Influence of chondrocytes differentiation stage on the capacity to generate cartilaginous tissue in vitro

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1 INTRODUCTION

1.1 CARTILAGE BIOLOGY

Cartilage is a specialized avascular connective tissue comprising of only one single type of cell called chondrocyte which is sparsely populated in a collagen and proteoglycan rich hydrated extracellular matrix (ECM). Based on the biochemical composition and structure of the ECM, the mechanical properties and structural characteristics of the tissue, three major types of cartilage (elastic cartilage, fibrous cartilage and hyaline cartilage) can be distinguished.

Elastic cartilage is found in the pinna of the ear, in the walls of the auditory and eustachian canals and tubes, as well as in the larynx and in the epiglottis. This type of cartilage with a more elastic property maintains tubes-like structures permanently open and provide intermediate mechanical stability. Elastic cartilage mostly consists of type II collagen matrix elements and elastic fiber bundles (elastin) which manifest in aligned fiber structures. This structural composition provides a tissue which is stiff yet elastic.

Fibrocartilage is most prominently found in areas which require greater tensile strength and support such as between intervertebral discs and at sites of tendons or ligaments connected to bone tissue. Typically, fibrocartilage is found at locations which are under considerable mechanical stress (i.e. tendon and ligaments) but still provides properties which allow flexible body movement. Accordingly, fibrocartilage mainly consists of type I collagen fibers which are aligned in thick fiber bundles and chondrocytes arranged in parallel rows between these fibers. The fibrous type of cartilage is usually associated with a dense connective tissue, namely the hyaline type cartilage which defines the third type of cartilage (1).

The *hyaline type cartilage* is the most abundant type of cartilage and is found in the nose, Larynx, trachea, bronchi, in the ventral ends of the ribs, and at the articular ends of the long bones. Characterized by the arrangement of the chondrocytes in multicellular stacks which prominently produce a type II collagen and a proteoglycan rich matrix, the hyaline type of cartilage provides the flexible support in nose and ribs but can also sustain mechanical load during body motion as shown at the surface of articular joints. This hyaline type of cartilage is lining as a thin layer of deformable, load bearing tissue at the bony ends of diarthrodial joints and is more specifically called articular cartilage (1).

1.1.1 Composition and structure of articular cartilage

The primary function of articular cartilage is the absorption and distribution of forces, generated during joint loading and to provide a lubricating tissue surface which prevents the abrading and degradation of the joint and the subchondral bone structure during joint motion. Indeed, the articular type of hyaline cartilage has to bear and tolerate enormous physical stress and load during its entire lifetime.

Despite the rather primitive composition of articular cartilage, characterized by chondrocytes entrapped in hydrated extracellular matrix molecules such as collagen type II, IV and VI, and proteoglycan aggregates, the tissue shows unique, highly defined structural organization to maintain its mechanical and functional integration.

Articular cartilage has two different structural characteristics: (i) cartilage zonation (ii) the organization of the extracellular matrix.

(i) The structure and composition of the entire articular cartilage tissue varies according to the distance from the tissue surface and reflects its functional role. Four different zones arranged as layers horizontally to the tissue surface can be distinguished and are characterized according to the extracellular matrix composition and cellular morphology (Figure 1).

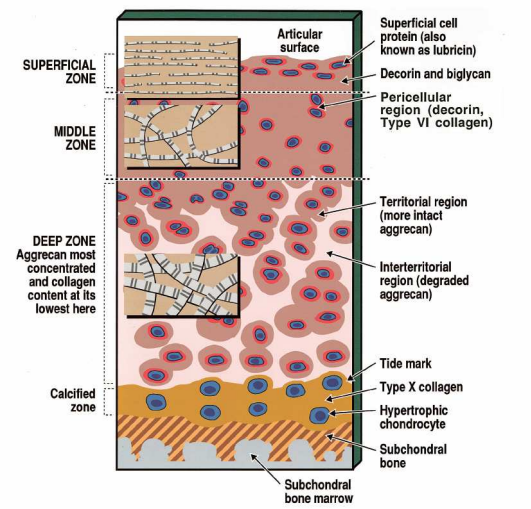

Figure 1. Schematic drawing of the general structure of human articular cartilage showing the zones, regions and relashionship with subchondral bone. The insets show the relative diameters and organization of collagen macrofibrils in different zones. (Reproduced from Poole CA (2); Composition and structure of articular cartilage).

In the superficial zone the layer of tissue is composed of flattened ellipsoid-shaped chondrocytes and a high concentration of thin collagen fibers arranged in parallel to the articular surface (3). In this layer the pericellular matrix structure mentioned below can not be found. The thin layer of cells is covered with an acellular sheet of collagen fibers (lamina splendes) which functions as a protective barrier between the synovial fluid and the cartilage tissue and controls the in- and egress of larger size molecules (4). Its rather low permeability regulates the diffusion transport of nutrients and oxygen to the underlying cartilage structures. Only within this zone chondrocytes synthesize and secret the superficial zone protein lubricin (5, 6) responsible to reduce surface friction during joint motion. The specific arrangement of the collagen fibrils which lay in parallel to the joint surface, provides a high mechanical stability of the tissue layer and mainly contributes to the tensile stiffness and strength of articular cartilage (7-10).

Below the superficial zone is the *midzone* where cell density is lower. This has the more typical morphologic features of a hyaline cartilage with more rounded cells and an extensive extracellular matrix rich in the proteoglycan aggrecan. The collagen fibers are synthesized at a lower quantity but show larger diameter fibrils which are aligned obliquely or randomly to the articular surface and describe an intermediate structure between the superficial zone and the adjacent deep zone.

In the *deep zone*, the chondrocytes have a round morphology and are arranged in cell columns perpendicular to the cartilage surface. The extracellular matrix contains a high content of

glycosaminoglycans and large diameter collagen fibers which form arcades perpendicular to the joint surface (11).

The partially *calcified zone* defines the boundary of cartilage tissue to the subchondral bone. This rather thin layer of calcified cartilage with intermediate mechanical properties functions as a buffer between the cartilage and bone tissue. The cells have a smaller volume and are partially surrounded by calcified cartilage matrix. The chondrocyte in this zone usually persist in a hypertrophic cell stage which correlates with the expression of collagen type X. Finally this boundary provides an optimal integration to the subchondral bone tissue and prevents vascular invasion.

(ii) In addition to this zonation, the matrix surrounding the chondrocytes of articular cartilage varies in its organization and can be divided in three compartments, such as the pericellular region adjacent to the cell body, the territorial region enveloping the pericellular matrix, and the interterritorial compartment which defines the space between these cellular regions (Figure 1)(1).

The *pericellular region* which is rich in proteoglycan, decorin, aggrecan, collagen type VI, and cell membrane associated molecules like anchorin and decorin (12-14) defines a narrow rim of a filamentous matrix network which fulfills the functions of the interlink between the chondrocyte cell body and the territorial matrix structure.

The *territorial region* describes an envelop surrounding the cells or cluster of cells with their pericellular matrix. Thin collagen fibrils (most prominently collagen type II) bind to the pericellular matrix and form a basket like structure which protects the cell from damage during loading and deformation of the cartilage tissue. Moreover these structures may also contribute to transmit mechanical signals to the chondrocytes during joint-loading (15, 16).

The *interterritorial region* confines the most volume of the articular cartilage tissue and contains intermolecular cross linked collagen fibrils (collagen type II), non collagen proteins and aggregates of glycoproteins (14). This extracellular matrix composition provides the tissue with its functional characteristic to absorb mechanical load.

Mechanical environment in mature cartilage

Chondrocytes and cartilage tissue during joint motion are exposed to body weight load which creates a rigorous mechanical environment for articular cartilage tissue such as direct compression, shear, and hydrostatic pressure. The function of articular cartilage to undergo tissue deformation is dependent on the specific arrangement of macromolecules in the extracellular matrix. Especially the organization of collagen fibers into a three dimensional arranged collagen network can balance the swelling pressure of the proteoglycan-water "gel" (17, 18). Cartilage is considered as a viscoelastic material composed of three principal phases: a solid phase composed of a dense, collagen fibrillar network and charged proteoglycan aggregates, a fluid phase of water and an ion phase with ionic species for neutralizing the charged matrix components (19, 20). Under physiological condition these three phases define an equilibrium where the extension of the proteoglycan-water gel volume is restricted by the firm collagen frame (21). The bound water in the cartilage tissue and finally the mechanical properties of the cartilage tissue are influenced by the interaction of water with the large, negatively charged proteoglycan aggregates (22). The negatively charged proteoglycans mostly driven by chondroitin sulphate residues are balanced by a high concentration of cations dissolved in the cartilage tissue (23).

In summary, the mechanical function of articular cartilage tissue bases on the matrix structure surrounding each single cell, the arrangement of the extracellular matrix fibres within the single zonal compartment and the proportional composition of the different extracellular matrix components.

1.1.2 Cartilage ontogeny

Articular cartilage as a part of the limb skeleton develops in a well defined and controlled multistep differentiation process of cells from the mesenchymal origin (24-26).

The establishment of the cartilage structure follows precise and distinct patterns of **cell differentiation** and **cell rearrangement** driven by environmental factors such as cell-cell and cell matrix interaction, growth factor and morphogen mediated signaling (27, 28) as well as defined biomechanical conditions (29).

The steps of development are divided in 3 phases (Figure 2). In the first phase mesenchymal precursor cells migrate from the lateral mesoderm towards the presumptive skeletogenic site and determine the cartilage anlagen (30). In the second phase, the epithelial-mesenchymal interactions results in the mesenchymal condensation. The pre-chondrogenic condensation is a prerequisite for the future establishment of the limb skeleton (31) and is associated with an increased cell to cell contact which facilitate the intercellular communication and the transfer of small molecules between the cells (32). It has been shown that such a high cell density is required to allow chondrogenic development (33) and that the level of cell condensation correlates with the stage of chondrogenic development (34, 35). Additionally, cell-matrix interactions appeared to play an important role in mesenchymal condensation (36). For example the integrin mediated binding of chondrocytes to collagen, has been shown to be essential for chondrocyte survival (37, 38). Finally, in the third phase, the overt differentiation of immature pre-chondrocytes into fully committed chondrocytes is manifested by an increased cell proliferation and by the up-regulation of cartilage specific matrix components like collagen type IIα1, IX and XI and aggrecan. In the final commitment of the chondrogenic phenotype the cells reduce their proliferative activity and maintain the functional integrity of the mature cartilage tissue (24-26).

The initial function of the cartilage during embryonic development is to give stability to the embryo and serve as a template for myogenesis and later for neurogenesis. Most of the embryonic cartilage is replaced by bone during a process called the endochondral ossification (39, 40). During the endochondral ossification, the chondrocytes progress to the hypertrophic phenotype, which is characterized by a massive enlargement of the cell, the onset of the expression of type X collagen (41), an increased expression and activity of the alkaline phosphatase and the carbonic anhydrase and a reduction in the synthesis of the type II collagens and proteoglycan. Protease inhibitors prevent vascular invasion are also reduced. Vascularization of the tissue takes place, and most or all of the hypertrophic chondrocytes undergo apoptosis followed by their replacement by osteoblasts which in turn will deposit bone matrix in the free lacunae. At the cell level, the entire endochondral ossification process can be seen as a sequential progression of the three chondrocytic phenotypes: the committed mesenchymal cell, the differentiated chondrocyte and the hypertrophic chondrocytes (42).

Within these developmental processes growth promoting factors act on the cell and contribute to establish a mature cartilage tissue.

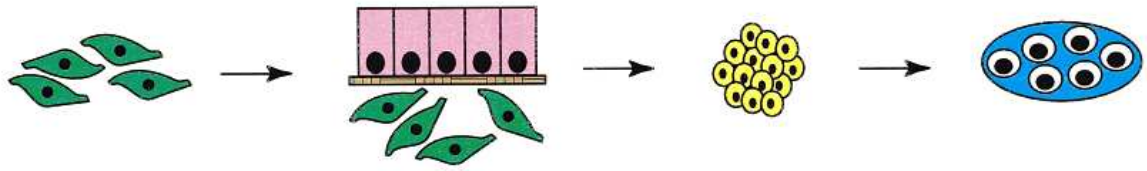

Figure 2. The three phases of the development of a skeletal element are (from left to right): (i) migration of preskeletal cells (green) to the site of future skeletogenesis, which is always associated with an epithelium (purple) and epithelial basement membrane (brown); (ii) interactions of those cells with epithelial cell products resulting in initiation of a condensation (yellow); (iii) overt differentiation of chondroblasts or osteoblasts (blue). Reproduced from Hall and Miyake (30)

Soluble growth factors in the cartilage development

Within the multi step cell differentiation process a number of growth factors and morphogenes are involved and essential during chondrocyte maturation and cartilage tissue formation. The most prominent growth factors belong to the transforming growth factor (TGF-β) superfamily which are responsible for chondrocyte proliferation (TGF-β1), terminal differentiation (TGF-β3; bone morphogenic protein; BMP) (43) or to promote cell-cell interaction in the early stage of chondrogenesis (BMP) (44). The insulin like growth factor 1 (IGF-1) which belongs to the IGF family of peptide hormones (including insulin) regulates many cellular functions during cartilage maturation such as induction of chondrocyte differentiation (45) and proliferation (46). In mature cartilage IGF-1 promotes and maintains the anabolic synthesis of proteoglycan and type II collagen (47) and inhibits the nitric oxide-induced de-differentiation of articular chondrocytes (48). Furthermore members of the fibroblast growth factor (FGF) family of morphogenes influence processes correlated with cell division and chondrocyte proliferation and have been shown to promote chondrocyte proliferation in a human growth plate ex vivo culture system (49).

Finally, only the combinatorial action of these growth and morphogenic factors specifically expressed in selective tissue areas in different developmental phases and at defined concentrations establishes the precise structure of the articular cartilage tissue.

Maintenance and aging of articular cartilage

Once the articular cartilage tissue structure is established, chondrocytes reduce their metabolic activity and persist in an anabolic and catabolic equilibrium of the matrix components. Although the two major extracellular matrix proteins, collagen type II and aggrecan, have a relatively long turnover time span (50), they have to be maintained in a balanced state of production and degradation. The key factors to maintain the equilibrium of tissue metabolism are found in the physicochemical environment of cartilage tissue such as: (i) mechanical load during joint motion; (ii) growth factor responsiveness of chondrocytes; (iii) the balanced molecular composition of the matrix (proportions of the matrix components). These factors contribute to the preservation of the functional properties of the mature articular cartilage surface.

After the third decade in human the properties of the weight bearing articular cartilage tissue significantly change with progressive age (51, 52). The process of cartilage aging has been shown to cause changes in the mechanical properties of articular cartilage (53), in the molecular composition, structure and organization of the extracellular matrix (54-56) and in the synthetic and metabolic activity of chondrocytes (57, 58). In advanced age individual the number of cells, the size of the cartilage tissue and the content of bound water diminish (59). The anabolic activity of chondrocytes required for the balance of cartilage tissue matrix homeostasis declines and thus the imbalance of matrix turn-over causes the loss of tissue matrix structure. Furthermore, in line with the decreased ability of chondrocytes to respond to a variety of extrinsic stimuli (e.g. growth factors) the sensitivity to catabolic regulative cytokines is enhanced in age. Moreover the imbalance of the tissue homeostasis can be moreover manifested by the increased expression of catabolic mediators such as matrix metalloproteinases (60). Finally, these change in the molecular structure of extracellular matrix components leads a softening of the cartilage tissue which increase the risk of synovial joint degeneration, often provoking the clinical syndrome of osteoarthritis (61).

However, not only the reduced tissue function in elderly individuals but also the generally low metabolic activity of cartilage tissue in combination might explain the limitation in the self-repair function of cartilage with increasing age (62, 63).

1.1.3 Articular Cartilage defects and self repair

Articular cartilage lesions, caused by trauma, osteochondritis dissecans or as a result of instability or abnormal loading are a common cause of disability, often associated with pain, reduction of joint mobility and loss of function and can ultimately lead to osteoarthritis. Articular cartilage has a very limited intrinsic healing capacity, related to the absence of vascularization and the presence of few and very specialize cells with low mitotic activity. According to the size of cartilage tissue damage in the cartilage surface, several grades of tissue injury can be distinguished which lead to different healing response (64-66).

In the case of partial thickness defects, the classical self-repair of injured cartilage tissue goes through conserved mechanisms of cell and tissue necrosis followed by the proliferation of surviving chondrocytes adjacent to the site of the lesion. Although these cells aggregate in clusters and demonstrate a temporary increased type II collagen synthesis, in long term the formed tissue shows a lost of hyaline like cartilage characteristics. Thus, these chondral lesions remain almost unchanged and can proceed towards osteoarthritic diseases (67).

In the case of full thickness defects, the lesion penetrates to the subchondral bone part gaining access to the cells that reside in the bone marrow space including the mesenchymal stem cells located therein. The repair response elicited by this type of defect results in the formation of a fibrocartilaginous tissue in the defect void. Anyhow, the decreased deposition of extracellular matrix components and the formed tissue with fibro-cartilage structures lack the strength, the mechanical properties and duration of the original articular cartilage tissue as it has been demonstrated in longer time follow-up studies (68, 69).

In conclusion, the two mechanisms of the spontaneous self healing show limitations in the quality and mechanical duration as compared to the native cartilage tissue and can increase the risk of

tissue and joint degeneration (61). Therefore, procedures to regenerate the functional properties of the cartilage surface are crucial to avoid the progression of secondary joint diseases.

1.1.4 Treatments for articular cartilage defects

The different strategies to treat cartilage defects vary from more conservative approaches, like physiotherapeutic measures or application of pharmaceuticals (i.e. corticosteroids, hyaluoronic acid and growth factors) towards more invasive (i.e. surgical) procedures.

Arthroscopic repair procedures

Arthoscopic *lavage* and *debridement* are often used to alleviate joint pain. Lavage involves irrigation of the joint during arthroscopy, while debridement is the removal of the damaged tissue from the joint. Both of these procedures are routinely used to alleviate joint pain however do not induce repair of articular cartilage (70, 71).

Arthroscopic surgical procedures such as *drilling***,** *abrasion* or *microfracture* used to induce repair of articular cartilage take advantage of the intrinsic repair response observed upon penetration of the subchondral bone in full thickness defects. These techniques rely upon the formation of a blood clot and mesenchymal progenitor cell invasion. However, the clinical outcome is varied, which is due, in part, to the unpredictable nature of the repair tissue formed, in addition to the age and activity levels of the patient (72-74).

Osteochondral Transfer

Osteochondral transplantation of autogenic and allogeneic tissue has been widely used to treat predominantly large osteochondral defects. Allogeneic material derived from cadaveric donors and it is indicated for large post traumatic defects of joints. Beaver et al. (75) reported satisfactory long-term results with these grafts, but the logistic of implanting a fresh allograft and the risk of transmitting infection reduce the indication for this procedure only to severe cases. Instead, autologous osteochondral graft implantation, involves the removal of cylindrical plugs of osteochondral tissue from non load bearing regions of the articular cartilage and their implantation into the prepared full

depth defect with press-fit fixation. This procedure is indicated in osteochondral defects of 3 to 5 cm^2 in young patient. It provides the re-establishing of a functional cartilage surface which can-absorb body weight load but has limitation in terms of poor tissue integration within the adjacent native cartilage tissue. Furthermore, the surgical intervention damages intact host tissue and might enhance the donor site morbidity (76, 77).

Even though such invasive procedures hold promise and showed acceptable results in some cases the outcome of these procedures shows generally limitations in terms of quality and reproducibility (78).

Cell based cartilage repair techniques

Given the intrinsic limitations of these techniques, innovative surgical approaches have been developed focused at obtaining the regeneration of hyaline cartilage and its functional integration with the surrounding tissues, as a means to restore the normal knee function and provide durable outcome.

Autologous chondrocytes implantation (ACI), first introduced by Brittberg in 1987 (79), as well as further improved and reviewed (65, 80, 81) has been proven to be clinically effective to restore the tissue structure of large full-thickness focal defects of the femoral condyle (82). This technique involves two surgical procedures: an arthroscopic harvesting of a small cartilage biopsy from a non weight-bearing area of the knee and the subsequent transplantation of in vitro expanded autologous chondrocytes, to defect site beneath a periosteal flap obtained from the tibia in the same surgical procedure. A large amount of evidence is currently available in the literature concerning the clinical results obtained with ACI indicating that the treatment is associated with improved health outcomes, such as pain reduction and improved joint functionality. Despite the promising clinical results obtained so far, the use of ACI is associated with a number of limitations essentially correlated with the complexity and morbidity of the procedure. While the *in vitro* expansion step is necessary to obtain sufficient cell number it induces well-known variations in biochemical and synthetic properties leading to cell de-differentiation and loss of chondrogenic phenotype and therefore the outcome in tissue quality is often limited (78, 79). In addition, the ACI technique is associated with a frequent occurrence of post operative periost hypertrophy.

With the intention to overcome these limitations, in a second generation techniques, such as matrix-induced autologous chondrocytes implantation (MACI), or the grafting of tissue engineered cartilaginous construct (TE), the cells are cultured in the same manner, but are then seeded directly onto a biomimetic scaffold which acts as a carrier for the cells. These approaches offers potential advantages, consisting in improved cell retention, the even distribution of cells and easily graft handling and earlier post-operative rehabilitation for the patient (83, 84).

In addition a tissue engineering approach with further *in vitro* maturation, before implantation, could induce cartilaginous extracellular matrix deposition achieving in principle a cartilaginous graft with biomechanical and biochemical properties closer to those of the native cartilage (85). The requirements and limitations of such in vitro tissue engineering approaches are discussed in the following chapter.

1.2 CARTILAGE TISSUE ENGINEERING

The term “tissue engineering” was first defined by Langer and Vacanti (85) as “an interdisciplinary field of science that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ". In line with the improved investigations, the activities in the field of tissue engineering broaded and the term “tissue engineering” required an extended definition which moreover emphasizes the "understanding of the principles in tissue growth, which then applied, leads to production of functional tissue replacements for clinical use" (86).

