the same authors claim that the rate of expansion in alginate would still be acceptable for practical autologous chondrocyte transplantations (150). However, for potential allogeneic applications (where the expansion demand would be far higher than for autologous applications), the suitability of such proliferation rate for the development of large scale cell expansion processes would need to be re-examined.

Microcarriers

Microcarriers are bead-like structures ranging from 100-200 μm in diameter that can be held in homogeneous suspensions in stirred bioreactors. In microcarrier cell culture technology anchorage-dependent animal cells are grown on the surface of small spheres which are maintained in stirred suspension culture. Cells attach and spread on the surface provided by the microcarriers and gradually grow into confluent layers. This technology is replacing conventional monolayer cell culture methods since the extremely high surface area to volume ratio afforded by microcarriers suppose a significant improvement for process scale-up. Apart

from this enhanced scalability, microcarrier cells culture technology allows efficient monitoring and culture control while maintains reduced processing costs and reduced risk of contamination (153, 154). Commercially available microcarriers (including porous and non-porous) have been made from a variety of materials such as DEAE Sephadex, collagen, glass and gelatine and their applications have included an extensive diversity of cells. For example, in the context of articular cartilage, human chondrocytes have been reported to proliferate and produce matrix components in microcarrier suspension culture by using both Cytodex-3 (denatured collagen coated. Amersham pharmacia biotech, Sweden) and Cellagen (collagen type I derived beads. ICN, US) non-porous microcarriers (92, 155, 156). Alternatively to non-porous microcarriers, macroporous microcarriers provides interior surface for cell attachment protecting the cells against the detrimental hydrodynamic forces (157, 158) and potentially improving the yield of expanded cells. One example of porous microcarriers is CultiSpher-G (Percell Biolytica, Sweden). CultiSpher-G is made of gelatine that is derived from collagen, simulating the *in vivo* environment more closely than with other commercially available microcarriers. One major advantages of CultiSpher-G over other macroporous microcarriers is that its gelatine matrix can be dissolved with a variety of proteolytic enzymes (such as dispase and trypsin) and consequently cell harvesting is facilitated and the need to separate cells and microcarriers eliminated. The use of CultiSpher-G have been already reported for a variety of animal cell including human nasal chondrocytes (93) and bovine chondroprogenitor cells (94). Figure 2 illustrates the expansion suitability of chondroprogenitor cells isolated from the superficial zone of bovine articular cartilages using macroporous microcarrier in spinner flask cultures. In addition, during microcarrier cultures, cells are capable of undergoing bead-to-bead migration (94), which allow subcultivation to be performed without a harvesting step, thus improving the scalability of the expansion process. Serial subcultivation of cells on microcarriers is one of the most cost-effective means of establishing animal cell culture production facilities and can be achieved using a series of culture vessels of increasing volume and capacity (154). The cells cultured in one vessel can be subsequently used to inoculate the following large vessel. This approach to scale-up would eliminate the large numbers of culture vessels, such as roller bottles or multi-trays that would be otherwise required to obtain sufficient cells. By employing microcarrier cultures it will be possible to obtain large number of cells for cartilage tissue engineering applications. Not only its satisfactory expansion potential, but more importantly the cost and operational advantages over traditional monolayer culture make this system a feasible alternative method for the extensive expansion of chondroprogenitor cells. In addition, large scale microcarrier culture
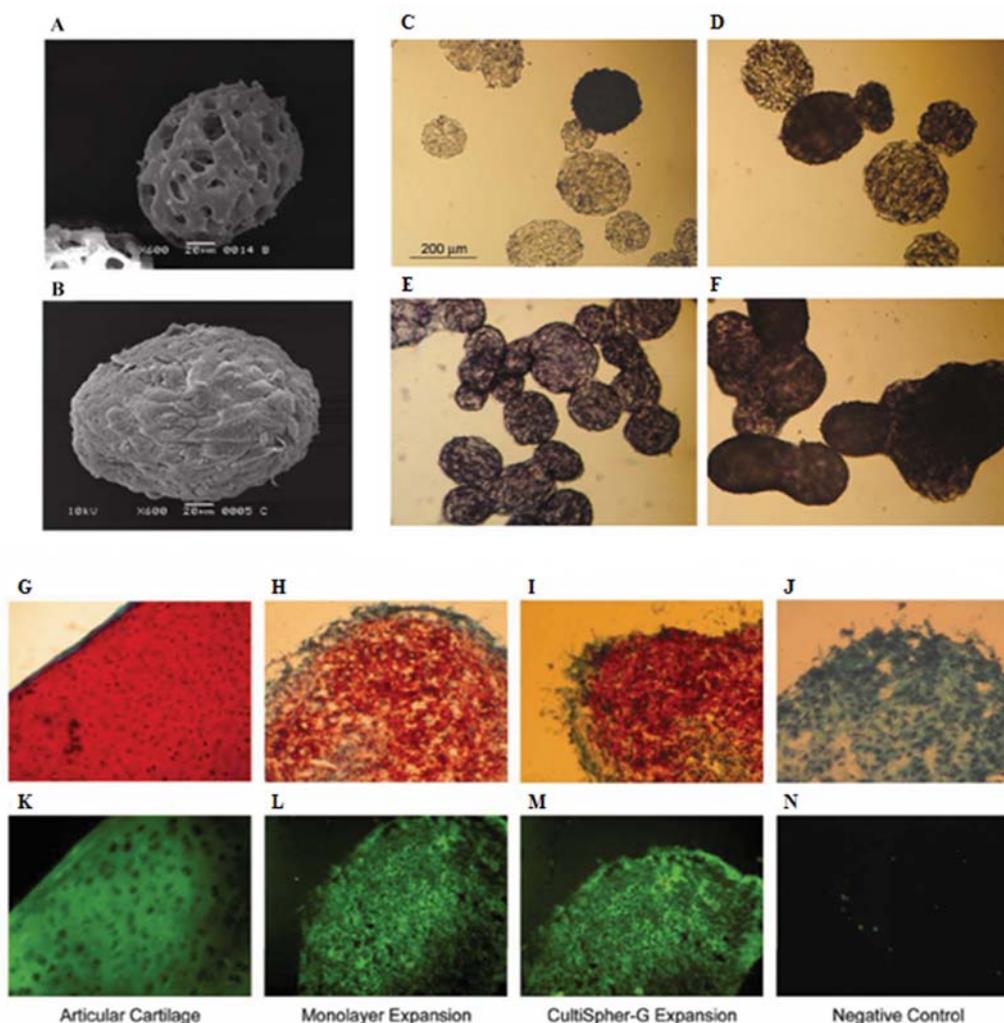
Fig. 2. Expansion of chondroprogenitor cells on macroporous microcarriers. Chondroprogenitor cells are able to attach and proliferate until confluence on macroporous microcarriers. (A) SEM micrographs of empty CultiSpher-G microcarriers and (B) confluent microcarriers after 7 day of culture. In addition, chondroprogenitor cells are capable to undergoing bead-to-bead migration, which allowed subcultivation to be performed without a harvesting step, thus improving the scalability of the expansion process. Panels C-F were taken from samples of spinner flasks that were seeded using as the inoculum cell-loaded CultiSpher-G microcarriers from a previous culture. Light micrographs of the cultured microcarriers were visualised at day 2 (C), day 4 (D), day 6 (E) and day 8 (F) after MTT staining. Finally, the expanded cell population was proven to maintain the ability to undergo chondrogenesis *in vitro*, an essential requisite for any proposed expansion method. Chondroprogenitor cells expanded either in monolayer or CultiSpher-G were subsequently cultured as pellets for 12 days for chondrogenesis induction. Histological sections of (G) bovine articular cartilage, (H) monolayer expanded cell pellet and (I) CultiSpher-G expanded cell pellet after safranin-O staining for GAG visualisation. Immunohistochemical sections of (K) bovine articular cartilage, (L) monolayer expanded cell pellet and (M) CultiSpher-G expanded cell pellet after incubation with monoclonal antibodies for collagen type II visualisation. (J and N) Negative controls obtained in the absence of TGFβ1 during the pellet culture period. This figure was adapted from reference (94) with permission.