Cartilage tissue engineering techniques have emerged as an innovative field of research with the potential to recreate three dimensional cartilaginous structures. Although cells have been cultured or grown outside the body for many years, the possibility of growing complex, three-dimensional tissues (literally replicating the design and function of human tissue) is a more recent development. Typically this is being attempted through the process of harvesting the patient’s own chondrocytes, expanding them, and after seeding them onto a biomaterial scaffold, culturing them possibly under controlled conditions in bioreactors allowing them to synthesize cartilaginous matrix (Figure 2). Successful engineering of cartilage grafts which follows a cell-scaffold based approach requires optimized *in vitro* culture condition. The success is dependent on three key elements: i) the selection of a cell source, able to produce a new tissue with hyaline like cartilage characteristics; ii) the choice of the biomaterial which allow cell seeding and promote the chondrogenic differentiation process; iii) the application of bio-inductive molecules supplemented in the culture growth media (i.e. growth factors, cytokines, hormones, vitamins, glucose and oxygen) or physical stimuli which enable the cells to differentiate and to re-organize a cartilage like matrix structure.

The three key elements per se but also approaches combining these parameters are currently under investigation and open a broad field of research where only an interdisciplinary approach might be able to overcome the current limitations of *in vitro* chondrocyte differentiation and cartilage tissue re-formation (78). In the following sections these requirements will be discussed regarding their potential and limitations to successfully engineer functional cartilage tissue grafts.

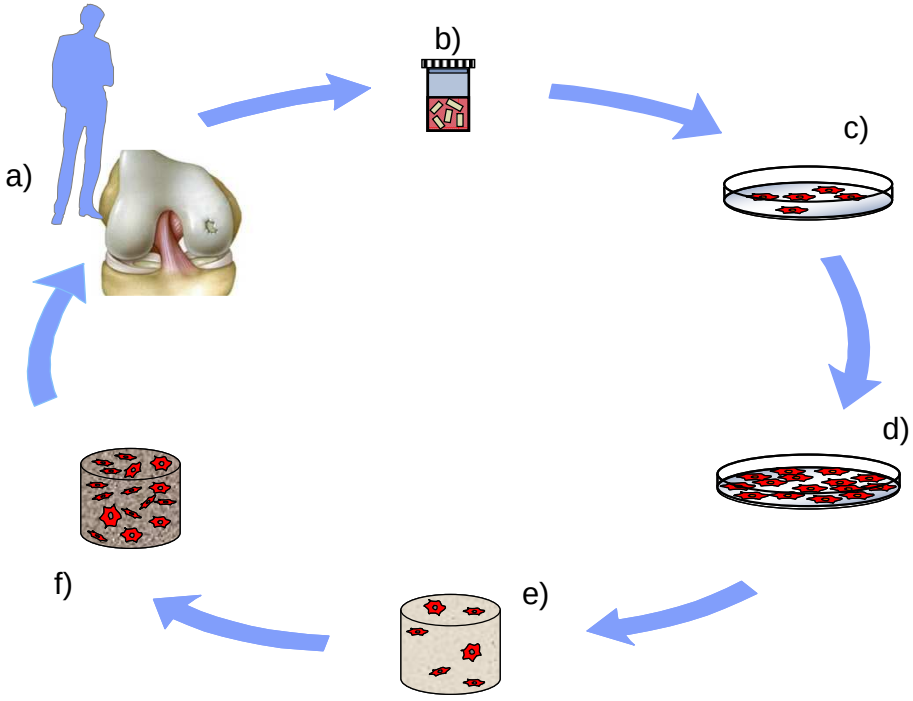

Figure 2. Representation of a typical articular cartilage lesion approach employing tissue engineered cartilage: a) Following a traumatic event, trauma could result in significant lesions to the cartilage surface, b) a biopsy is harvested arthroscopically and cells are obtained following a digestion procedure, c) cells are plated and expanded in monolayer culture, d) once the required number of cells is reached, cells are seeded onto biomaterials (scaffold), e) cell-scaffold constructs are hence cultured *in vitro* for an appropriate amount of time to promote cell redifferentiation and extracellular matrix deposition and finally f) the mature constructs is ready to be implanted to treat the wounded cartilage.

1.2.1 Cell sources to engineer cartilage tissue

Among the different parameters which influence the outcome of *in vitro* tissue engineering procedures, the selection and definition of a convenient cell type or cell source is the first issue to deal with. The indispensable demands on cells for cartilage tissue engineering are: (i) not to provoke hostile immune reaction (ii) not to induce tumorigenic development and (iii) to integrate within the site of insertion in a controlled way.

The requirements on these cells to moreover improve the quality of *in vitro* engineered cartilage tissue are: (i) to provide sufficient number of cells from the biopsy site which enables the culture of cells at a high cellular density to improve the induction of cartilage development in vitro; (ii) to harvest a population of cells which is able to properly recover a chondrogenic phenotype and (iii) to harvest the cells from body sites with low donor site morbidity caused by additional surgical interventions.

The use of xenogenic (animals derived) or allogeneic (human derived) cells and tissues could provide a source of cells with an almost unlimited availability and with a high accessibility to different populations of cells to most simply engineer tissue constructs *in vitro*. Anyhow, the use of an allogeneic or xenogenic cell source is usually correlated with possible adverse immunogenic effects (87).

The most evident choice for a non-immunogenic cell source is the use of autologous cells harvested from the patient's own tissue. These cells provide an optimal source which does not induce an immunogenic respond. For the implementation in cartilage tissue engineering the most promising attempts have been made by the isolation of bone marrow derived mesenchymal stem cells (progenitor from mesenchymal origin) or by the use of chondrocytes from cartilage tissue itself.

Mesenchymal Stem cells

Stem cells have the "capacity for self-renewal or unlimited self-renewal under controlled conditions" and "they retain the potential to differentiate into a variety of more specialized cell types" (88). Therefore these are cells with multipotent differentiation capacity (89). There are a number of stem-cell sources, such as embryonic stem cells and induced pluripotent stem cells. However, it is the adult mesenchymal stem cell (MSC) that is of most interest for articular cartilage repair (90). They represent an autologous supply of cells which can be easily harvested from a number of different tissues, including bone marrow, adipose tissue, muscle, periosteum and synovium (91). Many studies have compared these sources in terms of their chondrogenic ability, with several focusing on comparison between adipose tissue and bone marrow, of which bone marrow-derived cells have shown superior results (92). Bone marrow derived cells are the most readily available as they can be

easily harvested in a relatively non-invasive manner. They can be extensively expanded *in vitro* and kept in their undifferentiated properties when maintained in appropriate culture condition (93, 94). Subsequent culture of MSC in the presence of specific growth factors was shown to induce chondrogenic differentiation in three-dimensional micromass culture (95-97), or on polymeric cell carrier scaffolds (98), even so, MSC differentiated towards the chondrogenic lineage were shown to express markers specific of hypertrophic chondrocytes (99, 100) thus indicating a potential instability of the acquired chondrocytic phenotype. Despite a series of recent studies reporting the use of MSC for osteochondral defect repair in different animal models (101-103), the long-term efficacy of bone marrow derived MSC and their contribution to the regeneration of hyaline cartilage which does not remodel into bone in the long term, still has to be demonstrated.

Alternatively, allogeneic mesenchymal stem cells represent an option for cartilage repair. They have been shown to be immunoprivileged, therefore it is possible to deliver them in vivo without rejection (104, 105). They have also been shown to produce cytokines, which may help to modulate the repair process in vivo. Thus far, preclinical trials have been encouraging, but it remains to be seen whether this type of cellular therapy may become commonplace in clinical practice.

Also embryonic stem cells represent a promising cell source but many ethical issues need to be resolved prior to their clinical application.

Chondrocytes

Brittberg et al (79) published the "original method" of using chondrocytes in suspension under a periosteal patch for autologous implantation. Thus, chondrocytes are the cells of choice for all current ACI procedures.

The use of chondrocytes is attractive because these cells have been shown to be able to synthesize matrix containing Type II collagen and aggrecan (65). However, chondrocytes are in limited supply and they must be multiplied in culture to have an adequate number to support a repair.

As described in chapter 1.4, following a cartilage biopsy, primary articular chondrocytes can be successfully maintained and expanded in monolayer culture (106, 107). The application of different growth factors during the monolayer culture phase enables chondrocytes to proliferate, while they

progressively lose their typical differentiated phenotype and appear fibroblastic (108). However, it has been shown that while the exposure of chondrocytes to a variety of growth factors (i.e. TGFβ-1; bFGF-2) enhance the de-differentiation of chondrocytes, can additionally improve the capacity to re-gain a differentiated phenotype during subsequent culture in a permissive chondrogenic environment (109). Beside the treatment of the cells with soluble chondrogenic inducer the maintenance of the cells in a 3-dimensional environment at a high cellular density during the phase of chondrogenic re-differentiation can additionally promote the differentiation process (110).

Nevertheless, considering the implementation of chondrocytes harvested from adult individuals in cartilage engineering approaches, the resulting tissue quality shows limitations in terms of donor variability which might be influenced by the clinical background, the disease history or the age of the individual. In particular, the age of the individual, significantly reduces the capacity of the ex vivo cultured chondrocytes to respond to growth stimulation and thus the quality of the cartilage tissue produced from cells of elderly donors could be inferior (111, 112).

The critical issues associated with the use of autologous articular chondrocytes are: the acquirement of the biopsy from the individual causing morbidity at the donor site and the following small number of available cells. In particular, the harvesting of a cartilage biopsy in the joint represents an additional injury to the cartilage surface, and might be detrimental to the surrounding healthy articular cartilage (113). To circumvent this problem an alternative approach would be based on the use of chondrocytes obtained from non-articular cartilage tissues. For instance, biopsies of nasal or ear cartilage can be harvested by a less invasive procedure than excising tissue from distinct areas of the joint. The potency of morbidity is also reduced by the fact that the donor site (ear and nose) is not subjected to high levels of physical forces, as in the joint. Various studies have been shown that chondrocytes derived from human nasal septum or ear cartilage proliferate and generate cartilaginous tissue after monolayer expansion with similar or superior capacity to those derived from articular cartilage (114-116). However, to demonstrate whether the tissue generated by non-articular chondrocytes is adequate for articular cartilage tissue repair, extensive data from in vivo orthotopic experimental studies and from *in vitro* loaded models will be needed.

Both chondrocytes and mesenchymal stem cells are troubled with fibroblastic de-differentiation and terminal differentiation to a hypertrophic phenotype in vivo. It is therefore likely that these cells types will require some degree of modulation to be used successfully. This may be provided by the use of specific biomaterials (scaffolds) or by the addition of media supplements (growth factors).

1.2.2 Scaffolds for tissue generation

The purpose of using biomaterial scaffolds for tissue-engineered constructs is to mimic the 3-dimensional environment of the extracellular matrix, provide structural support to the regenerate and surrounding tissues, and provide an increased surface area to volume ratio for cellular migration, adhesion and differentiation (117).

A large number of scaffold designs and concepts were tested experimentally, in animal models and received the approval in clinical applications (118).

An ideal scaffold material or architecture must provide the following characteristics: (i) bio-degradable with a controlled degradation and absorption rate which allows tissue in-growth; (ii) biocompatible and not provoke a hostile immune response; (iii) a three-dimensional structure with defined porosity and interconnectivity to allow cell invasion, tissue growth and transport of nutrients and metabolic waste; (iv) mechanical stability for *in vitro* handling and subsequent implantation within surgical procedures; (v) and provide a suitable surface chemistry or the ability to absorb proteins to improve chondrocyte attachment, proliferation, or differentiation and thus to promote and support tissue specific development (118, 119).

The two most commonly used solid scaffold architectures reported in the literature are porous sponges and non-woven fiber meshes (120). There are several biomaterial options used for articular cartilage tissue engineering, which can be natural or synthetic (121).

Scaffolds based on natural biopolymeric compounds mimic and resemble the natural cartilage environment. Further they can be subdivided into protein-based matrices such as collagen and fibrin and carbohydrate-based matrices such as alginate, agarose, chitosan and hyaluronan (122). The presentation of bioactive surface structures can induce signals to the entrapped chondrocytes and

potentially stimulate the chondrogenic differentiation process which leads to the cartilage tissue neogenesis (123).

Various synthetic polymer scaffold materials have been validated in cartilage tissue engineering such as polylactic- or polyglycolic acids (124-126), polycaprolactones, polycarbonates or co-polymer containing ethylene-terephthalate (127, 128). In contrast to the advantage to provide initial mechanical stability, non-immunogenicity and bio-resorbability these scaffold polymers have been shown to potentially provoke adverse cytotoxic effects due to the release of acidic products (129). Moreover synthetic polymers per se would not have biological properties to induce cartilage tissue regeneration.

1.2.3 Media supplements and culture environment

Soluble mediators are mostly involved during the event of cartilage growth, metabolism and development, such as in the mesodermal differentiation of the cartilaginous skeleton in the embryo, the process of endochondral bone formation and the onset of articular cartilage "repair" (25). As a common basis of various approaches considered for cell-based engineering of cartilage tissue, it is known that during the *in vitro* culture of chondrogenic cells, specific growth factors, cytokines, hormones or enzymatic co-factors (e.g. vitamins) can enhance cell proliferation, migration or cell differentiation, and in consequence allow to obtain sufficient cells with the potency to re-induce cartilaginous tissue structures.

In general, growth factors and cytokines are cell-secreted molecules and when bound to cell membrane receptors can induce intracellular signaling pathways which lead to cell adhesion, proliferation or promote cell differentiation, by the up or down regulation of target genes.

As compared to the morphogenic action in vivo, several growth factors and mitogens are applied in *in vitro* tissue engineering approaches.

Basic Fibroblast growth factor (bFGF) within the FGF family is the most widely investigated in articular cartilage repair. It is stored bound to heparin sulphate proteoglycan in the extracellular matrix. It's an important mitogen that stimulates RNA and DNA synthesis in chondrocytes (130). Many *in vitro* studies have shown that FGF plays a key role in chondrocytes proliferation (130),

promotes the de-differentiation process of primary chondrocyte in monolayer culture (131) and prevents chondrocytes from terminal differentiation (132).

Transforming growth factor-β (TGF-β) is a member of the TGF superfamily, which also includes bone morphogenetic proteins (BMPs). It is secreted in an inactive form, bound to a latency-associated peptide from which it dissociates before becoming active and binding to its target receptor (133). Growth media supplementation with TGF-β induces chondrogenic differentiation as shown in a pre-chondrogenic cell line (134) or in MSC micromass pellet culture (99), and has been reported to promote cell proliferation and to up-regulate aggrecan and type II collagen when applied synergistically with insulin or insulin like growth factor-1 (IGF-I) in chondrocyte alginate culture (135).

Platelet-derived growth factor (PDGF) is a known chemo-attractant, stimulating macrophages and fibroblasts during healing. It is stored in platelets; hence it was recognized as a key growth factor in the microfracture technique attracting cells to the defect site. Not only it is a chemo-attractant, in vitro studies have also showed it to have an impact on chondrocytes and mesenchymal stem cell differentiation, enhancing matrix production and preventing the progression toward endochondral maturation (136-138).

IGF-I is the main anabolic growth factor of articular cartilage. It has been shown to increase proteoglycan and collagen type II synthesis as well as provide chondrocyte phenotypic stability. It is stored in the extracellular matrix, bound to proteoglycans via IGF-1 binding proteins. It's likely that the interaction between it and the binding protein regulate its activity, as an increase in catabolic activity causes proteolysis of these proteins, thereby modulating its release (139).

Indeed, there are evidences that the combination of several specific growth factors during the phases of chondrocyte expansion and subsequent 3D micro-mass culture can have additive effects on the cell proliferation or chondrocyte re-differentiation process (140).

Moreover, historically, monolayer expansion of chondrocytes is supplemented with Fetal Calf Serum (FCS) to support attachment and proliferation (141, 142).

However, the use of animal serum for the generation of grafts for clinical use should be seriously considered since such supplements carry the remote possibility of prion or viral transmission

and of immune reaction against animal proteins. Substitution of animal serum with autologous serum would address these issues and bring human cartilage engineering nearer to a safe clinical application. Previous studies comparing chondrocytes growth with human serum and FCS generally reported superior proliferation rate of chondrocytes in human serum (143-147) and also a reduced variability due to the donor age of the patient (147) .

To re-establish a proper matrix structure during the re-differentiation process in 3-dimensional chondrocyte culture, enzymatic co-factors can additionally be supplemented. For instance, ascorbic acid known as a co-factor for proline and lysine hydroxylase is required for the assembly and stabilization of collagen fibrils (148).

Therefore, in this work we evaluated how the addition to the culture medium of specific growth factors (TGFβ-1/FGF-2/PDGF) during monolayer expansion can modulate the proliferation of human articular chondrocytes seeded at different cell density and in presence of reduced percentage of Human Serum. Moreover we assessed the ability of expanded articular chondrocytes under different conditions (different cell seeding density; percentage of Human Serum; monolayer vs 3D expansion), to re-gain a chondrogenic cell phenotype during the phase of re-differentiation (exposed to TGFβ-1/ insulin/ ascorbic acid) in 3 dimensional cell culture system (i.e. micro mass pellet and collagen type II scaffolds).

1.2.4 *In vitro* culture of chondrocytes: expansion strategies

Chondrocyte-based cartilage repair techniques, including the mentioned ACI, require that autologous articular chondrocytes isolated from a small biopsy are efficiently expanded prior to being grafted in the defect. However, this approach is limited by the difficulty of obtaining sufficient autologous chondrocytes. Finding conditions that permit fast amplification of chondrocytes while maintaining their capacity to generate cartilaginous tissue is an objective of different research groups. The main obstacle for an efficient propagation of chondrocytes derived from the fact that the cell *in vitro* expansion is intrinsically associated with cellular de-differentiation and reduced ability to re-differentiate.

De-differentiation occurs when chondrocytes are cultured under conditions allowing them to attach and spread on a two-dimensional surface. In this environment the spherical phenotype is gradually lost and the cells acquire an elongated fibroblast-like morphology.

These morphologic alterations are accompanied by profound biochemical changes, as indicated by the reduction or total loss of synthesis of aggrecan and type II, IX and XI collagen (cartilage-specific proteins) and the increase in synthesis of versican and type I, III and V collagen (proteins associated with an undifferentiated mesenchymal cell phenotype).

To overcome the limitation of a low initial number of cells and dedifferentiation, various studies have been performed in which chondrocytes are multiplied in monolayer cultures to increase the number of cells and then transferred into an environment supporting a spherical morphology; however their original phenotype is not fully re-acquired (108, 149-151).

In an effort to overcome this problem different strategies have been proposed to expand chondrocytes under conditions maintaining their original phenotype. These include the culture of chondrocytes within 3D gels, sponges or meshes, encapsulated in alginate beads or at the surface of microcarrier beads (152-154). The culture techniques allow chondrocytes to remain round in shape and to continue the expression of cartilage-specific genes, but are limited in the extent of proliferation achieved (155).

One alternative approach to obtain a large number of chondrocytes capable to generate cartilaginous tissues, in contrast to the above described strategy, consists in keeping cells in a more 'plastic' state, thus enhancing their ability to re-differentiate. In particular, the growth factor combination transforming growth factor beta-1, fibroblast growth factor-2 and platelet-derived growth factor-BB (TFP) was shown to increase cell proliferation rate, accelerate the process of cell de-differentiation, and to enhance the re-differentiation capacity of the expanded cells (109).

Another approach to enhance the post-expansion differentiation capacity of chondrocytes is based on the coating of cell culture dishes with specific substrates. In these regards, Barbero et al. (156) found that chondrocytes expanded in type II collagen exhibited higher capacity to generate cartilaginous tissues as compared to cells expanded in plastic culture dishes.

1.2.5 Redifferentiation and implantation

During the monolayer expansion, the chondrocytes dedifferentiate and gradually lose their phenotype. However the feasibility to engineer cartilage tissue starting from expanded chondrocytes is based on the fact that, at least to some extent, the differentiation process may be reversed when cells are transferred into three dimensional environments (157) under appropriate culture conditions (140, 147). The ability of redifferentiate is particularly important when the cell-constructs are going to be transplanted back into the patient.

After implantation, engineered tissue unlike native tissue must continue to grow and remodel in a chemical environment that is likely to contain potent catabolic mediators stemming from inflammatory responses resulting from the disease or the surgical intervention it-self.

During an inflammatory response, cartilage remodeling process is initiated and cytokines such as Interleukin (IL)-1 and Tumor Necrosis Factor (TNF)-α, are produced in response by resident cells and infiltrating inflammatory cells (chondrocytes, monocytes, neutrophils). In particular, IL-1 (α and β) is a pro-inflammatory cytokine that plays important role in normal physiology of cartilage tissue, including stimulation of the turnover of extracelllular matrix. IL-1 isoforms have been shown to have harmful effects on chondrocytes: they (i) inhibit the synthesis of the major physiological inhibitors of pro-degradative enzymes (158), (ii) stimulate the production of prostaglandins, free radicals and NO (159), (iii) inhibit the synthesis of matrix components such as type II collagen and proteoglycans (160-163), (iv) inhibit the chondrocyte differentiation- phenotype by suppressing the expression of Sox-9 (160, 164), (v) inhibit the chondrocytes proliferation and induce cell death (164).

These post-operative inflammatory responses have particular relevance in the context of cell based-cartilage repair, considering that therapeutic cell preparations (single cell suspension, cell-seeded matrixes or cartilaginous tissues) once grafted in the joint defect will become exposed to a biochemical environment rich in catabolic mediators and could have long-lasting detrimental effects (165).

It’s therefore important to define the appropriate stage of maturation for cell-based constructs and the consequential state of cell redifferentiation at which the chondrocytes result less susceptible to such mediators guaranteeing superior clinical outcome.

1.2.6 Automated tissue engineering system for clinical application

A bioreactor is an apparatus in which biological and biochemical processes develop under finely designed environmental and operating conditions such as pH, temperature, pressure, nutrient supply, waste removal and biomechanical stimuli (166).

Recently, bioreactor culture systems have been applied to the tissue engineering field, providing reliable models to study tissue biology under controlled experimental conditions. Moreover, the use of bioreactors introducing high level of control, reproducibility and automation can be applied to the manufacturing process of tissue engineered products for clinical applications.

Currently, in the case of the manufacturing process for cultured tissues aiming at autologous transplantation, such as autologous chondrocytes implantation (ACI), a minimum of biopsies is harvested from patients. The isolated cells after shipment to special GMP production facilities are subjected to ex vivo expansion in a series of monolayer cultures. After the cells are expanded sufficiently, cells or three-dimensional grafts are returned to the hospital for implantation. Obviously, the resulting process of manufacturing is burdened by limitations such as the lack of standardization with the risk of biological contamination due to the numerous manual handling steps. Additionally, high cost due to the implementation of special room facilities in compliance with Good Manufacturing Practice (GMP) requirements are associated with manufacturing. Therefore, the application of cell-based tissue engineering approaches in the routine clinical practice critically depends on the development of innovative bioreactor systems.

An ideal bioreactor would be a single closed and automated system in which starting from a patient's biopsy, are performed all the different processing phases: cell isolation, expansion, seeding on scaffold and differentiation.

A controlled closed bioreactor system would therefore facilitate the streamlining and automation of the numerous labor-intensive steps involved in the *in vitro* engineering of 3D cartilage tissue in a manner that is reproducible, safe, clinically effective as well as economically acceptable and cost-effective (167).

One example is the Autologous Clinical Tissue Engineering System (ACTES), under development by Millenium Biologix (www.millenium-biologix.com), is a closed bioreactor system

aimed at fully automating the processes cartilage biopsy digestion, chondrocytes expansion and cell resuspension. However associated to a bioreactor system like ACTES, there are several constraints such as a fixed expansion surface, the possibility of a single passage and the requirement to yield a minimum amount of cells (12 Millions) and the necessity to reduce the percentage of autologous serum.

We aimed our study to define suitable operating conditions for systems like ACTES. In particular we aimed our study at determining whether a clinical relevant number (≥12 millions) of human articular chondrocytes can be generated in 1 passage starting from variable initial seeding densities and using low percentages of human serum, assuming a defined culture surface, currently in use in the ACTES system.

1.3 AIMS OF THE THESIS

Cartilage lesions resulting from trauma or degenerative diseases are one of the major factors leading to joint disease and disability. Articular cartilage being an avascular tissue has limited capacity for self repair.

To overcome these limitations, tissue engineering techniques have emerged as an innovative field of research with the potential to recreate three dimensional cartilaginous tissues.

Such approach generally rely on the expansion of a limited population of chondrocytes derived from a small cartilage biopsy, intrinsically associated with cellular de-differentiation (108), and on the ability of the expanded cells to efficiently and reproducibly re-differentiate and generate cartilaginous tissue.

The objective of my research was to study different clinically relevant aspects of the biology of human articular chondrocytes, related to different phases in the process of engineering of a functional cartilage tissue (Figure 4). In particular, my thesis was aimed at determining:

- whether the supplementation of growth factors enhancing chondrocyte proliferation and re-differentiation capacity allows a reproducible and efficient clinical-scale expansion of human articular chondrocytes (HAC) starting from variable initial seeding densities and using low percentages of human serum (**Study 1;** Francioli *et al*., Tissue Eng (2007));

- whether the quality of cartilaginous tissues generated by HAC on three-dimensional biomimetic scaffolds can be enhanced by direct expansion on the scaffold, as compared to standard growth on plastic, or by increasing cell seeding density (**Study 2;** Francioli *et al*., Tissue Eng (*submitted*));

- how the extent of maturation of HAC-based cartilaginous tissues modulates the profile of chemokine production and the inflammatory/catabolic response to IL-1β (**Study 3;** Francioli *et al*., Clin Orthop Relat Res (*in preparation for submission*)).

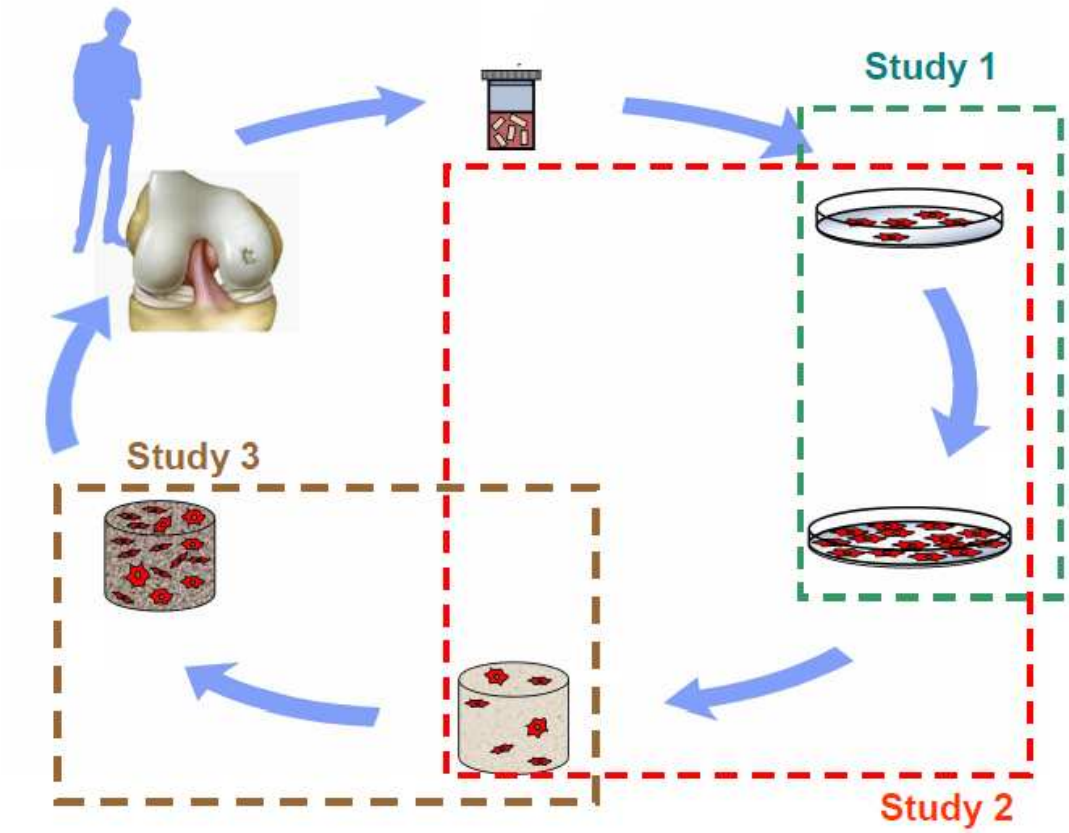

Figure 4. Diagram of a typical tissue engineering approach for cartilage repair. The dotted squares indicate the phases of the process to which the different studies presented in this thesis are related.

2 PAPERS

2.1 PAPER I: Growth Factors for Clinical-Scale Expansion of Human Articular Chondrocytes: relevance for Automated Bioreactor System

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Growth Factors for Clinical-Scale Expansion of Human Articular Chondrocytes: Relevance for Automated Bioreactor Systems

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ABSTRACT

The expansion of chondrocytes in automated bioreactors for clinical use requires that a relevant number of cells be generated, starting from variable initial seeding densities in one passage and using autologous serum. We investigated whether the growth factor combination transforming growth factor beta 1/fibroblast growth factor 2/platelet-derived growth factor BB (TFP), recently shown to enhance the proliferation capacity of human articular chondrocytes (HACs), allows the efficiency of chondrocyte use to be increased at different seeding densities and percentages of human serum (HS). HACs were seeded at 1,000, 5,000, and 10,000 cells/cm^2 in medium containing 10% fetal bovine serum or 10,000 cells/cm^2 with 1%, 5%, or 10% HS. The chondrogenic capacity of post-expanded HACs was then assessed in pellet cultures. Expansion with TFP allowed a sufficient number of HACs to be obtained in one passage even at the lowest seeding density and HS percentage and variability in cartilage-forming capacity of HACs expanded under the different conditions to be reduced. Instead, larger variations and insufficient yields were found in the absence of TFP. By allowing large numbers of cells to be obtained, starting from a wide range of initial seeding densities and HS percentages, the use of TFP may represent a viable solution for the efficient expansion of HACs and addresses constraints of automated clinical bioreactor systems.

INTRODUCTION

ARTICULAR CARTILAGE has a limited capacity for self-repair, and if left untreated, cartilage defects can lead to the early onset of osteoarthritic degenerative changes. Promising approaches to induce cartilage repair are based on the implantation of autologous chondrocytes directly after expansion[1] or after loading and culture on specific scaffolds.[2–5] The manufacture of cell-based products for cartilage repair typically requires shipment of a small cartilage biopsy to Good Manufacturing Practice (GMP) facilities, where chondrocytes are isolated, expanded in culture flasks, and shipped back to the healthcare center. These procedures are, however, associated with complicated logistics and, because of the predominance of manual handling steps, to limited standardization and ultimately high costs.

The use of bioreactors within the hospital or healthcare center for automated culture of autologous cells would eliminate logistical issues of transferring specimens between locations, reduce the need for large and expensive GMP facilities, and minimize operator handling, with the final possible result of simplifying, reducing the cost, and increasing the standardization of chondrocyte-based cartilage repair techniques. One example of this, Autologous Clinical Tissue Engineering System (ACTES), under development by Millenium Biologix (www.millenium-biologix.com), is a closed bioreactor system aimed at fully automating the processes of cartilage biopsy digestion, chondrocyte expansion, rinsing,

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and concentration in a defined medium volume.[6] However, in a bioreactor system like ACTES, cells have to be expanded in only one passage, and because of the fixed area of the culture surface and the variable size of cartilage biopsies, the initial cell seeding densities may be highly variable between production batches. Moreover, the system should use human autologous serum at low percentages, to avoid use of animal-derived factors and to reduce the dependence of the culture on a non-standardized factor such as autologous serum. With these constraints, the bioreactor system should yield at least 12 million chondrocytes, because typical autologous chondrocyte implantation is indicated to treat cartilage defects up to 12 cm^2 in size by injection of at least 1 million cells per cm^2 defect.[7]

With the ultimate goal of defining suitable operating conditions for systems like ACTES, in the present study we aimed at determining whether a clinically relevant number (≥12 million) of human articular chondrocytes (HACs) can be generated in 1 passage starting from variable initial seeding densities and using low percentages of human serum, assuming a culture surface area of 250 cm^2, currently in use in the ACTES system. Specific growth factors, namely transforming growth factor beta 1 (TGFβ-1), fibroblast growth factor 2 (FGF-2), and platelet-derived growth factor type BB (PDGF-BB) (TFP), previously shown to increase the proliferation efficiency of HACs,[8,9] were tested to reduce the variability in the yield of HACs under the different conditions. Because the quality of HAC expansion is related not only to the number but also to the cartilag-forming capacity of the expanded cells, HACs grown under the different experimental conditions were assessed in a micromass pellet culture model typically used to investigate the chondrogenic capacity of various cells types.[10,11]

MATERIALS AND METHODS

Human serum

Pools of human serum (HS) were purchased by Blutspendezentrum SRK Bern AG, and serum from 3 healthy donors was isolated from Blutzentrum, University Hospital Basel, Basel, Switzerland. The sera were aliquoted and stored at −20°C until ready for use. Heat inactivation of the sera was not performed.

Cartilage sample collection and cell isolation

Full-thickness human articular cartilage biopsies were obtained postmortem (within 24 h after death) from the lateral condyle of knee joints of 5 individuals (mean age 42.7, range 27–61) with no history of joint disease, after informed consent by relatives and in accordance with the local ethics committee of University Hospital Basel, Switzerland. The biopsies used, although they were not derived from a traumatic joint, would be relevant for our target clinical application (e.g., treatment of cartilage injuries), considering the established similarity between chondrocytes surrounding a cartilage defect or from normal cartilage.[12] Cartilage tissues were minced and then digested with 0.15% type II collagenase (10 mL solution/g tissue, 300 U/mg, Worthington Biochemical Corporation, Lakewood, NJ) for 22 h, and the isolated chondrocytes were then seeded and expanded as described below.

Cell expansion

Cell seeding at different densities. Freshly isolated HACs were seeded at 1,000, 5,000 or 10,000 cells/cm^2 with Dulbecco's modified Eagle medium (DMEM) containing 4.5 mg/mL D-glucose, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.29 mg/mL L-glutamate (basic medium) supplemented with 10% fetal bovine serum (FBS) without (CTR) or with the addition of growth factors (1 ng/mL TGFβ1, 5 ng/mL FGF-2, and 10 ng/mL PDGF-BB) (TFP)[13] and expanded in monolayer for 1 passage in a humidified 37°C/5% carbon dioxide (CO_2) incubator. Medium was changed twice a week.

The densities 1,000 cells/cm^2 and 5,000 cells/cm^2 were used considering the size of a cartilage biopsy usually provided from patients undergoing autologous chondrocyte implantation (100–500 mg),[7] a normal yield after tissue digestion (2.6×10^6 cells/g)[14] and the available cell expansion surface in ACTES system (250 cm^2),[6] whereas 10,000 cells/cm^2 was considered to be a standard cell-seeding density.[13,15]

When HACs reached confluence, they were rinsed with phosphate buffered saline, detached by treatment with 0.3% type II collagenase, followed by 0.05% trypsin/0.53 mM ethylenediamine tetraacetic acid (EDTA), counted, and used to generate pellets as described below.

Use of different percentages of human serum. Freshly isolated HACs were seeded at 10,000 cells/cm^2 with basic medium supplemented with 1%, 5%, or 10% HS without or with supplementation of the growth factor combination TFP and expanded in monolayer for 1 passage in a humidified 37°C/5% CO_2 incubator. Cells from 1 additional individual (male, 51 years old) were also cultured with 1%, 5%, or 10% serum obtained from 3 different donors.

Three-dimensional pellet cultures

To assess the quality of the expanded chondrocytes, HACs were induced to re-differentiate in pellet cultures as previously described.[8] Briefly, HACs were suspended in basic medium supplemented with 10 µg/mL insulin, 5.5 mg/mL transferrin, 5 ng/mL selenium, 0.5 mg/mL bovine serum albumin, and 4.7 mg/mL linoleic acid (Sigma Chemical, St Louis, MO); 0.1 mM ascorbic acid 2-phosphate; 1.25 mg/mL human serum albumin; 10^{-7} M dexamethasone; and 10 ng/mL TGF-β1 (chondrogenic medium). Aliquots of 5×10^5 cells/0.5 mL were centrifuged at 250 g for 5 min in 1.5-mL

polypropylene conical tubes (Sarstedt, Nümbrecht, Germany) to form pellets, which were placed onto an orbital shaker (Bioblock Scientific, Frenkendor, Switzerland) at 30 rpm. Pellets were cultured for 2 weeks, with medium changes twice per week, and subsequently processed for histological, immunohistochemical, and biochemical analysis as described below. Each analysis was performed independently on at least 2 entire pellets for each primary culture and expansion condition.

Analytical methods

Proliferation rate. Proliferation rate was calculated as the ratio of $\log_2(N/N_0)$ to T, where N_0 and N are the numbers of cells respectively at the beginning and end of the expansion phase, $\log_2(N/N_0)$ is the number of cell doublings, and T is the time required for the expansion.

Histological and Immunohistochemical analysis. Pellets were fixed in 4% formalin for 24 h at 4°C, embedded in paraffin, cross-sectioned (5 µm thick), and stained with Safranin O for sulfated glycosaminoglycans (GAGs). Sections were also processed for immunohistochemistry to visualize collagen type II (II-II6B3, Hybridoma Bank, University of Iowa, Iowa City, IA), as previously described.[16]

Biochemical analysis. Pellets were digested with protease K (0.5 mL of 1 mg/mL protease K in 50 mM Tris with 1mM EDTA, 1 mM iodoacetamide, and 10 µg/mL pepstatin-A) for 15 h at 56°C, as previously described.[17] GAG amounts were measured spectrophotometrically using dimethylmethylene blue,[18] with chondroitin sulfate as a standard, and normalized to the deoxyribonucleic acid (DNA) amounts, measured spectrofluorometrically using the CYQuant cell proliferation assay Kit (Molecular Probes, Eugene, OR) and with calf thymus DNA as a standard. GAG contents are reported as µg GAG/µg DNA.

Statistical analysis. All values are presented as means ± standard deviation of measurements from 4 to 5 independent experiments (with cells from the 4–5 different individuals). The coefficient of variation (standard deviation as a percentage of the mean), was used to assess the variability of a certain parameter under different culture conditions. Differences between experimental groups were assessed using Mann-Whitney tests and considered statistically significant at $p < 0.05$.

RESULTS

Cell seeding at different densities

Cell proliferation. This set of experiments was performed using medium containing 10% FBS.

In CTR medium, HACs seeded at 10,000 and 5,000 cells/cm^2 proliferated at comparable rates. Cells reached confluence within 15 to 19 days of culture and underwent a similar number of doublings (2.2 and 2.9, respectively) (Table 1). However, HACs seeded at 1,000 cells/cm^2 required more than 3 weeks to reach confluence, and during this time they underwent 2.2 times as many doublings as cells seeded at the highest density (Table 1). In general, a decrease in cell density paralleled enhanced HAC expansion; the total cell doublings were 4.8, 2.9, and 2.2 for cells seeded at the densities of 1,000, 5,000 and 10,000 cells/cm^2, respectively (Table 1).

In medium containing growth factors (TFP medium), at any seeding density, chondrocytes proliferated at a remarkably and significantly higher rate (up to 3.5-fold), reached confluence in a shorter time (up to 2.0-fold) and underwent more doublings (up to 1.9-fold) than HACs cultured in CTR medium (Table 1). As observed for cells cultured in CTR medium, doublings of HACs expanded with TFP increased by decreasing seeding density (7.6, 5.4, and 3.9 for the seeding densities of 1,000, 5,000 and 10,000 cells/cm^2, respectively). Expansion with TFP resulted in reduced variability in the proliferation rates of HACs seeded at the different seeding densities, with the coefficient of variation decreasing from 27.5 to 14.6.

We then used the doublings measured under the different conditions to estimate the number of HACs that would be obtained if chondrocytes were seeded in the culture surface of the ACTES system (equal to 250 cm^2). The required number for clinical-scale expansion (12 million cells) would not

Table 1. Expansion of Human Articular Chondrocytes (HAC) in Relation to Seeding Density

	1000 cells/cm^2			*5000 cells/cm^2*			*10000 cells/cm^2*		
	Doublings	*Expansion time (days)*	*Proliferation rate*	*Doublings*	*Expansion time (days)*	*Proliferation rate*	*Doublings*	*Expansion time (days)*	*Proliferation rate*
CTR	4.8 ± 0.4*	24.3 ± 3.1*	0.20 ± 0.04*	2.9 ± 0.8	18.7 ± 2.1	0.17 ± 0.05	2.2 ± 0.6	15.0 ± 2.6	0.15 ± 0.04
TFP	7.6 ± 0.8*°	12.0 ± 1.6*°	0.64 ± 0.05*°	5.4 ± 0.7*°	10.6 ± 1.8*°	0.51 ± 0.06°	3.9 ± 0.6°	7.6 ± 0.5°	0.52 ± 0.07°
TFP/CTR	1.6	0.5	3.2	1.9	0.6	3.0	1.8	0.5	3.5

HAC were seeded in monolayer at different densities in medium containing 10% fetal bovine serum and cultured without growth factors (CTR) or with transforming growth factor beta 1, fibroblast growth factor 2, and platelet-derived growth factor type BB (TFP) for 1 passage. For the calculation of the parameter see the Materials and Methods section. Results are means ± standard deviations of values from 4 to 5 independent donor cells populations. *$p < 0.05$ from 10,000 cells/cm^2; °$p < 0.05$ from CTR.

be reached for any of the seeding densities in CTR medium, whereas expansion with TFP would yield more than 40 millions of cells for each seeding density condition (Fig. 1A).

Chondrogenic capacity. Because not only the number of cells obtained after expansion, but also their quality, is a clinically relevant issue, we evaluated the chondrogenic capacity of cells expanded in the different conditions in a pellet culture model. HACs seeded at 10,000 and 5,000 cells/cm^2 produced tissues that stained similarly with Safranin O, whereas cells seeded at 1,000 cells/cm^2 generated pellets with a lower staining intensity (Fig. 2A). Biochemical analysis of the pellets confirm this trend, although because of the large donor-to-donor variability, no statistically significant difference in the GAG contents of the generated tissues could be measured between the experimental conditions. Expansion with TFP reduced the variability in the GAG contents of pellets produced by cells seeded at different densities histologically and biochemically, with the coefficient of variation for the GAG/DNA contents decreasing from 37.5% to 24.5% (Fig. 2B).

Cell expansion using different percentages of HS

Cell proliferation. This set of experiments was performed using an initial seeding density of 10,000 cells/cm^2.

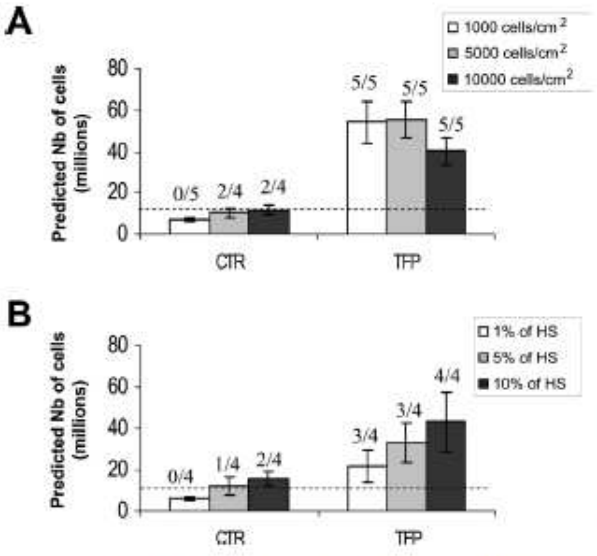

FIG. 1. Predicted number of human articular chondrocytes (HACs) that would be obtained after expansion in a culture surface comparable to that of Autologous Clinical Tissue Engineering System (250 cm^2) under different conditions of initial seeding densities (**A**) or percentage of human serum (HS) (**B**). Results are means ± standard deviations of values from 4 to 5 independent donor cells. Fractions over each bar represent the ratio of cultures that reached a minimum of 12 million to total cultures analyzed.

In CTR medium, HACs cultured with 10% and 5% HS proliferated at comparable rates. Cells reached confluence within 9.5 to 10.5 days of culture and underwent a similar number of doublings (2.5 and 2.1, respectively) (Table 2). However, cells expanded with 1% HS required more time to reach confluence (1.4-fold) and underwent fewer doublings (2.1-fold) than chondrocytes cultured with 10% HS (Table 2). Chondrocytes displayed a higher proliferation rate overall (1.9-fold) when cultured with 10% HS than with 10% FBS (see Table 1 and 2).