is a very well documented and accepted technology. Industrial scale microcarrier applications have been proved to be reliable and cost-effective for the manufacture of both human and animal health care products. Virus vaccines, interferons, plasminogen activators and urokinases, cytokines, hormones and a variety of factors such as platelet derived growth factors (PDGF), epithelial growth factor (EGF), tumor necrosis factor (TNF), erythropoietin (EPO), colony stimulating factor (CSF) and others are currently produced from such microcarrier facilities (154). Some of these products are already available on the market as human and animal diagnostics and therapeutics. Many are undergoing evaluation and clinical trials and even more are in the developmental phase.

Conclusion

A continuous production of large scale quantities of chondrocytes will be shortly required for the manufacture of engineered cartilages, especially if allogeneic tissue engineering products are to be used for the treatment of cartilage defects. However, routine tissue culturing methodologies can hardly cope with the scale of cell production that would eventually be required. In this review we have addressed two of the major constrains for the successful production of large number of articular chondrocytes: the necessity of alternative sources of stem/progenitor cells and the development of feasible large scale cell expansion processes.

In the process of cartilage tissue engineering, it is important to ensure that the expanded cell population retains its phenotypic function. Chondrocytes derived from articular cartilage biopsies have only a limited proliferative potential. They dedifferentiate upon repeated passaging and the number of cell divisions chondrocytes undergo *in vitro* decreases with age. The issue of phenotype expression and differentiation has led to the investigation of the potential use of stem/progenitor cells as a source for tissue engineering. These have included mesenchymal stem cells, which are capable of differentiating into bone, cartilage, tendon and muscle. More recently, a new population of chondroprogenitor cells isolated form the superficial zone of the articular cartilage has been identified and partially characterized. Chondroprogenitor cells are able to differentiate and produce cartilage-like extracellular matrix upon extensive expansion *in vitro*, providing a promising alternative source of cells. This enhanced expansion potential of chondroprogenitor cells together with their ability to form cartilage after extensive culturing could constitute a step forward for cartilage repair as it may enable the generation of large cell banks for use in tissue engineering applications.

The second fundamental issue addressed in this review is the necessity of feasible large scale expansion protocols. Despite the improvements introduced by the use of large scale operation units, monolayer systems present very low ratios of surface to volume, which inevitably make them inefficient in term of scalability. If production of articular chondrocytes is to be significantly increased, the number of culture units has to be remarkably increased, making the process time consuming and laborious. The result is that the expansion process may not be cost effective. The introduction of three-dimensional alternative systems could potentially provide the improved ratio of surface to volume necessary to cope with the scale of cell expansion required for allogeneic tissue engineering applications. One of these alternatives is the use of cell-seeded microcarriers for cell expansion. Both mature chondrocytes as well as chondroprogenitor cells have been already shown to growth satisfactorily in a diversity of microcarrier materials. Additionally, chondroprogenitor cells were shown to be capable of undergoing bead-to-bead migration, which allow subcultivation to be performed without a harvesting step, thus improving the scalability of the process. Finally, the microcarrier-expanded cell population has been also proven to maintain the ability to undergo chondrogenesis *in vitro*, an essential requisite for any proposed expansion method. Therefore, not only due to its satisfactory expansion potential, but more importantly the operational advantages over traditional monolayer cultures make microcarrier cultures a feasible alternative method for extensive cell expansion.

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