In TFP medium, HACs cultured at any HS percentage proliferated at a higher rate than those cultured in CTR medium, although because of large donor-to-donor variability, statistical significance was established only for the lowest serum concentration. At 1% HS, TFP-expanded chondrocytes reached confluence in a shorter time (1.6-fold) and underwent more doublings (2.3-fold) than CTR-expanded cells (Table 2). Expansion with TFP resulted in less variability in the proliferation rates of HACs cultured with the different

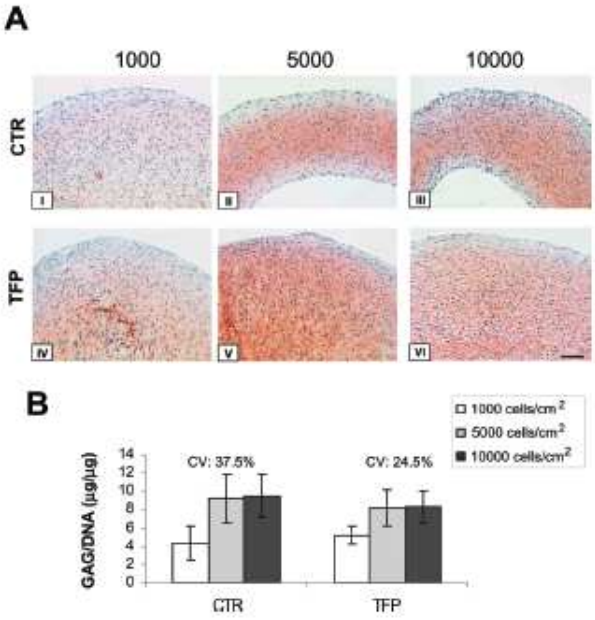

FIG. 2. Glycosaminoglycan (GAG) accumulation in pellets generated by human articular chondrocytes (HACs) after expansion under different initial seeding density conditions. (**A**) Safranin O staining of representative pellets generated by HACs seeded at 1,000 (I and IV), 5,000 (II and V), or 10,000 (III and VI) cells/cm^2 and expanded in CTR (I, II, and III) or TFP (IV, V, and VI) medium. Bar = 100 µm. (**B**) GAG content normalized to the amount of deoxyribonucleic acid (DNA) (GAG/DNA) in the pellets generated by HACs expanded under the different conditions. Results are means ± standard deviations of values from 4 to 5 independent donor cell populations. Coefficients of variation (CVs) over each bar are the standard deviations (as % of the mean) of the GAG contents measured at the different cell-seeding densities. Color images available online at www.liebertpub.com/ten.

TABLE 2. EXPANSION OF HUMAN ARTICULAR CHONDROCYTES (HACs) IN RELATION TO HUMAN SERUM (HS) PERCENTAGE

	1% HS			*5% HS*			*10% HS*		
	Doublings	*Expansion time (days)*	*Proliferation rate*	*Doublings*	*Expansion time (days)*	*Proliferation rate*	*Doublings*	*Expansion time (days)*	*Proliferation rate*
CTR	1.2 ± 0.5*	13.5 ± 2.6*	0.12 ± 0.1*	2.1 ± 0.9	10.5 ± 3	0.23 ± 0.2	2.5 ± 0.6	9.5 ± 2.1	0.29 ± 0.1
TFP	2.7 ± 1.2°	8.5 ± 1.3°	0.34 ± 0.2°	3.5 ± 1.1	8.5 ± 1.3	0.42 ± 0.2	3.9 ± 0.9	8.2 ± 1.5	0.49 ± 0.16
TFP/CTR	2.3	0.6	2.8	1.7	0.8	1.8	1.6	0.9	1.7

HACs were seeded in monolayer at 10,000 cells/cm^2 and cultured with different HS concentrations in medium without growth factors (CTR) or with transforming growth factor beta 1, fibroblast growth factor 2, and platelet-derived growth factor type BB (TFP) for 1 passage. For the calculation of the parameter, see the Materials and Methods section. Results are means ± standard deviations of values from 4 independent donor cell populations. *$p < 0.05$ from 10% HS; °$p < 0.05$ from CTR.

concentrations of HS, with the coefficient of variation decreasing from 45.3 to 22.2.

In CTR medium, cells cultured with 1% HS would not yield the required number of cells (12 million) if cultured under the same conditions in the culture surface of the ACTES system (250 cm^2), whereas in TFP medium, more then 20 million chondrocytes would be generated for each HS percentage (Fig. 1B).

Chondrogenic capacity. HACs expanded in CTR medium with 10% and 5% HS produced tissues similarly stained with Safranin O, whereas cells expanded with 1% HS generated pellets with a lower staining intensity (Fig. 3A). Biochemical analysis of the pellets confirmed this trend, but because of the large donor-to-donor variability, no statistically significant difference in the GAG contents of the generated tissues could be established between the experimental conditions. Expansion with TFP reduced variability in the GAG contents of pellets produced by cells cultured with different HS percentages, with coefficient of variation decreasing from 11.6% to 4.4% (Fig. 3B). Tissues that contained large amounts of GAG generally contained higher amounts of collagen type II protein, as assessed using immunohistochemical staining (Fig. 3C).

To determine whether HS from different donors could promote cell proliferation and re-differentiation capacity differently, chondrocytes from 1 additional cartilage biopsy were expanded in TFP medium with serum obtained from 3 different donors and then cultured in pellets. For each HS percentage condition used (1%, 5%, and 10%), cells proliferated at similar rates (coefficient of variation < 6.0). However, a greater percentage of HS used during the expansion resulted in enhanced variability in the GAG contents of pellets generated by cells expanded with serum from different donors, with the coefficients of variation of 4.7, 19.5, and 46.1 for 1%, 5%, and 10% HS, respectively (Fig. 4).

DISCUSSION

With the final goal of validating culture conditions for clinical-scale expansion of HACs, we investigated the proliferation capacity of HACs cultured starting from a wide range of initial seeding densities (1,000, 5,000, and 10,000 cells/cm^2) and HS percentages (1%, 5%, and 10%) in medium not supplemented and supplemented with growth factors. We found that expansion with CTR medium was not reproducible and did not yield a clinically relevant number of chondrocytes (≥12 millions) at each seeding density condition and at the lowest percentage of HS. Using TFP medium, instead, proliferation capacity was strongly enhanced such that 22 to 55 million chondrocytes could be generated in the different experimental conditions tested. Moreover, medium supplementation with TFP reduced variability in proliferation and cartilage-forming capacity of cells expanded under the different experimental conditions.

In clinical practice, the dimension of a cartilage biopsy and therefore the number of chondrocytes available for *ex vivo* expansion will vary from donor to donor. Therefore, having the constraint of a fixed surface area, have initially evaluated the effect of seeding density on HAC proliferation capacity. To the best of our knowledge, this is the first study evaluating the growth capacity of articular chondrocytes of human origin at different initial seeding densities. Similar investigations have been performed using chondrocytes from non-articular sites[19,20] or of animal origin.[21] Our results indicate that HACs seeded at decreasing densities proliferated in CTR medium with greater rates, such that a maximum number of population doublings (4.8) was measured for HACs seeded at 1,000 cells/cm^2. Mandl *et al.* observed a similar relationship between initial seeding density and proliferation capacity with human *auricular* chondrocytes.[20] The authors reported that, using a density of 3,500 or 7,500 cells/cm^2, a 20-fold increase in cell number was reached between passage 2 and 3 for most donors. We have demonstrated that human *articular* chondrocytes seeded at 1,000 cells/cm^2 undergo almost 8 doublings (corresponding to more than a 250-fold increase in cell number) in less than 2 weeks in just 1 passage while maintaining their differentiation capacity, but only in the presence of TFP. Thus, the use of TFP during HAC expansion allows a clinically relevant number of cells to be obtained also if initially seeded at low densities (and therefore starting from a cartilage biopsy of small size), reduces the time required for cell expansion, and reduces the

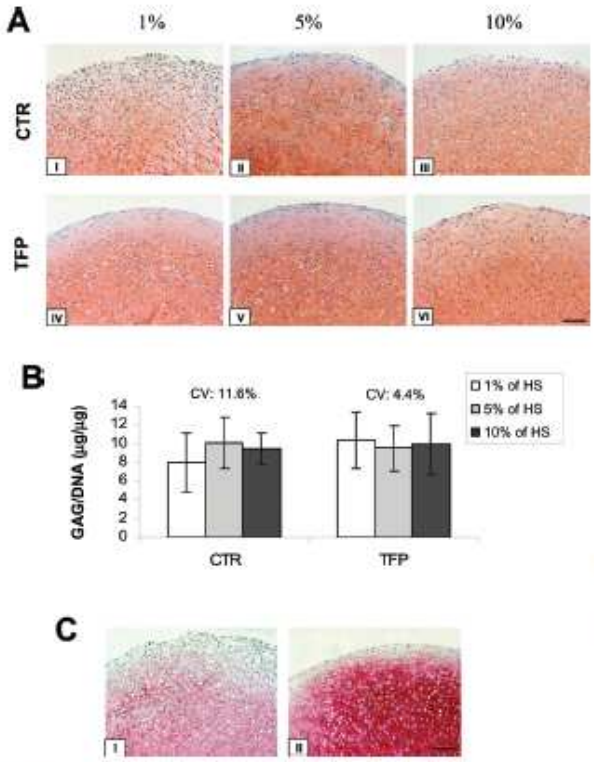

FIG. 3. Glycosaminoglycan (GAG) and collagen type II accumulation in pellets generated by human articular chondrocytes (HACs) after expansion with different percentages of human serum (HS). (**A**) Safranin O staining of representative pellets generated by HACs expanded with 1% (I and IV), 5% (II and V), or 10% (III and VI) HS in medium without growth factors (CTR) (I, II, and III) or with transforming growth factor beta 1, fibroblast growth factor 2, and platelet-derived growth factor type BB (TFP) (IV, V, and VI). Bar = 100 µm. (**B**) GAG content normalized to the amount of deoxyribonucleic acid (DNA) (GAG/DNA) in the pellets generated by HACs expanded under the different conditions. Results are means ± standard deviations of values from 4 independent donor cell populations. Coefficients of variation (CVs) over each bar are the standard deviation (as % of the mean) of the GAG contents measured at the different HS percentages. (**C**) Collagen type II immunohistochemistry staining of representative pellets generated by HACs expanded with 1% HS in CTR (I) or TFP medium (II). Bar = 100 µm. Color images available online at www.liebertpub.com/ten.

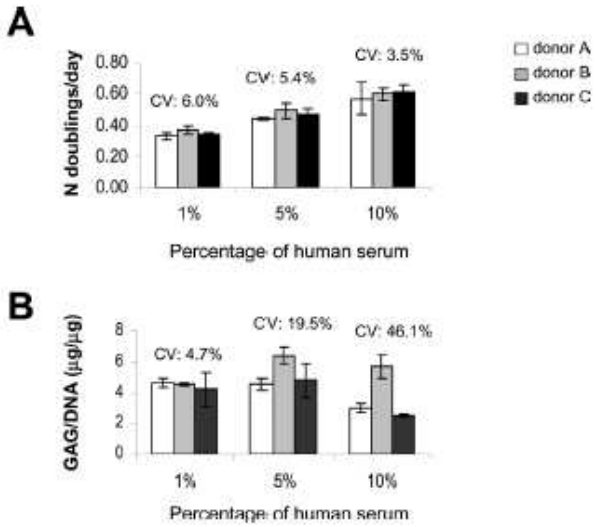

FIG. 4. Serum-related differences in the proliferation and glycosaminoglycan (GAG) accumulation of human articular chondrocytes (HACs). HACs from one cartilage biopsy were expanded in medium containing transforming growth factor beta 1, fibroblast growth factor 2, and platelet-derived growth factor type BB (TFP) and serum obtained from 3 different donors (A, B, and C) at increasing percentages. (**A**) Proliferation rates of HACs. (**B**) GAG contents of pellets generated by HACs. Coefficients of variation (CVs) over each bar are the standard deviations (as % of the mean) of the proliferation rates or GAG contents of cells expanded with serum from the different donors.

variability in proliferation rates of cells initially seeded at different densities. TFP-expanded chondrocytes generated pellets with high GAG contents even if they underwent a much larger number of doublings than cells expanded without growth factors. With the prospective of a clinical application, contact of human cells with FBS must be minimized, because proteins from ruminant materials have been implicated in prion transmission,[22,23] and European legislation does not recommend their use. In the second part of our study, we therefore evaluated the effect of using HS on HAC proliferation capacity. Our observation that 10% of a *pool* of HS supported higher proliferation of chondrocytes than 10% FBS is in agreement with previous results obtained using *autologous* HS[24–26] and may be due to the higher concentration of epidermal-derived growth factor and PDGF measured in human serum.[25] Using an initial cell seeding density of 10,000 cells/cm^2, we then showed that HAC expansion with TFP, but not with CTR medium, yielded more than 20 million cells for any HS percentage. Because the proliferation rate of HACs was higher using lower cell-seeding densities (Table 1), it is likely that TFP will allow the required cell numbers to be obtained for any HS percentage even at the lowest seeding densities, although this remains to be experimentally determined. When HS from different donors was used, similar and low coefficients of variation in HAC proliferation rate were measured at any HS percentage, whereas coefficients of variation in the GAG content of resulting pellets were dramatically greater with HS percentage. This result confirms the importance of assessing not only chondrocyte yields but also their chondrogenic capacity and also indicates the need for a reduction in the percentage of HS used to increase reproducibility of HAC quality. In this context, the use of TFP during HAC expansion, by supporting sufficient cell yields even at low percentages of HS, could also contribute to reducing the variability

in the number and quality of expanded autologous chondrocytes.

From a regulatory standpoint, the use of recombinant growth factors for the production of a therapeutic product requires the appropriate level of qualification. Safety concerns related to the use of TFP during HAC expansion should have minor relevance, because extensive washing of the cell preparation will remove these components so that they would be virtually absent in the final graft material. However, chondrocyte populations generated using TFP might need to be further analyzed for specific genetic alterations possibly related to senescence or DNA methylation before their clinical use.

In conclusion, our study showed that the use of TFP represents a viable solution for the reproducible and efficient clinical-scale expansion of HACs. Although our findings were based on the use of conventional tissue culture plates, implementation of the principle would be particularly relevant for automated processing systems for clinical use, which have higher constraints than typical manual culture in dishes. The concept of using specific growth factors in clinical-grade bioreactors could be applied for the propagation of cells from different human sources to reduce variability in the number and quality of expanded cells and, ultimately, in the clinical outcome of cell-based regenerative techniques.

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2.2 PAPER II: Effect of three-dimensional expansion and cell seeding density on the cartilage-forming capacity of human articular chondrocytes in type II collagen sponges

Effect of three-dimensional expansion and cell seeding density on the cartilage-forming capacity of human articular chondrocytes in type II collagen sponges.

Francioli SE; Candrian C; Martin K; Heberer M; Martin I; Barbero A.

ABSTRACT

Chondrocytes for tissue engineering strategies are typically expanded in monolayer (2D), leading to cell de-differentiation but allowing to generate large cell numbers for seeding into scaffolds. Direct chondrocyte culture in scaffolds, instead, would reduce cell de-differentiation but also the total proliferation extent and thus the potential seeding density. This study investigates whether the quality of cartilaginous tissues generated in vitro by human articular chondrocytes (HAC) on type II collagen sponges is enhanced (i) by direct expansion on the substrate (3D), as compared to standard 2D, or (ii) by increasing cell seeding density, which in turn requires extensive 2D expansion. 3D expansion of HAC on the scaffolds, as compared to 2D expansion for the same number of doublings, better maintained the chondrocytic phenotype but did not enhance the cartilage-forming capacity of the expanded cells. Instead, increasing the HAC seeding density in the scaffolds allowed enhanced chondrogenesis, even if seeded cells had to be expanded and de-differentiated more extensively in 2D in order to reach the required cell numbers. The present study indicates that a high seeding density of HAC in 3D scaffolds is more critical for the generation of cartilaginous constructs than the stage of cell differentiation reached following expansion.

INTRODUCTION

Articular cartilage is a highly specialized tissue, characterized by unique biomechanical properties and, on the other hand, a poor regenerative potential (1, 2). As a consequence, when a traumatic lesion or degenerative disease occurs, the defect is filled with a fibrous tissue which is not capable to withstand the high compressive and shear forces acting in the joint, often leading to the development of early osteoarthritis (2). Among the many surgical options for the treatment of such lesions, implantation of autologous chondrocytes is gaining increasing popularity, especially for critically sized defects (3, 4). These procedures include autologous cartilage implantation (ACI) (5-8), the more recent matrix-mediated ACI (MACI) (9), or the grafting of tissue engineered cartilaginous constructs (TE) (10, 11), typically based on cultivation of cells on porous scaffolds. The latter approach offers potential advantages, consisting in improved cell retention, easier graft handling upon implantation, and earlier post-operative rehabilitation for the patient (12).

Cells for TE strategies are typically expanded in two-dimensional (2D) culture surfaces to increment their original number. Such culture phase is known to cause cellular de-differentiation, a process during which chondrocytes gradually lose their spherical shape and acquire an elongated fibroblast-like morphology. These morphologic alterations are accompanied by profound biochemical changes, as indicated by the reduction or total loss of synthesis of aggrecan and type II collagen (cartilage-specific proteins) and the increase in synthesis of proteins associated with an undifferentiated mesenchymal cell phenotype, including smaller proteoglycans (e.g., versican) and type I collagen (13-15).

The feasibility to engineer cartilage tissues starting from expanded chondrocytes is based on the fact that, at least to some extent, the dedifferentiation process may be reversed when cells are transferred onto three dimensional (3D) scaffolds (13, 16-18), especially if seeded at a high density (19, 20) . Thus, one possible approach to cartilage TE relies on extensive 2D expansion of chondrocytes, allowing to obtain a large number of cells and thus to increase the cell seeding density in the scaffold. In this regard, the use of specific culture supplements during chondrocyte expansion was previously shown to support a more efficient cell growth while maintaining the redifferentiation capacity (21, 22).

An alternative strategy for the utilization of the small number of chondrocytes obtained from a cartilage biopsy consists in their limited expansion in 2D or even in the culture onto 3D porous scaffolds directly

following cell isolation (23-25). In principle, this technique would allow a better maintenance of the chondrocytic phenotype but reduces the extent of cell proliferation, and thus the cell seeding density.

With the ultimate goal to improve the efficiency of utilization of a limited number of autologous chondrocytes, we assessed the quality of cartilage tissues engineered by *human* articular chondrocytes (HAC) using different strategies related to their expansion. In particular, we compared the outcome of HAC expansion (i) to the same extent onto a 3D porous scaffold or in 2D (Study 1), and (ii) to different extents in 2D, followed by seeding in 3D scaffolds at different densities (Study 2). The 3D scaffolds used in this work were made of type II collagen (i.e., the main component of the articular cartilage), which were previously shown to support maintenance of the chondrocytic phenotype and to promote the re-differentiation of de-differentiated chondrocytes from several species (24, 26-29).

MATERIALS AND METHODS

Type II collagen scaffolds

Type II collagen scaffolds, prepared from porcine cartilage using proprietary methods, were obtained as pre-fabricated sponges (Chondrocell; Geistlich Biomaterials, Wolhusen, Switzerland). The average pore size of the scaffold was previously reported as 86µm with a porosity of 85% (29). The collagen matrices were produced as sterile sheets, from which disks of 1.2mm thickness and 8mm diameter were cut out with a biopsy punch under sterile conditions.

Cartilage biopsies and Chondrocytes isolation

Full-thickness human articular cartilage biopsies were obtained post mortem (within 24 hours after death) from the lateral condyle of knee joints of 8 individuals (mean age: 46, range 37 and 65 year old) with no history of joint disease, after informed consent by relatives and in accordance with the local ethics committee (University Hospital Basel, Switzerland).

Cartilage tissues were minced in small pieces and digested upon 22-hours incubation at 37°C in 0.15% type II collagenase (10 mL solution/g tissue, 300 U/mg, Worthington Biochemical Corporation, Lakewood, NJ, USA) and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum, 4.5mg/mL D-glucose, 0.1mM non essential amino acids, 1 mM sodium pyruvate, 100mM HEPES buffer, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.29mg/mL glutamine (*complete medium*).

The isolated chondrocytes were then counted using trypan blue, seeded, expanded and cultured as described below.

Chondrocytes culture

Study 1: Cell expansion directly in 3D scaffolds or in 2D to the same extent

Freshly isolated (P0) human articular chondrocytes (HAC) were loaded on the type II collagen disks at the density of $1x10^5$ cells/scaffold, corresponding to $1.6x10^3$ cell/mm^3. This cell density, lower than the one used in our previous work (30, 31), was determined based on the number of cells potentially available following digestion of a typical size cartilage biopsy (i.e., about 300 mg) and considering a typical size of the target graft (i.e., about 5 cm^2). HAC were cultured in *complete medium* supplemented with 1 ng/mL of transforming growth factor β1 (TGF β1), 5 ng/mL of fibroblast growth factor 2 (FGF-2), and 10 ng/mL of Platelet-Derived Growth Factor-BB (all from R&D Systems, Minneapolis, MN) (*expansion medium*) in a humidified 37°C/5% CO_2 incubator with change medium twice a week, as previously described (21). This specific combination of growth factors was previously shown to markedly enhance proliferation and post-expansion chondrogenic differentiation of HAC (32). As 2D expansion control, aliquots of $1x10^5$ HAC (i.e., the same amount of cells loaded on the sponges) were seeded in 6-well plates and cultured in *expansion medium*. In order to match the number of doublings by 3D and 2D expanded cells, growth curves were first analyzed in the different culture systems. HAC seeded 3D constructs and 2D cultures were thus harvested at different time points (4, 7, 9 and 12 days), cells were counted and the number of doublings calculated.

Constructs were harvested and analyzed via real time RT PCR to assess HAC differentiation stage or cultured for additional 4 weeks in complete medium supplemented with 0.1 mM ascorbic acid, 10 µg/ml Insulin and 10 ng/ml Transforming Growth Factor-β3 (chondrogenic medium), with medium changes twice a week (differentiation phase). HAC expanded in 2D were detached and also analyzed via real time RT-PCR or seeded in the type II collagen sponges at a density of $1.5x10^6$ cells/construct (equal to $25.0x10^3$ cell/mm^3, that corresponded to the density measured in constructs following 3D expansion) and cultured for additional 4 weeks in chondrogenic medium as described above. At the end of the differentiation phase, each construct was divided into two halves which were independently processed for histological, immunohistochemical, biochemical or gene expression analyses, as described below.

Study 2: Cell seeding at different densities, following 2D expansion to different extents

Freshly isolated chondrocytes were plated in tissue culture flasks at a density of 10^4 cell/cm^2 and cultured in expansion medium. When sub confluent (passage 1, P1) cells were detached by sequential treatment with 0.3% type II collagenase, followed by 0.05% trypsin/0.53mM EDTA, and serially replated at $5x10^3$ cells/cm^2 for 4 additional times (passage 5, P5).

P1 and P5 expanded cells were loaded statically onto type II collagen sponges either at $1.5x10^6$ cells/scaffold (*low density*, equal to that in Study 1) or at $4x10^6$ cells/scaffold (*high density*, equivalent to $66.0x10^3$ cells/mm^3) (20). Cell-seeded constructs were statically cultured in chondrogenic medium for 4 weeks and processed as described below.

Analytical Methods

Histological analyses

The constructs were rinsed with PBS, fixed in 4% formalin, embedded in paraffin, and cross-sectioned (7 µm thick). Sections were stained with Safranin-O for sulfated glycosaminoglycans (GAG) (33) or processed for immunohistochemistry to visualize collagen type II (II-II6B3, Hybridoma Bank, University of Iowa, USA), as previously described (34).

Biochemical Analysis

Tissue constructs were then digested in 1mL of proteinase-K (1mg/mL proteinase-K in 50mM Tris with 1mM EDTA, 1mM iodoacetamide and 10µg/mL pepstatin-A) for 15h at 56°C. GAG amounts were measured spectrophotometrically after reaction with dimethylmethylene blue(35), with chondroitin sulphate as a standard. The amount of DNA was measured spectrofluorometrically using the CyQuant cell proliferation assay Kit (Molecular Probes, Eugene, OR), with calf thymus DNA as a standard (36).

Real-time quantitative RT-PCR assays

RNA was extracted from expanded HAC and from tissue construct using 500µL Trizol (Life Technologies, Basel, Switzerland), according to the Manufacturer's protocol. RNA was treated with DNAse-I using the DNA-freeTM Kit (Ambion, USA) and quantified spectrofluorimetrically. cDNA was generated from total RNA by using 500 µg/ml random hexamers (Catalys AG, CH) and 1 µL of 50 U/ml StratascriptTM reverse transcriptase (Stratagene, NL), in the presence of dNTPs. PCR reactions were performed and monitored

using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer/Applied Biosystems, Rotkreuz, Switzerland). The PCR master mix was based on AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems). Cycle temperatures and times as well as primers and probes used for the reference gene (GAPDH) and the genes of interest (type II collagen), were as previously described (22). For each cDNA sample, the threshold cycle (Ct) value of GAPDH was subtracted from the Ct value of the target gene, to derive ΔCt. The levels of expression of type II collagen were calculated as $2^{\Delta Ct}$. Each sample was assessed at least in duplicate for each gene of interest.

Statistical analysis

For each analysis, at least triplicate samples for each condition and donor were assessed. Statistical evaluation was performed using SPSS software version 7.5 software (SPSS, Sigma Stat). Values are presented as mean ± standard deviation (SD). Differences between groups were assessed by Mann-Whitney test and considered statistically significant when P values were lower than 0.05.

RESULTS

Study 1: Cell expansion directly in 3D scaffolds or in 2D to the same extent

Cell proliferation and dedifferentiation

Freshly harvested HAC were seeded and cultured in *expansion medium* in type II collagen sponges (3D expansion) or in monolayer (2D expansion) (see experimental design, Fig.1A). HAC within the scaffolds continued to proliferate over a 9-days period, reaching $1.5x10^6$ cells/scaffold and thus undergoing approximately 4 doublings (Fig. 2A). Cell number did not further increase with additional culture time. 2D cultured HAC grew exponentially within the considered time frame, reaching numbers 10.1-fold higher than those obtained following 3D expansion at the end of the observation time of 12 days. Based on these experiments, in order to approximately match the number of doublings performed during 3D culture, HAC were hereafter for Study 1 expanded in 2D for 7 days, corresponding to 4 doublings (Fig. 2A). Following 4 doublings, 3D expanded HAC expressed 13.7-fold higher levels of type II collagen mRNA than the 2D expanded population, with only a slight reduction from freshly isolated cells (Fig. 2B), indicating a better preservation of the differentiated phenotype.

Chondrogenic redifferentiation

Constructs derived from 3D expanded HAC or from cells seeded following 2D expansion were cultivated for additional 4 weeks in chondrogenic medium (differentiation phase). Histological analyses indicated that 2D and 3D expanded HAC deposited extracellular matrix faintly stained for Safranin O in scattered areas (Fig. 3A). Biochemical analysis quantitatively confirmed similar amounts of GAG ($p > 0.05$) in the constructs generated by 2D or 3D expanded chondrocytes (Fig. 3B).

In summary, results from Study 1 indicated that (i) 2D expanded HAC seeded onto the type II collagen sponges at low density had limited chondrogenesis and (ii) 3D expansion did allow for a better maintenance of the chondrocytic phenotype of HAC but not for a superior chondrogenesis. Since cell density was in both groups lower than what we previously typically used to obtain higher quality cartilaginous tissues(30, 31), we then decided to investigate the effect of cell seeding density on chondrogenesis within type II collagen sponges. For these experiments we used the 2D expansion strategy, since that would allow to obtain larger cell numbers also from a clinically relevant size biopsy.

Study 2: Cell seeding at different densities, following 2D expansion to different extents

Cell proliferation and dedifferentiation

HAC were expanded in 2D for 1 passage (P1) or 5 passages (P5) (see experimental design in Fig.1B). During this expansion phase, cells underwent respectively 4.6±1.7 or 17.3±1.6 doublings (Fig. 4A). RT-PCR analysis indicated that type II collagen was 488.6-fold less expressed in P5 as compared to P1 expanded chondrocytes, confirming that de-differentiation further progressed during expansion time (Fig. 4B).

Chondrogenic redifferentiation

After 2D expansion, P1 and P5 chondrocytes were loaded onto type II collagen sponges either at $1.5x10^6$ cells/scaffolds (*low density*) or at $4x10^6$ cells/scaffolds (*high density*) and cultured in chondrogenic medium for 4 weeks (see experimental design in Fig.1B). Histological analysis indicated that P1 HAC seeded at *low density* generated tissues faintly stained for GAG, consistent with results of Study 1, while those generated by P1 HAC seeded at *high density* formed cartilaginous tissues more intensely stained for GAG. Interestingly, a similar tendency was observed for the highly de-differentiated P5 chondrocytes, with the

cells seeded at *high density* (but not at *low density*) depositing extracellular matrix intensely stained for GAG (Fig. 5A). Staining for type II collagen was negative in the constructs generated at *low density* (data not shown), and intensely and uniformly distributed in constructs generated by P1 or P5 chondrocytes seeded at *high density* (Fig. 5B). Biochemical quantitative characterizations of the tissues were consistent with their histological and immunohistochemical appearance. Both P1 and P5 HAC accumulated higher amounts of GAG when seeded at higher density in the sponges (respectively 1.6- and 1.5-fold) (Fig. 5C). RT-PCR analysis indicated higher type II collagen expression by cells cultured at *high density* vs *low density* condition (9.3- and 4.1-fold respectively for P1 and P5 cells). The expression of type II collagen was higher in constructs generated by P1 expanded chondrocytes at both seeding densities (3.9- and 8.8-fold for low and high density respectively).

In summary, results from Study 2 indicate that *high density* seeding of HAC in type II collagen scaffolds enhanced chondrogenesis and cartilaginous matrix deposition, even if cells were expanded and de-differentiated to a higher extent.

DISCUSSION

In this study we demonstrated that 3D expansion of human articular chondrocytes (HAC) on type II collagen scaffolds, as compared to expansion in 2D, better maintains their chondrocytic phenotype but does not enhance their cartilage-forming capacity. Instead, increasing the HAC seeding density in the scaffolds allowed enhanced chondrogenesis, even if seeded cells had to be expanded and de-differentiated more extensively in 2D in order to reach the required cell numbers.

Several studies have shown that chondrocytes from different animals can be expanded in 3D even if at lower extent that those expanded in monolayer, but maintaining better the chondrocytic phenotype. Lin et al (23) reported that *porcine* articular chondrocytes cultured on alginate scaffolds within a perfusion system underwent a 60-fold increase in their number within 4 weeks culture while maintaining the expression of the chondrocitic genes type II collagen, aggrecan and Sox-9. Instead, cells cultured for the same time in 2D proliferated more extensively (expansion factor of 800) but losing the expression of the same genes (23). Similarly, *canine* chondrocytes cultured on type II collagen sponges have been shown to increase in cell number (but only by 1.4-fold in 14 days) while maintaining the round phenotype (29). Interestingly, using a

type I collagen matrix, Roche et al (37) showed that fetal *bovine* epiphyseal chondrocytes increased in cell number (by 4-fold in 4 weeks) only in the matrix seeded at low density (i.e., 10^6 cells per scaffold, corresponding to $2.1x10^3$ cell/mm^3) but not in those seeded at high density (i.e., 10^7 cells per scaffold, corresponding to $2.1x10^4$ cell/mm^3). These results overall indicate that 3D expansion can maintain the chondrocytic phenotype, though the extent of cell proliferation depends on the experimental conditions, e.g. cell source, scaffold type, culture type (static vs dynamic) and cell seeding density used.

To date, no study demonstrated whether a better maintenance of a chondrocytic phenotype corresponds to an enhanced tissue forming capacity. In our work, a very low amount of *human* articular chondrocytes (10^5 cells, which is the average number of chondrocytes available following digestion of a regular size cartilage biopsy (8)) was seeded on the type II collagen sponges. Interestingly, HAC proliferated in the scaffolds almost maintaining their native phenotype even in the presence of strong mitogenic/de-differentiating factors (21, 22), in contrast to control cells expanded in 2D for the same number of doublings, which reduced the type II collagen expression of about 2 orders of magnitude. However, under our experimental conditions, both 3D and 2D expanded HAC generated poor cartilaginous tissues. While it remains to be assessed whether functionalized scaffolds (38), strong enhancers of chondrogenesis (e.g.: BMPs) (39, 40) or in vivo implantation could improve the obtained cartilage quality, results from Study 1 overall indicate that the maintenance of the chondrocytic phenotype is not a condition sufficient to guarantee functionality of HAC *in vitro*.

Since high cellular density is known to favour the onset of chondrogenesis and tissue development by chondroprogenitors (41) , we then tested whether the extent of cartilage formation by HAC could be enhanced by seeding the scaffolds at high density. Roche et al (37) previously reported higher accumulation of GAG in collagen sponges seeded with high number of chondrocytes (10^7 cells) as compared to those seeded with a lower number (10^6 cells). However in such study P0 bovine chondrocytes were used, thus not allowing to assess the benefit of high cell density culture in a clinical scenario were a large number of freshly harvested human chondrocytes can not be used. In our study, the number of HAC used for the *high density* condition (i.e., $4x10^6$) could be easily obtained starting from the few cells which can be isolated from a small cartilage biopsy (42), if expansion could be extended for a few passages. The extensively dedifferentiated phenotype of P5 expanded cells did not appear to interfere with the

chondrogenic program, provided that high cell density was initially established. Indeed, the extent of HAC passaging (e.g., P1 vs P5) and consequent stage of de-differentiation did not modify the accumulation of type II collagen following 3D culture. This is a further proof of the extensive plasticity exhibited by HAC expanded in culture under suitable conditions (32).

Our results confirm previous findings that supplementation of chondrocytes with specific factor(s) during their monolayer expansion allows to generate large number of cells with high cartilage forming capacity at high density culture conditions (21, 22, 32, 43).

In contrast to our finding, Kang et al. (44) reported that passage 5 *rabbit* chondrocytes seeded onto non-woven PGA fiber-based scaffolds failed to produce cartilaginous tissues *in vitro* or ectopically in nude mice. The discrepancy with our results may be attributed to the different origin of the chondrocytes used and to the different conditions used for the monolayer expansion of chondrocytes.

Conclusion

In conclusion, the present study indicates that a high seeding density of HAC in 3D scaffolds is more critical for the generation of cartilaginous constructs than the stage of cell differentiation reached following expansion. Expansion of freshly isolated HAC directly onto 3D scaffolds, as compared to 2D surfaces, did not enhance the extent of cartilaginous matrix deposition, likely because it did not allow for a sufficient cell growth and thus increase in cell density. The identification of innovative strategies (e.g., co-culture of native chondrocytes with undifferentiated cells, use of bioreactor cultures or smart biomaterials) enabling to enhance cell growth in the constructs should be further investigated to allow bypassing the typical phase of HAC expansion in 2D.

Disclosure Statement

No competing financial interests exist.

ACKNOWLEDGMENTS

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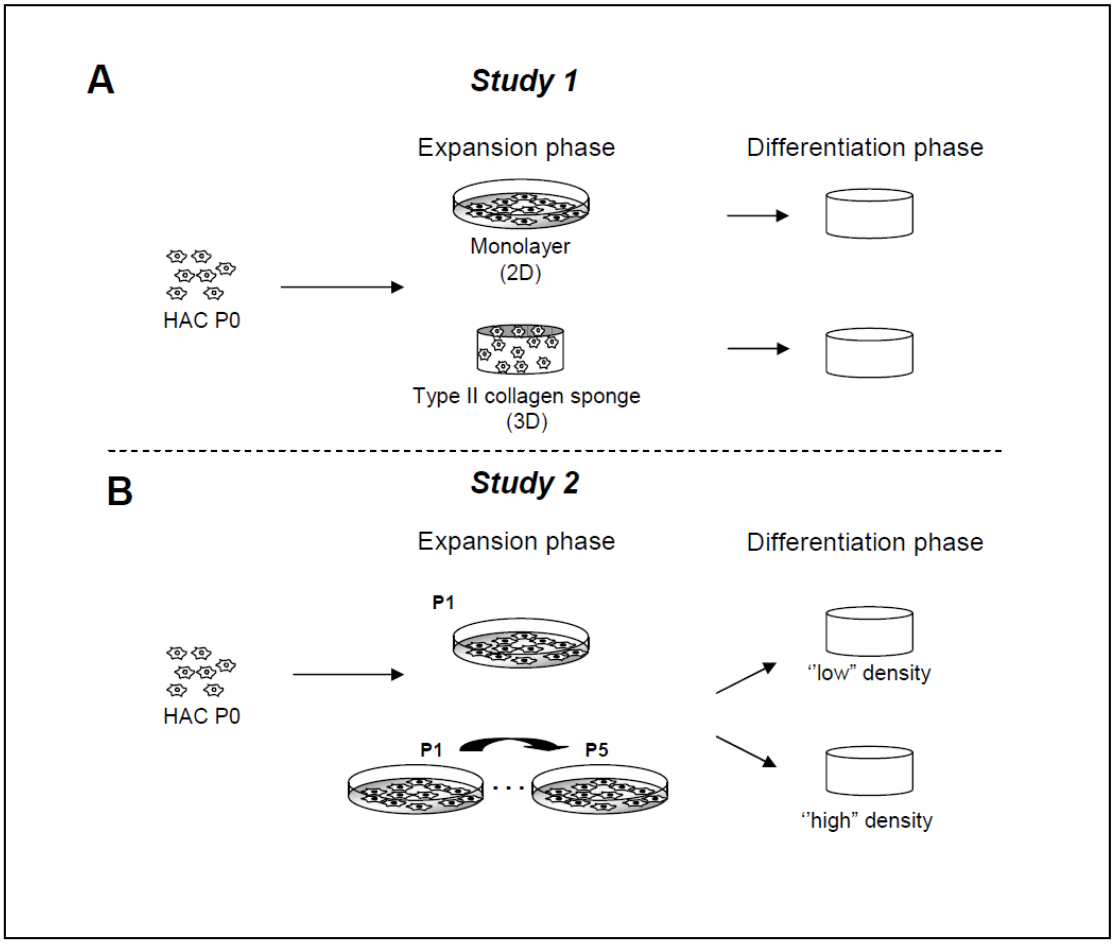

Figure 1. Experimental design. (A) *Study 1*. Freshly isolated (P0) human articular chondrocytes (HAC) were cultured in *expansion medium* either on type II collagen sponges at a low density (105 cells/scaffold) or in 2D wells (105 cells/well). Expansion time in the 2 culture strategies was selected such that HAC underwent the same number of doublings prior to induction of differentiation for additional 4 weeks. **(B) *Study 2*.** P0 HAC were expanded in 2D for 1 passage (P1) or 5 passages (P5) and then seeded into type II collagen scaffolds at *low density* (1.5x106 cells/ scaffold) or at *high density* (4.0 x106 cells/ scaffold). Resulting constructs were then cultured for 4 weeks in chondrogenic medium.

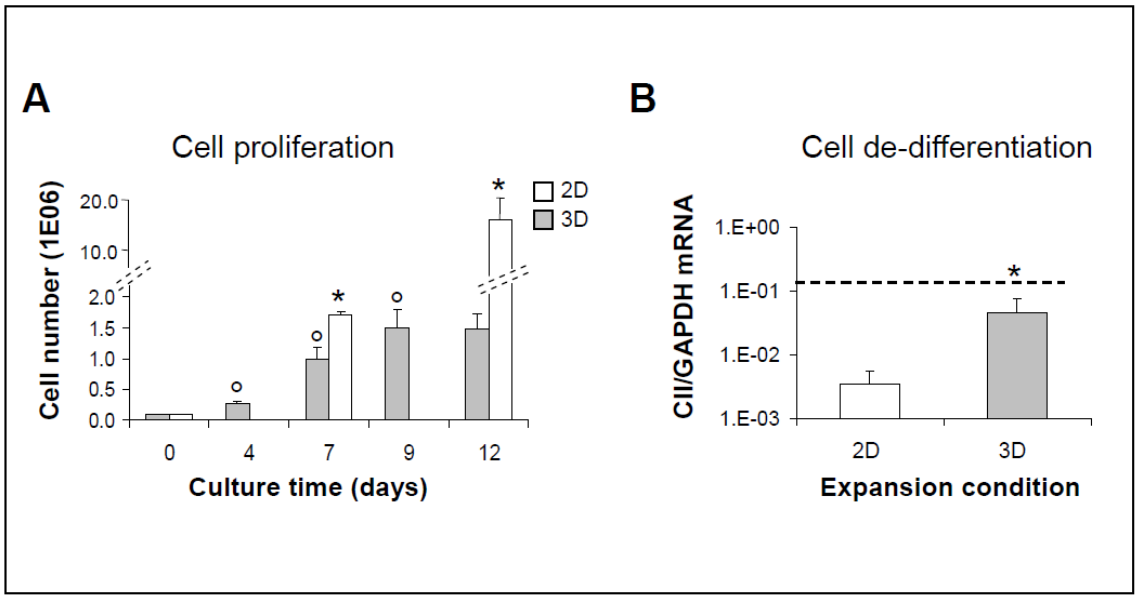

Figure 2. *Study 1*: Cell proliferation and dedifferentiation during expansion. (**A**) Number of cells counted in the type II collagen scaffolds (3D) or in the wells (2D) after different times of culture in *expansion medium*. (**B**) Real time RT-PCR analysis of the expression of type II collagen (CII) mRNA by chondrocytes expanded in 2D or 3D. Levels are expressed as fold of difference from Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Values are mean ± SD of measurements obtained from 3 different experiments. ° = signifi cantly different from the previous culture time; * = significantly different from the corresponding 3D experimental group. The dotted line indicates the CII expression level measured in P0 cells (average of 3 donors).

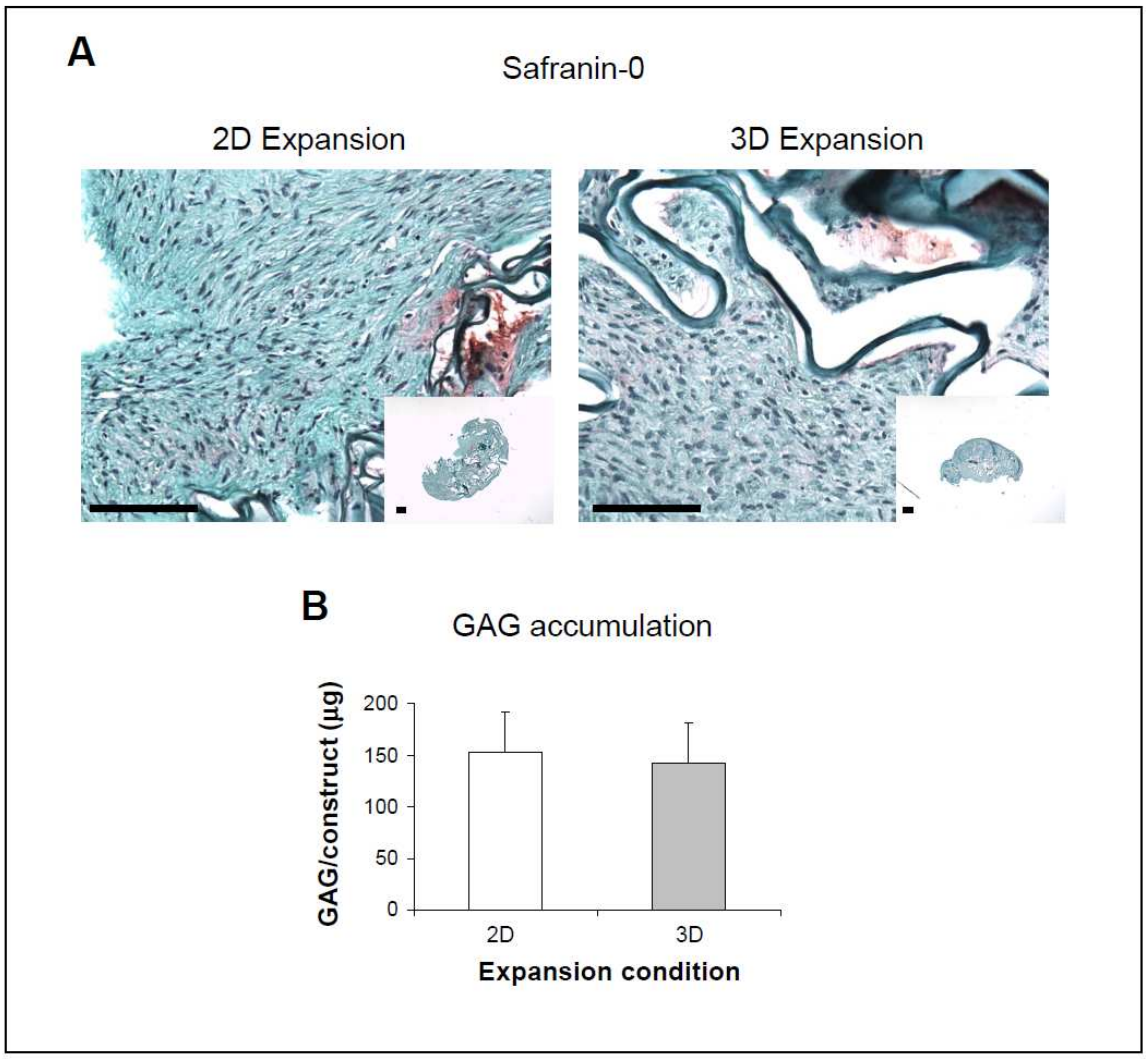

Figure 3. *Study 1*: Chondrogenic redifferentiation. (**A**) Safranin O staining of representative constructs generated by HAC expanded in 2D or in type II collagen sponges (3D) and cultured for 4 weeks. Scale bars = 100mm. The insets are low magnification images of the entire constructs. (**B**) Sulphated glycosaminoglycan (GAG) content of the constructs reported as total GAG/construct (µg). Values are mean ± SD of measurements obtained from 4 different experiments.

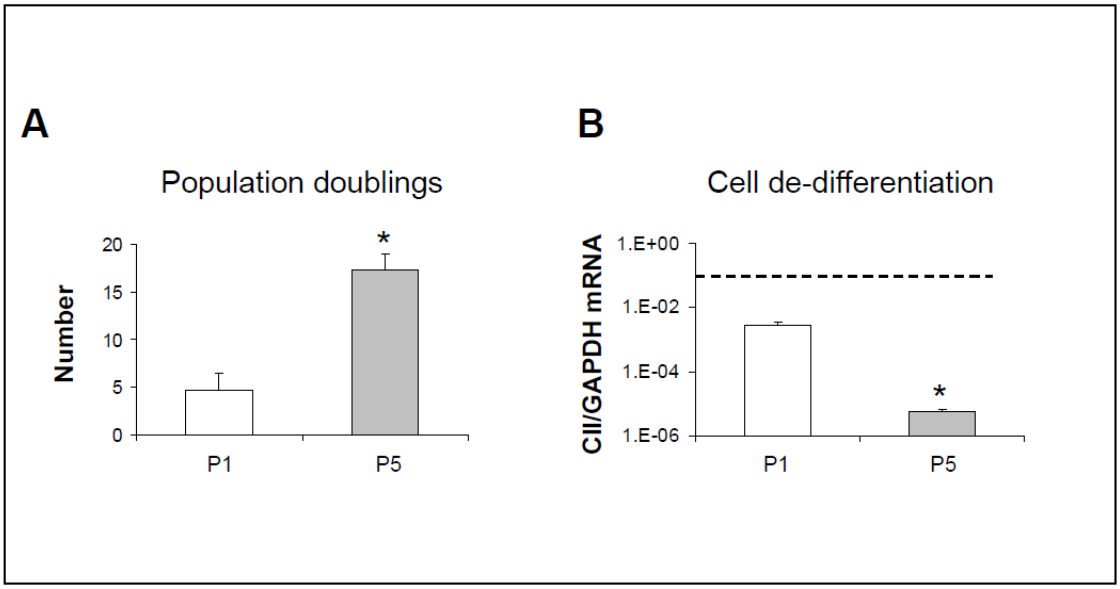

Figure 4. *Study 2*: Proliferation and dedifferentiation. (**A**) Number of population doublings by HAC cultured in 2D for 1 passage (P1) or 5 passages (P5). **(B)** Real time RT-PCR analysis of the expression of type II collagen (CII) mRNA by P1 or P5 chondrocytes. Levels are expressed as fold of difference from Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Values are mean ± SD of measurements obtained from 4 different experiments. * = significantly different from the corresponding P1 condition. The dotted line indicates the CII expression level measured in P0 cells (average of 3 donors).

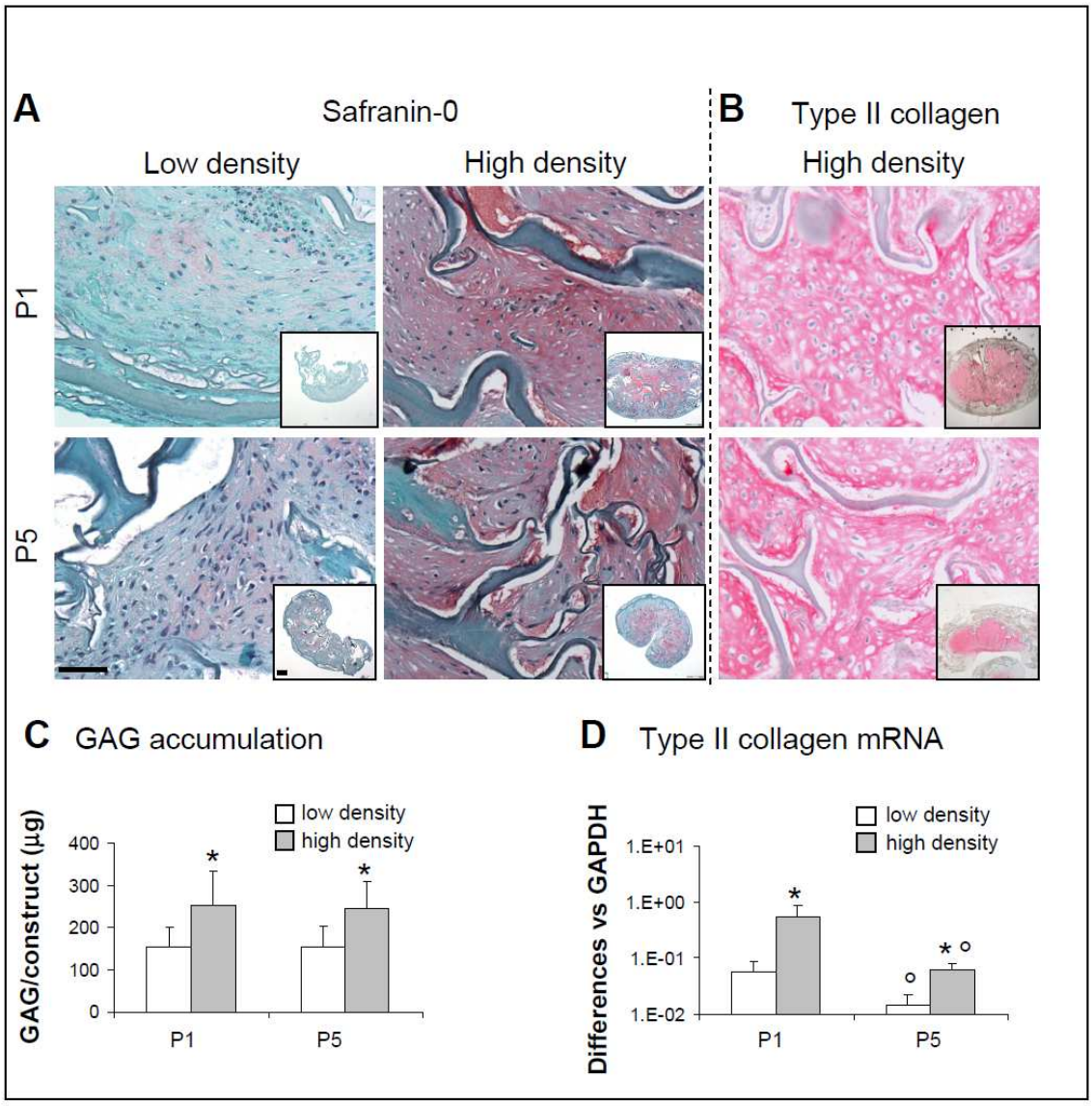

Figure 5. *Study 2*: Chondrogenic redifferentiation. (**A**) Safranin O (**A**) and type II collagen (**B**) staining of representative constructs generated by chondrocytes expanded for 1 passage (P1) or 5 passages (P5) and seeded at *low density* (1.5x106 cells/scaffold) or *high density* (4.0x106 cells/scaffold) onto type II collagen sponges. Scale bars = 100mm. The insets are low magnification images of the entire constructs. (**C**) Sulphated glycosaminoglycan (GAG) content of the constructs reported as total GAG/construct (µg). (**D**) Real time RT-PCR analysis of the expression of type II collagen (CII) mRNA. Levels are expressed as fold of difference from Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Values are mean ± SD of measurements obtained from 4 different experiments. * = significantly different from the corresponding low density condition; ° = significant ly different from the corresponding P1 conditions.

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2.3 PAPER III: Production of cytokines and response to IL-1β by human articular chondrocytes at different stages of tissue maturation

Production of cytokines and response to IL-1β by human articular chondrocytes at different stages of tissue maturation

S. Francioli, C. Cavallo, S. Miot, J. Frueh, M. Jakob, B. Grigolo, A. Barbero, I. Martin

ABSTRACT

INTRODUCTION: Ex-vivo cultured chondrocytes express a variety of pro-inflammatory chemokines and cartilage degenerative enzymes, whose production is enhanced by increasing cell passaging and by stimulation with IL-1β. These responses have particular relevance in the context of cell based-cartilage repair, considering that therapeutic cell preparations (single cell suspension, cell-seeded matrixes or cartilaginous tissues) once grafted in the joint defect will become exposed to a biochemical environment rich in catabolic mediators. The cell preparation in which the chondrocytes are less susceptible to such mediators might guarantee superior clinical outcome. We aimed the study at investigating how the extent of maturation of human-based cartilaginous tissues modulates the profile of chemokine production and the inflammatory/catabolic response to IL-1β.

MATERIALS AND METHODS: HAC isolated from knee cartilage biopsies of 6 individuals were expanded in monolayer and then cultured in pellets. Pellets were evaluated for spontaneous release and mRNA expression of MCP-1, IL-8 and TGF-β at different experimental times (3, 6, 9, 15, 21 and 27 days). In another experimental set, pellets were cultured for 1 or 14 days (immature and mature pellets respectively) and thereafter exposed to IL-1β for 3 days. Control pellets were cultured for 4 or 17 days without IL-1β. Content of cartilaginous matrix was assessed biochemically (GAG), histologically (Safranin-O) and immunohistochemically (type II collagen). MCP-1, IL-8 and TGF-β as well as specific metalloproteases (MMPs) were quantified in culture surnatants or in tissues by real time RT-PCR.

RESULTS: By increasing culture time, the spontaneous release of pro-inflammatory chemokines IL-8 and MCP-1 decreased (respectively 12.0-fold and 5.5-fold) while the anabolic factor TGFβ increased (5.4-fold). This trend was also confirmed by real time PCR.

Following IL-1 exposure, GAG content in mature pellets was reduced by 40% and type II collagen immunostaining slightly reduced. Both cartilaginous proteins become undetectable in IL-1β stimulated immature tissues. As compared to immature pellets, mature pellets responded to IL-1β by releasing (i) lower amounts of MMP-1 (2.9-fold) and MMP-13 (1.7-fold), (ii) increased amounts of IL-8 and MCP-1 (1.5- and 5.0-fold respectively), and (iii) enhanced levels of TGF-β (by 7.5-fold). RT-PCR analysis generally confirmed these trends.

CONCLUSION: Our results indicate that the extent of maturation of cartilaginous tissues profoundly modulates the profile of chemokine production and the catabolic response to IL-1β. The described findings are relevant in the optimization of chondrocyte culture for cell-based cartilage repair applications.

INTRODUCTION

Damaged or diseased articular cartilage has no ability to self-repair, therefore, frequently leads to progressive debilitation resulting in a marked decrease in the quality of life.

A well established technique used in the clinic for the repair of cartilage or osteochondral defects, Autologous Chondrocytes Implantation (ACI), consists in the injection of a suspension of expanded autologous chondrocytes into the defect (1). The recently introduced alternative matrix-mediated ACI (MACI) (2), or the grafting of tissue engineered cartilaginous constructs (TE) (3, 4) allow in principle a superior handling and could offer the possibility of earlier post operative loading. However the effective clinical performance has not yet been proven to be superior and criteria to determine appropriate maturation stage are not yet available.

All the above mentioned techniques (ACI, MACI and TE) require monolayer expansion of chondrocytes and therefore the use of dedifferentiated cells (5). It's well established that de-differentiated chondrocytes share some phenotypic and genotypic traits with chondrocytes derived from patients with osteoarthritis (OA) (6-8). In particular, it has been shown that expanded and OA chondrocytes exhibit similar enhanced expression of de-differentiated markers (e.g.: versican, type I collagen and cathepsin B) as compared to native normal chondrocytes (9-12). Moreover, it has been reported that, similarly to OA chondrocytes, ex-vivo cultured chondrocytes express a variety of pro-inflammatory chemokines/chemokine receptors and cartilage degenerative enzymes (13-18), whose production is enhanced by increasing cell passaging and by stimulation with IL-1β and TNF-α (19, 20).

IL-1 isoforms have been shown to have harmful effects on chondrocytes: they (i) inhibit the synthesis of the major physiological inhibitors of pro-degradative enzymes, (ii) stimulate the production of prostaglandins, free radicals and NO (Ref), (iii) inhibit the synthesis of matrix components such as type II collagen and proteoglycans (21-24), (iv) inhibit the chondrocyte differentiation- phenotype by suppressing the expression of Sox-9 (21, 25), (v) inhibit the chondrocytes proliferation and induce cell death (25).

These IL-1-mediated effects have particular relevance in the context of cell based-cartilage repair, considering that therapeutic cell preparations (single cell suspension, cell-seeded matrices or

cartilaginous tissues) once engrafted in the joint defect will become exposed to a biochemical environment that is likely containing catabolic mediators deriving from the diseased joint or from the surgical intervention itself (25-27). The cell preparation in which the chondrocytes are less susceptible to the IL-1 effects might guarantee an improved clinical outcome. The presence of abundant extracellular matrix surrounding the chondrocytes in an engineered cartilage graft in principle may protect the cells from the IL-1 insult. In this regard, (27) using native *bovine* chondrocytes have recently shown that mature engineered cartilage constructs (having native level of GAG content and Young's modulus) were capable to counteract IL-1α mediated catabolic effects. However, this method must be investigated using clinically relevant cells (expanded adult *human* chondrocytes) which are known to have a reduced chondrogenic capacity as compared to freshly harvested bovine.

We aimed the study at investigating how the extent of maturation of *human*-based cartilaginous tissues modulates (i) the profile of chemokine production and (ii) the inflammatory/catabolic response to IL-1β.

For this purpose, HAC have been cultured in a standard pellet culture (28, 29) for different time points in medium promoting chondrogenesis with or without IL-1β. Production/expression of pro-inflammatory chemokines (IL-8, MCP-1), anabolic factors (TGF-β) and degradative mediators (MMP-1 and MMP-13) have been analysed.

Our results will be discussed in the context of defining appropriate maturation stage of a cartilage graft for cell-based cartilage repair approaches.

MATERIALS AND METHODS

Cartilage biopsy, chondrocytes isolation and expansion.

Full-thickness human articular cartilage biopsies were obtained post mortem (within 24 hours after death) from the lateral condyle of knee joints of 6 individuals (mean age: 44 year, range 32-65 year) with no history of joint disease, after informed consent by relatives and in accordance with the local ethics committee (University Hospital Basel, Switzerland).

Cartilage tissues were minced in small pieces and digested upon 22-hours incubation at 37°C in 0.15% type II collagenase (10 mL solution/g tissue, 300 U/mg, Worthington Biochemical Corporation,

Lakewood, NJ, USA). The isolated human articular chondrocytes (HAC) were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum, 4.5mg/mL D-glucose, 0.1mM non essential amino acids, 1 mM sodium pyruvate, 100mM HEPES buffer, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.29mg/mL glutamine (complete medium) and then alive cells counted using trypan blue exclusion and plated in tissue culture flasks at 10^4 cells/cm^2 in complete medium supplemented with 1 ng/mL of transforming growth factor-1 (TGF-β1), 5 ng/mL of fibroblast growth factor-2 (FGF-2) (expansion medium) in a humidified 37°C/5% CO2 incubator. The growth factor combination was selected based on the previously reported ability to increase human chondrocyte proliferation and capacity to redifferentiate (30, 31).

After approximately 10 days, when cells were about 80% confluent, first passage cells were rinsed with phosphate buffered saline (PBS), detached using 0.05% trypsin/0.53 mM EDTA and replated at 5×10^3 cells/cm^2. After an additional week, when cells were again about 80% confluent, second passage cells (corresponding to a total of 8.1± 1.0 doublings) were detached and induced to redifferentiate in pellet cultures as described below.

3D-pellet culture

The chondrogenic capacity of expanded chondrocytes was investigated in pellet cultures using a defined serum free medium, as previously described (31). Cells were suspended in DMEM supplemented with ITS+1 (Sigma Chemical, St. Louis, MO; i.e., 10 µg/mL insulin, 5.5 mg/ml transferrin, 5 ng/mL selenium, 0.5 mg/ml bovine serum albumin, 4.7 mg/mL linoleic acid), 0.1 mM ascorbic acid 2-phosphate, 1.25 mg/mL human serum albumin, 10^{-7} M dexamethasone and 10 ng/mL TGF-β1 (chondrogenic medium). Aliquots of 5×10^5 cells/0.5 ml were centrifuged at 250×g for 5 min in 1.5 mL polypropylene conical tubes (Sarstedt, Nümbrecht, Germany) to form spherical pellets, which were placed onto a 3D orbital shaker (Bioblock Scientific, Frenkendorf, Switzerland) at 30 rpm. Pellets were evaluated for spontaneous release and mRNA expression of cytokines at different experimental times: 3, 6, 9, 15, 21 and 27 days.

In another experimental set, pellets were cultured for 1 or 14 days and therefore exposed to 1ng/mL IL-1β for 72 hours. Control pellets were cultured for 4 or 17 days without IL-1β. The IL-1β concentration was selected based on a preliminary study (32). Pellets were processed for histological,

immunohistochemical, biochemical and gene expression analyses, while surnatants were collected and evaluated at different time points for cytokines and MMPs release.

Analytical Methods

Histological and Immunohistochemical analyses

Pellets were rinsed with PBS, fixed in 4% formalin, embedded in paraffin, and cross-sectioned (5 µm thick). Sections were stained with Safranin-O for sulfated glycosaminoglycans (GAG) (33). The immunohistochemical analyses for IL-8, MCP-1, TGF-β and type II collagen were performed using the following primary antibodies: mouse monoclonal anti-human IL-8 and TGF-β (R&D Systems, Minneapolis, MN, USA), mouse monoclonal anti-human MCP-1 (PeproTech INC, Rocky Hill, USA) and mouse monoclonal anti-human against type II collagens (II-II6B3, Hybridoma Bank, University of Iowa, USA). Paraffin sections were deparaffinized and rehydrated. Immunostaining for Type II collagen was performed as previously described (33, 34). Tissue sections for MCP-1 and TGF-β were treated with 0.1 % hyaluronidase (Sigma) in PBS at 37°C for 10 minutes for epitope unmasking. After washes, the slides were incubated for 1 hour at room temperature (RT) with primary antibodies diluted 1:10 for both MCP-1 and TGF-β in PBS with 1% Bovine Serum Albumin (BSA). The slides for IL-8 after deparaffination and rehydration were incubated for 1 hour at RT with the primary antibodies diluted 1:10 in PBS with 1% BSA. After washes performed in PBS with 1% BSA, the slides were incubated with biotinylated immunoglobulins against various animal species (BioGenex, San Ramon, CA, USA) for 20 minutes at room temperature. Then samples were incubated with a phosphatase-labeled streptavidin Kit (Biogenex) for 20 minutes at room temperature, and then washed. The reactions were developed using fast red substrate (Biogenex). Negative controls were performed by omitting the primary antibody. Slides were counterstained with hematoxylin and mounted in glycerol gel. Histological sections were visualized with a Nikon Eclipse 90i microscope equipped with NIS (Nikon Imaging Software) elements (Nikon Inc).

Biochemical Analysis

Pellets were digested in 1mL of proteinase-K (1mg/mL proteinase-K in 50mM Tris with 1mM EDTA, 1mM iodoacetamide and 10µg/mL pepstatin-A) for 15h at 56°C. GAG amounts were measured spectrophotometrically after reaction with dimethylmethylene blue (35), with chondroitin sulphate as a

standard. The amount of DNA was measured spectrofluorometrically using the CyQuant cell proliferation assay Kit (Molecular Probes, Eugene, OR), with calf thymus DNA as a standard (36).

Quantification of released cytokines

IL-8 and MCP-1 chemokines were determined by the use of specific immunoassays (Pharmingen, San Diego, CA), standardized in the laboratory of Istituti Ortopedici Rizzoli Bologna. Briefly, for each cytokine's determination, two monoclonal antibodies of different epitope specificity were used to prepare the sandwich ELISA. Ninety-six-well, polystyrene plates (EIA microplate, ICN, Costa Mesa, CA) were coated with 50 µl of purified mouse anti-human IL-8 or MCP-1 adjusted at a concentration of 1 and 4 µg/ml respectively in sodium carbonate buffer pH 9.5, and incubated overnight at 4° C. After washing the plates, serial diluitions of recombinant human IL-8 or MCP-1 and appropriate diluted samples were added to the wells (100 µl/well) and incubated for 2 hours at room temperature followed by a 1 hour incubation with 100 µl/well of biotinylated mouse anti-human IL-8 or rabbit anti-human MCP-1. After a further 30 minutes incubation at room temperature with 100 µl/well of streptavidin-horseradish peroxidase conjugate, the bound antibodies were detected by adding 1,2 *o*-phenylenediamine and H_2O_2 as substrate, running the reaction for 10 minutes before terminating it with 2 M H_2SO_4 and the absorbance measured at 492 nm. All the experiments described here were performed in duplicate. TGF-β concentrations in surnatants were evaluated by commercial ELISA kits following the manufacture's instructions (R&D). Values of TGF-β measured in the chondrogenic medium were subtracted from those measured in the surnatants. The amount of each released cytokine was normalized to the DNA content of the tissue and expressed as pg / ng.

Quantification of released matrix metalloproteinases

Matrix metalloproteinases (MMP) were quantified in media collected from cultured pellets by using the MultiAnalyte Profiling MMP base Kit (Fluorokine® MAP: LMP000) complemented with the specific MMPs (MMP-1: LMP901; MMP-13: LMP511, R&D Systems, Minneapolis, USA). The assay was performed on a Luminex 100™ analyzer following the manufacturer's instructions. The amount of released MMPs was normalized to the DNA content of the tissue.

Real-time quantitative RT-PCR assays

RNA was extracted from expanded HAC and from pellets using 500µL Trizol (Life Technologies, Basel, Switzerland), according to the Manufacturer's protocol. RNA was treated with DNAse-I using the DNA-freeTM Kit (Ambion, USA) and quantified spectrofluorimetrically. cDNA was generated from total RNA by using 500 µg/ml random hexamers (Catalys AG, CH) and 1 µL of 50 U/ml StratascriptTM reverse transcriptase (Stratagene, NL), in the presence of dNTPs.

TGF-β, IL-8, and MCP-1 gene expression was analyzed by Real-Time RT-PCR. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. PCR primers sequence for GAPDH was obtained from published references (37), while PCR primers sequences for TGF-β, IL-8 and MCP-1 were designed using the PRIMER3 software (Steve Rozen, Helen J, Skaletsky 1998 Primer 3) and were all chosen to span exon junctions. Specific primer pairs, annealing temperatures and references are reported in Table 1. Real-Time PCR was run in a LightCycler Instrument (Roche Molecular Biochemicals, Mannheim, Germany) using the QuantiTect™ SYBR® Green PCR Kit (Qiagen, GmbH, Germany) with the following protocol: initial activation of HotStarTaq™ DNA Polymerase at 94°C for 15 minutes, 45 cycles of 94°C for 15 s, 56 ÷ 60°C for 20 s and 72° for 10 s. The increase in PCR products was monitored for each amplification cycle by measuring the increase in fluorescence caused by the binding of SYBR Green I dye to dsDNA. The threshold cycle (CT) values (i.e. the cycle number at which the detected fluorescence reaches a threshold value in the range of exponential amplification) were determined for each sample and specificity of the amplicons was confirmed by melting curve analysis and agarose gel electrophoresis. All values were normalized to GAPDH.

Statistical analysis

For each analysis, at least duplicate samples for each condition and donor were assessed. Statistical evaluation was performed using SPSS software version 7.5 software (SPSS, Sigma Stat). Values are presented as mean ± standard deviation (SD). Differences between groups were assessed by Mann-Whitney test and considered statistically significant when P values were lower than 0.05.

RESULTS

Spontaneous production of cytokines during redifferentiation of HAC

Chondrocytes cultured in pellet for different times (i.e.: 3, 6, 9, 15, 21 and 27 days) were analysed for spontaneous protein release in the medium via ELISA assays. IL-8 and MCP-1 showed similar secretion kinetics: the amounts of these cytokines were highest at day 3, they dropped down (by 3.7- and 3.4-fold respectively) after additional 3 days' culture, and got close to the limit of detection following additional culture time. Contrarily, TGF-β amount in the culture medium remained very low or close to the limit of detection up to 15 days' culture, it drastically increased at 21 day and remained constant at day 28 (Figure 1A). Production of IL-8, MCP-1, and TGF-β were also assessed in pellets at different culture times via RT-PCR and immunohistochemically. RT-PCR analyses were in agreement with the ELISA results: IL-8 and MCP-1 mRNA decreased during the culture time up to 22.9- fold ($p<0.05$) and 5.3-fold ($p>0.05$), whereas TGF-β mRNA increased significantly by 10.0-fold from 3 to 27 days of culture (Figure 1B). Immunohistochemical analysis confirmed that as compared to pellets cultured for 3 days, those cultured for 27 days accumulated lower amounts of IL-8 and MCP-1 and higher amounts of TGF-β (Figure 1C).

Effects of IL-1β exposure on GAG and type II collagen accumulation in mature and immature pellets.

HAC were cultivated in pellets for 1 or 14 days and subsequently additionally cultured for 72 hours with or without (control) further addition of 1 ng/mL IL-1β. Hence, pellets cultured for a total time of 4 days (immature) or 17 days (mature) were analysed to assess differences in the content of the main cartilaginous matrix protein GAG and type II collagen.

As expected, pellets matured with increasing culture time accumulating larger amounts of GAG and type II collagen as shown histologically and immunohistochemically by enhanced staining intensities for both macromolecules (Figure 2A-B). Biochemical analysis confirmed significantly higher GAG content (2.4-fold) in mature vs immature pellets (Figure 2C). IL-1β exposure to immature pellets resulted in extensive loss of cartilaginous matrix as evidenced histologically by total absence of staining for GAG and type II collagen (Figure 2A-B) and biochemically by a reduction of GAG at negligible levels (Figure 2C). In mature pellets IL-1β exposure caused also a certain extent of loss of

cartilaginous components as evidenced histologically, immunohistochemically and biochemically (GAG content reduced by 1.7-fold, $p<0.05$). However chondrocytes in the IL-1 treated tissues remained surrounded by a compact extracellular matrix rich in GAG and type II collagen (Figure 2).

Effects of IL-1β exposure on MMPs release by mature and immature pellets.

The levels of MMP-1 and MMP-13 were determined in the surnatants from control untreated and IL-1β treated immature and mature pellets. These two MMPs were selected because they were observed to be significantly enhanced by HAC under exposure to high oxygen tension (condition not favouring chondrogenesis)(38).

The release of MMP-1 and MMP-13 in unstimulated pellets decreased with increased cultured time so that the amount of these enzymes were respectively 2.9-fold ($p<0.05$) and 1.7-fold ($p<0.05$) higher in immature vs mature tissues. In both immature and mature pellets, the exposure to IL-1β markedly and significantly increased the release of MMP-1 (6.7- and 3.2-fold respectively) and MMP-13 (9.3- and 2.8-fold respectively). However, the amounts of both degradative enzymes were significantly higher in the media from immature vs mature pellets (2.1- and 2.0-fold respectively for MMP-1 and MMP-13) (Figure 3).

Effects of IL-1β exposure on the production of cytokines by mature and immature pellets.

Quantification of the cytokines in the surnatants

The amounts of IL-8 and MCP-1 were low in the surnatants of unstimulated immature pellets and become close to the level of detection in mature pellets. TGF-β amounts, instead increased in mature pellets (2.0-fold, $p>0.05$). Stimulation with IL-1β drastically enhanced the release of IL-8 and MCP-1. Surprisingly, mature tissue respond to IL-1β releasing superior amounts of both chemokines so that the final amounts of IL-8 and MCP-1 in the surnatants of such pellets were 1.5-fold ($p<0.05$) and 5.0-fold ($p<0.05$) higher than those of immature pellets (Figure 4A). TGF-β release was also enhanced to higher extent by mature pellets in response to IL-1β: the amount of this cytokine was 6.2-fold higher in the surnatants of mature vs immature pellets (Figure 4B)

Expression of the cytokines genes in the tissues

RT-PCR results were generally in agreement with ELISA results. As compared to immature tissue, mature tissue expressed (i) lower amount of IL-8 (12.4-fold, $p<0.05$), lower amount of MCP-1 (2.1-

fold, $p>0.05$) and higher amount of TGF-β (4.2-fold, $p>0.05$). Stimulation with IL-1β caused an up-regulation of IL-8 and MCP-1 that was more pronounced in mature tissues. Consequently these two chemokines were respectively 1.8-fold ($p>0.05$) and 28.0-fold ($p<0.05$) highly expressed in mature vs immature tissues. TGF-β mRNA expression was only slightly increased (by 3.9-fold, $p>0.05$) following IL-1β stimulation (Figure 4B) in both mature and immature tissues.

Accumulation of IL-8 and MCP-1 in the tissues

Immunohistochemistry for IL-8 and MCP-1 was performed in immature or mature pellets, treated or not with IL-1β. Staining for both chemokines appeared more intense in immature vs mature tissues. However considering that staining was mainly intracellular and that immature pellets were more cellular and contained much less extracellular matrix than mature pellets, a direct comparison was difficult. Instead, clearly, the intensity of staining for both chemokines increased in mature pellets following IL-1β exposure (Figure 5).

DISCUSSION

In this study, we demonstrated that the profile of chemokine production and response to IL-1β by monolayer expanded human articular chondrocytes (HAC) cultured in pellets correlated with cell differentiation stage. By increasing culture time, spontaneous production and release of the pro-inflammatory chemokines IL-8 and MCP-1 decreased, while the anabolic factor TGF-β increased. As compared with immature pellets, mature pellets responded to IL-1β by producing less MMP-1 and -13, more IL-8, MCP-1 and TGF-β.

Several studies reported that unstimulated cultured chondrocytes produce various chemokines involved in the recruitment of inflammatory cells (15, 18, 39). However to the best of our knowledge differences in the basal chemokine expression profile by human articular chondrocytes at different stages of in vitro differentiation have been so far never reported. Our finding that IL-8 and MCP-1 by expanded HAC decreased with increasing culture time under re-differentiating conditions is consistent with the large plasticity of such cells (30, 40). The concomitant down regulation of pro-inflammatory chemokines with the up-regulation of the anabolic factor TGF-β by re-differentiating HAC suggest

that chondrocytes cultured in 3D in presence of chondrogenic stimuli would in principle be more suitable for cartilage repair approach as compared to single cell suspension or cell-seeded matrices.

It is important to consider that once engrafted in the joint defect chondrocytes will become exposed to a biochemical environment that is likely containing catabolic mediators deriving from the diseased joint or from the surgical intervention itself (25-27). The responses of articular chondrocytes to IL-1 isoforms or TNFα have been investigated by several scientists (20, 41) as a model system to study processing occurring in degenerative cartilage diseases. In particular it was shown that such molecules strongly enhance the expression of a large number of mediators contributing to cartilage degradation like matrix metalloproteinases (19, 39) and inflammatory cytokines (42, 43), as well as inhibit the synthesis of key extracellular matrix proteins such as type II collagen and proteoglycan (21-24). For such studies, however primary undifferentiated chondrocytes or cartilage explants derived from OA patients were used. Due to the fact that the responses of chondrocytes to IL-1 vary drastically from normal vs osteoarthritic chondrocytes and from differentiated vs passaged chondrocytes (20), the results reported in the aforementioned studies could not be directly translated to a cell-based clinical scenario. In our study monolayer expanded HAC were cultured in pellets for one day or 2 weeks and then treated for 72 hours with IL-1 β to study effects occurring by chondrocytes within MACI and TE products following their implantation in the inflamed cartilage defect. 72 hour IL-1 exposure mimics the duration of the inflammation caused by the surgical intervention.

We observed that IL-1 β treatment caused a reduction in the amount of cartilaginous matrix which was more pronounced in the immature tissues compared to the mature ones. These result might be the consequence of an enhanced tissue degradation mediated by MMPs, in fact we showed that IL-1β treatment caused an increased production of MMP-1 and MMP-13 more pronounced in immature pellets. Although the activity of such MMPs in the present study was not assessed, previously studies reported that IL-1 β enhances the activity of such enzymes (19, 44, 45).

A surprising result of our study was that mature pellets (vs immature pellets) responded to IL-1β by expressing higher amounts of IL-8 and MCP-1 mRNA and releasing larger amount of these two chemokines. The reason for this is unclear. It is possible that this effect might be partially mediated by TGFβ. In fact, it was previously observed that such factor acts synergistically with IL-1β enhancing

the secretion of IL-8 by human articular chondrocytes (42). Superior levels of TGFβ found in mature pellets (vs immature pellets) indeed, can result from an enhanced expression by chondrocytes as demonstrated in this study and by an increased accumulation of such factors in the matrix of the pellets following prolonged culture with our chondrogenic medium containing high concentration of TGFβ-1. Therefore one possible mechanism occurring following IL-1β treatment of mature pellets is that IL-1β induces an upregulation of degradative enzymes (as observed in the present study by enhanced release of MMPs) which in turn causes a massive release of the TGF-β stored in the matrix. In addition, one important aspect that has to be considered is that some of the chemokines produced by chondrocytes not only mediate inflammatory and degradative actions, they are also shown to induce chemotaxis of mesenchymal stem/progenitors cells (MSC) (46). Interestingly, IL-8 is one of such chemokines promoting MSC chemotaxis (47). We can therefore speculate that "mature" chondrocytes once grafted in the defect might promote a more pronounced recruitment of MSC and that such cells can actively participate to the repair process.

We are also aware that the model used in the present study, have not been validated to directly predict the reparative ability of cell preparation at different stages of maturation when implanted in cartilage lesions. In the absence of an orthotopic model where to test the performance of human chondrocytes for cartilage repair beyond the patient, further studies may investigate the HAC performance under conditions better resembling the injured joint (e.g., more physiologic oxygen tension, exposure to loading, presence of MSC).

In summary, the cytokine expression profile and IL-1β-mediated catabolic effects by human articular chondrocytes are strongly modulated by their differentiation stage. The fact that engineered tissues with high extent of maturation following IL-1β treatment (i) still possess abundant cartilaginous matrix and (ii) release lower amounts of MMPs suggest that the implantation of more mature cartilaginous tissues could guarantee superior graft survival and functional outcome. The observed up-regulation of MCP-1 and IL-8 by chondrocytes prolonged cultured with TGFβ-1 highlights the importance of the selection of the growth factors for the preparation of autologous cells for cartilage repair applications.

RNA template	***Primer sequences***	Annealing temperature (°C)	References*
GAPDH	5'-TGG TAT CGT GGA AGG ACT CAT GAC 3'-ATG CCA GTG AGC TTC CCG TTC AGC	60	Blanco et al.
TGF-β	5'-CTGAGGTATCGCCAGG 3'-CGCGTGCTAATGGTGGA	58	PRIMER 3
IL-8	5'-ACTTCTCCACAACCT 3'-CCAAACCTTTCCACCC	56	PRIMER 3
MCP-1	5'-AGCCACCTTCATTCC 3'-GCTTCTTTTGGGACACTTGCT	56	PRIMER 3

Table 1. Real-Time RT-PCR primers description.
*Primer sequences were obtained from published references where indicated or designed using PRIMER 3.

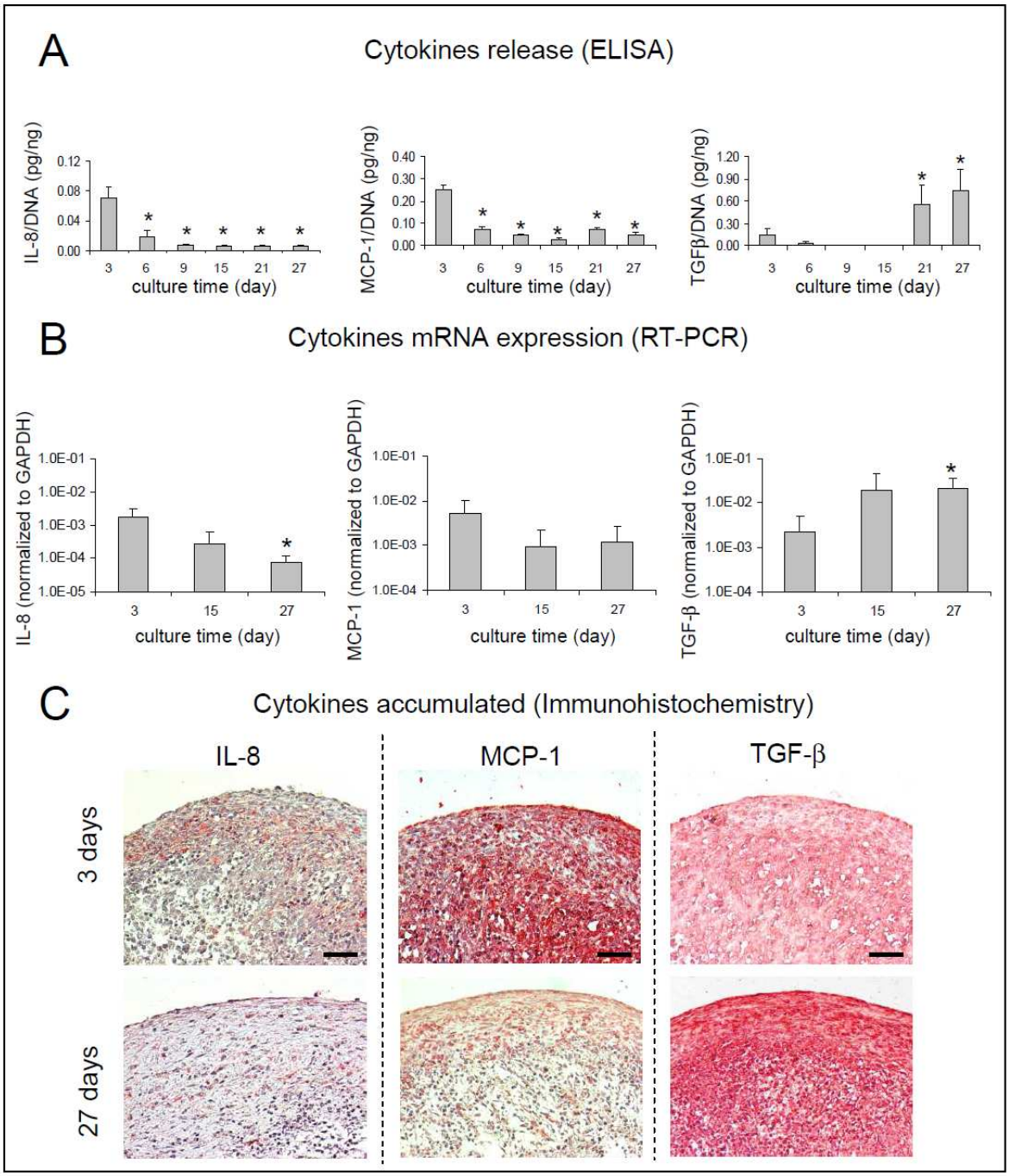

Figure 1. IL-8, MCP-1, TGF-β (A) protein levels in the surnatants, (B) messenger mRNA expression, (C) protein expression in tissue by immunohistochemistry, of HAC cultured in pellet system for different experimental times. Values are expressed as mean ± standard deviation (SD) of measurements obtained from 6 different donors. * = $p<0.05$ vs 3 days. Bar = 200µm

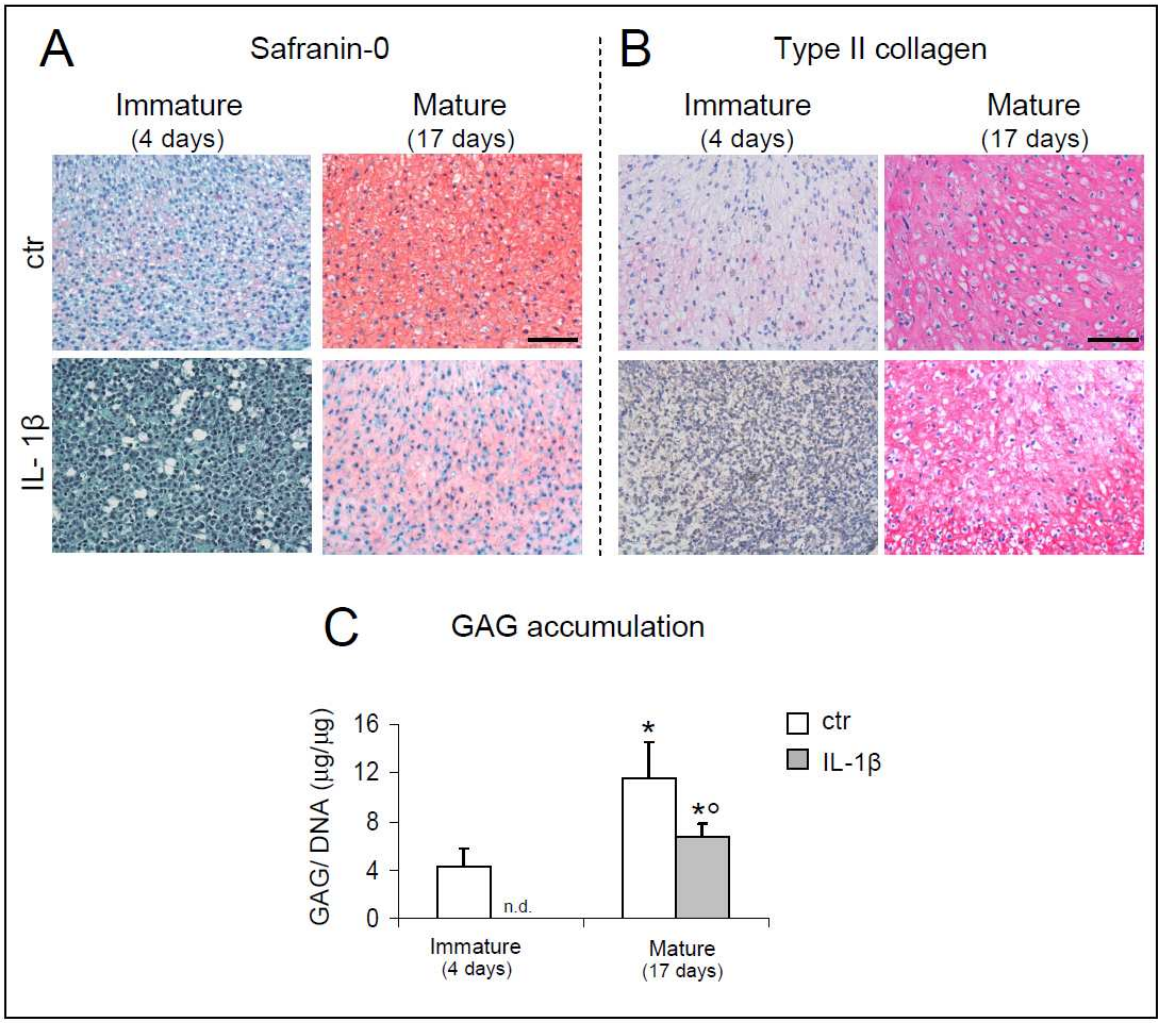

Figure 2. Effects of 72 h IL-1β exposure on immature (4 days) and mature (17 days) pellet tissues. (A) Safranin-O stainings of representative pellets. Bars = 200µm. (B) Sulphated glycosaminoglycan content normalized to the amount of DNA. Values are mean ± SD of measurements obtained from 7 different experiments.
* = $p<0.05$ vs 4 days (same culture condition); ° = $p<0.05$ vs ctr (same culture time).

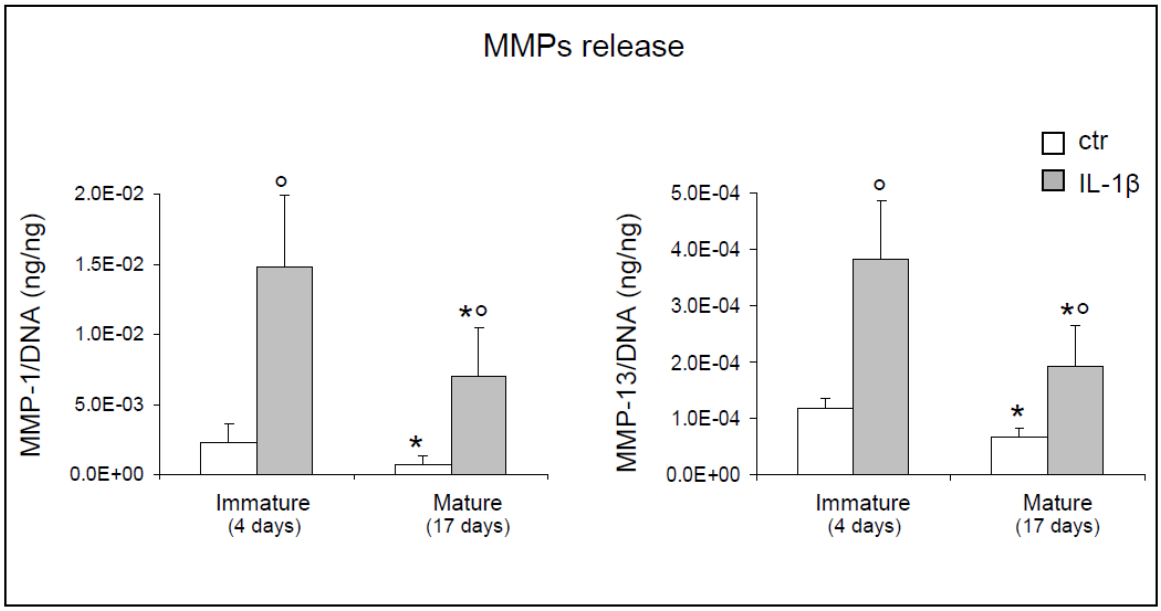

Figure 3. MMP-1 and MMP-13 protein levels in the surnatants produced by immature (4 days) and mature (17 days) pellet tissues with and without stimulation with IL-1β for 72 h. Values are expressed as mean ± standard deviation (SD) of measurements obtained from 4 different donors.
* = p<0.05 vs 4 days (same culture condition); ° = p <0.05 vs ctr (same culture time).

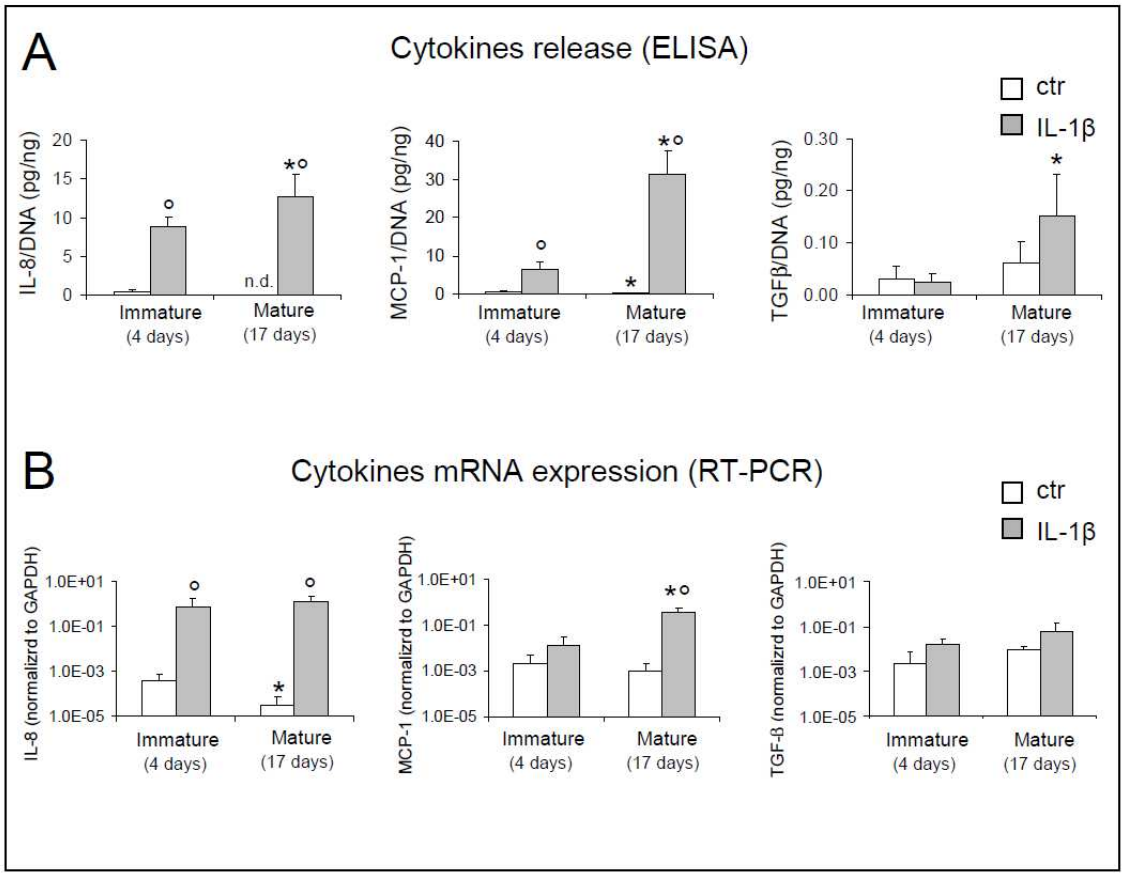

Figure 4. IL-8, MCP-1 and TGFβ (A) mRNA expression (B) protein release by immature (4 days) and mature (17 days) pellet tissues with and without stimulation with IL-1β for 72 h. mRNA levels are expressed as fold of difference from Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Values are expressed as mean ± standard deviation (SD) of measurements obtained from 3 different donors. * = $p<0.05$ vs 4 days (same culture condition); ° = $p<0.05$ vs ctr (same culture time).

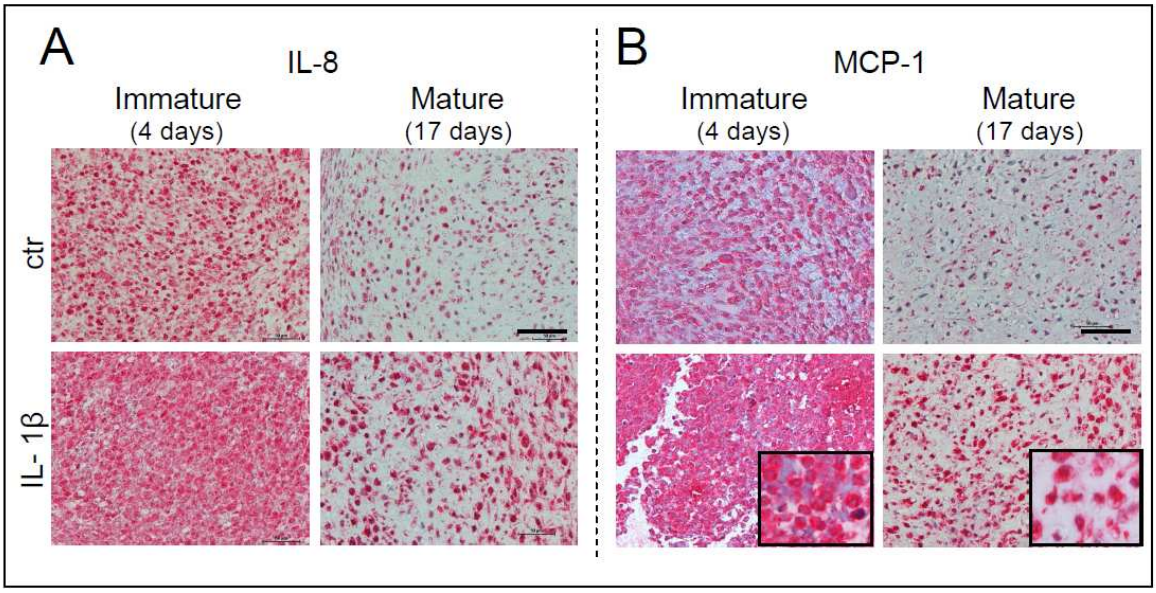

Figure 5. Immunohistochemistry for IL-8 and MCP-1 protein expression by immature (4 days) and mature (17 days) pellet tissues with and without stimulation with IL-1β for 72 h. Bar = 200μm. Pictures in the inserts are higher magnification (400X)

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3 SUMMARY

Regenerative medicine holds great promise for the reconstitution of damaged tissues and organs. Treatment for articular cartilage injuries is a prime target for regenerative techniques, as spontaneous healing is poor.

Among the many surgical options for the treatment of such lesions, implantation of autologous chondrocytes is gaining increasing popularity, especially for critically sized defects (79, 91). These procedures include autologous cartilage implantation (ACI) (65, 80, 81, 168), the more recent matrix-mediated ACI (MACI) (169), or the grafting of tissue engineered cartilaginous constructs (TE) (170, 171), typically based on cultivation of cells on porous scaffolds.

The main aim of my PhD was to study different aspects of human articular chondrocytes (HAC) biology and to evaluate their relevance for the generation of a tissue engineered construct with properties compatible with utilization in clinical cartilage repair.

Study 1

Main results

In the first study we showed that the use of the specific growth factor combination TGFβ-1/FGF-2/PDGF (TFP) during the expansion of human articular chondrocytes (HAC) allows to obtain (i) a clinically relevant number of competent cells also if initially seeded at low densities (and therefore starting from a cartilage biopsy of small size), (ii) reduced variability in proliferation and cartilage forming capacity of cells expanded under different conditions of seeding densities and human serum percentages.

Relevance

Cartilage biopsies are variable in size while harvesting an excess of it is a detrimental loss that should be minimized. Consequently, the number of chondrocytes obtainable from a biopsy is variable

and relatively low for a clinical application. In addition, the final cell-based products vary in size according to the needs of individual patients (79). Therefore, the monolayer expansion, which is the core process of all the above mentioned cell-based techniques, is affected by these variables, resulting in low reproducibility.

Moreover, in the last years, efforts to commercialize cell-based therapies are raising the need for capable, reproducible, scalable, automated manufacturing systems.

The use of bioreactors within the hospital for automated culture of autologous cells would eliminate logistical issues of transferring specimens between locations, reduce the need for large and expensive GMP facilities and minimize operator handling with the final possible result of simplifying, reducing the cost and increasing the standardization of the entire process (166, 167).

In our study we have defined culture conditions allowing to overcome some constraints related to the implementation of closed automated bioreactor system for the generation of implantable autologous cartilage products in which the cells isolable from cartilage biopsies of different sizes have to be expanded in only one passage on a fixed culture surface. Moreover, the system should use human autologous serum at low percentages, to avoid use of animal derived factors and to reduce the dependence of the culture on a non-standardized factor such as autologous serum.

The concept of using specific growth factors in clinical-grade bioreactors could be applied for the propagation of cells from different human sources to reduce variability in the number and quality of expanded cells and, ultimately, in the clinical outcome of cell-based regenerative techniques.

Study 2

Main results

In this study we demonstrated that expansion of freshly isolated HAC, in biomimetic three-dimensional scaffold (type II collagen sponge), as compared to expansion in monolayer, better maintains the chondrocytic phenotype but does not enhance the cartilage-forming capacity of HAC. Instead, increasing the HAC seeding density in the scaffolds allowed enhanced chondrogenesis, even if the seeded cells had to be expanded more extensively in 2D in order to reach the required cell

numbers. Therefore, high seeding density of HAC in 3D scaffolds is more critical for the generation of cartilaginous constructs than the stage of cell differentiation reached following expansion.

Relevance

Study 2

Main results

In this study we demonstrated that expansion of freshly isolated HAC in a biomimetic three-dimensional scaffold (type II collagen sponge), as compared to expansion in monolayer, better maintain the chondrocytic phenotype but does not enhance the cartilage-forming capacity. Instead, increasing the HAC seeding density in the scaffolds allowed enhanced chondrogenesis, even if the seeded cells had to be expanded more extensively in 2D in order to reach the required cell numbers. Therefore, high seeding density of HAC in 3D scaffolds is proposed to be more critical for the generation of cartilaginous constructs than the stage of cell differentiation reached following expansion.

Relevance

Monolayer expansion, the core process of cell-based cartilage repair techniques is associated with cell de-differentiation and loss of the chondrocytic phenotype. Therefore to overcome the limitations of a low initial number of cells and dedifferentiation, various studies have been performed in which chondrocytes are multiplied in monolayer cultures to increase the number of cells and then transferred at high density to a long term three dimensional culture system to regain their phenotype (172, 173). An alternative strategy for the utilization of the small number of chondrocytes obtained from a cartilage biopsy consists in their limited expansion in 2D or even in the direct culture onto 3D porous scaffolds (174-176). In principle, this technique would allow a better maintenance of the chondrocytic phenotype while reducing the extent of cell proliferation and thus the cell seeding density.

We showed that the extensively dedifferentiated phenotype of chondrocytes did not appear to interfere with the chondrogenic program, provided that high cell density was initially established. Indeed, the extent of HAC passaging and consequent stage of de-differentiation did not modify the cartilage-forming capacity of these cells following 3D culture. This is a further proof of the extensive

plasticity exhibited by HAC expanded in culture under suitable conditions (109, 147). Moreover our results clearly indicate that a high seeding density of HAC in 3D scaffolds is more critical for the generation of implantable cartilaginous constructs than the stage of cell differentiation reached following expansion.

Study 3

Main results

In this study, we demonstrated that the profile of chemokine production and the response to inflammatory/degradative stimuli by monolayer expanded HAC correlated with the differentiation stage of cells cultured in micromass pellets. In particular, cells within more mature tissues produced higher amounts of the pro-inflammatory chemokines IL-8 and MCP-1 and releases lower amounts of MMPs following IL-1β stimulation.

Relevance

Expanded chondrocytes exhibit enhanced expression of de-differentiated markers (e.g.: versican, type I collagen and cathepsin B) as compared to native normal chondrocytes (108, 149, 151, 177). Moreover, it has been reported that, similarly to OA chondrocytes, ex-vivo cultured chondrocytes express a variety of pro-inflammatory chemokines/chemokine receptors and cartilage degenerative enzymes (178-183), whose production is enhanced by increasing cell passaging and by stimulation with IL-1β and TNF-α (184, 185). These responses have particular relevance in the context of cell based-cartilage repair, considering that therapeutic cell preparations (single cell suspension, cell-seeded matrixes or cartilaginous tissues) once grafted in the joint defect will become exposed to a biochemical environment rich in catabolic mediators. The cell preparation in which the chondrocytes are less susceptible to such mediators might guarantee superior clinical outcome. Our findings that chondrocytes embedded in a more abundant cartilaginous matrix release lower levels of pro-inflammatory chemokines and exhibit less IL-1β-mediated catabolic effects suggest that the implantation of more mature cartilaginous tissues could guarantee superior graft survival and functional outcome.

CONCLUSION

In conclusion, during my PhD we demonstrated that the phenotype of human articular chondrocytes can be strongly modulated following the exposure to specific biological and physicochemical cues. The findings described in this work have therefore allowed to improve the knowledge on the biology of cartilage cells and to define specific conditions to improve the efficiency of utilization of chondrocytes for clinical applications.

My findings resulted indeed essential in the definition of the culture conditions to use in a recently proposed internal phase I, prospective, uncontrolled clinical trial aimed at demonstrating safety and feasibility in the use of engineered cartilage grafts. The specific surgical target of the trial is the reconstruction of a two layer defect of the alar lobule using a tissue engineered nasal cartilage graft and a local flap, following resection of a nonmelanoma skin cancer.

For such trial, in fact, nasal articular chondrocytes isolated from a small cartilage biopsy in the nose will be expanded in monolayer with medium containing low percentage of autologous serum and with the previously described growth factor combination (study 1). Cells will be then seeded at high density onto collagenous matrices (study 2) and cultured under differentiation conditions allowing to generate mature cartilage graft (study 3).

During my PhD I worked in a unique environment where the scientific approach, clinical and industry point of views were combined in order to facilitate the achievement of a final and common goal. Indeed the interaction with different collaborating partners (i.e., the company Millenium Biologix AG and orthopaedic surgeons) helped me to remain focused on investigating scientific relevant questions which could bring to a final cell-based product, pertinent for a clinical application.

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CURRICULUM VITAE

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EDUCATION

PhD, University of Basel, CH **Expected completion December 2009**
Subject: Influence of chondrocyte differentiation stage on the capacity to generate functional cartilaginous tissue in vitro: relevance for cell-based cartilage repair technique

Master Degree in Biology, University of Insubria, Varese, I 2002
Subject: The use of cellular cultures in toxicity tests required by European laws: toxicological patterns due to trace metals

WORK HISTORY

Tissue Engineering, ICFS, University Hospital, Basel **Apr 2004 onwards**
PhD: Development of strategies for the generation of cartilage and osteochondral grafts/substitutes, based on autologous cells, 3D scaffolds and use of bioreactor system.
Responsible: Prof. Dr. Ivan Martin

Pharmacology, Biozentrum, Basel Fachhochschule Beider Basel (FHBB), Muttenz Apr. - Dec.2003
Research Assistant: Development of a cell-based reporter gene assay for the measurement of drug induction in human beings.
Responsibles: Prof. Urs A. Meyer, Prof. Daniel Gygax

Immunobiology of HIV, DIBIT , San Raffaele Scientific Institute, Milan, Italy Sep 2002 – Mar 2003
Research Assistant: Characterization of specific humoral responses of risk population aim at defining new possible therapies against the HIV virus.
Responsible: Dr. Lucia Lopalco

Toxicology, Joint Research Center (JRC) ECAVM Unit, Ispra, VA, Italy **2001 - 2002**
Master thesis: The goal was to approach to the prediction of systemic toxicity and human hazard using biological *in vitro* tests in compliance to the GLP Principles.
Responsibles: Dr. Enrico Sabbioni; Dr. Maria P. Pilar

PUBLICATIONS

Francioli SE, Martin I, Sie CP, Hagg R, Tommasini R, Candrian C, Heberer M, Barbero A. Growth Factors for Clinical-Scale Expansion of Human Articular Chondrocytes: Relevance for Automated Bioreactor Systems.Tissue Eng. 2007 Jun;13(6):1227-34.

Tampieri A, Sandri M, Landi E, Pressato D, Francioli SE, Quarto R, Martin I. Design of graded biomimetic osteochondral composite scaffolds. Biomaterials. 2008 Sep;29(26):3539-46. Epub 2008 Jun 5.

E. Kon, M. Delcogliano, G. Filardo, M. Fini, G. Giavaresi, SE. Francioli, I. Martin, D. Pressato, E.Arcangeli, R. Quarto, M. Sandri and M. Marcacci. Orderly osteochondral regeneration in a sheep model using a Novel Nano-composite multi-layered. Biomaterial. 2008 Sep; 29(26):3539-46.

C. Candrian, A. Barbero, E. Bonacina, S. Francioli, M.T. Hirschmann, S. Milz, V. Valderrabano, M. Heberer, I. Martin, M. Jakob.
A novel implantation technique for engineered osteo-chondral grafts. Knee Surg Sports Traumatol Arthrosc. 2009 Mar 21.

Francioli SE, Martin I, Sie CP, Hagg R, Tommasini R, Candrian C, Heberer M, Barbero A. Growth Factors for Clinical-Scale Expansion of Human Articular Chondrocytes: Relevance for Automated Bioreactor Systems. Tissue Eng. 2007 Jun; 13(6):1227-34.

Francioli SE, C. Candrian, Heberer M, Martin I, Barbero A. Effect of three-dimensional expansion and cell seeding density on the cartilage-forming capacity of human articular chondrocytes in type II collagen sponges. ***Submitted.***

Francioli SE, C. Cavallo, Heberer M, B. Gigolo, Martin I, Barbero A. Effects of culture time and exposure to IL-1b on the expression of degenerative cartilage molecules by human articular chondrocytes. ***In advanced preparation.***

CONGRESS PRESENTATIONS

D. Wirz , V. Buergin, S. Francioli, A. Barbero, I. Martin, B. Goepfert, A.U. Daniels. 90° peel off test for measuring osteochondral bonding in tissue-engineered constructs Forschungstag 2008 (UHBS)

Francioli S., Miot S., Frueh J., Cavallo C., Jakob M., Grigolo B., Martin I., Barbero A. Production of cytokines and response to IL-1b by human articular chondrocytes at different stages of tissue maturation. Abstract presented at the Tissue Engineering Regenerative Medicine International Society (TERMIS) in Porto, Portugal (June 2008).

Francioli S., Barbero A., Candrian C., Tampieri A., Pressato D., Martin I., Wendt D. Engineering osteochondral composite grafts using human de-differentiated articular chondrocytes and an innovative three-layered composite scaffold. Abstract presented at the European Conference on Biomaterials (ESB) meeting in Brighton, UK (Sept 2007).

Francioli S., Candrian C., Martin K., Heberer M., Martin I, Barbero A. Effect of three-dimentinal expansion and cell seeding density on the cartilage-forming capacity of human articular chondrocytes in type II collagen sponges. Abstract presented at the International Cartilage Repair Society (ICRS) meeting in Warsaw, Poland (September 2007) and at the Tissue Engineering Regenerative Medicine International Society (TERMIS) meeting in London, UK (Sept 2007).

Candrian C., Vonwil D., Bonacina E., Barbero A., Miot S., Wendt D., Francioli S., Wolf F., Farhadi J., Wirz D., Daniels D., Jakob M., Heberer M., Martin I. Response to cyclic loading of human engineered cartilage based on nasal or articular chondrocytes. Abstract presented at the Orthopedics Research Society (ORS) meeting in San Diego, USA (Feb 2007).

S. Francioli, A. Barbero, C. Sie, R. Hagg, R. Tommasini, I. Martin Growth factors for human articular chondrocytes expansion: relevance for automated tissue engineering bioreactors. Abstract presented at ETES meeting in Munich, Germany (Sept 2005)