UNIVERSITÉ DE MONTREAL

OPTIMISATION ET CARACTÉRISATION DE LA CULTURE DE CHONDROCYTES EN SUSPENSION

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RÉSUMÉ

La recherche sur le cartilage et les cellules le composant, les chondrocytes, permet de mieux comprendre la survenance et le développement des maladies articulaires comme l'arthrose, mais aussi d'élaborer de nouvelles stratégies thérapeutiques. Les chondrocytes, qui sont responsables à la fois de la synthèse et de la dégradation de ce tissu, peuvent être isolés et cultivés *in vitro*, afin d'être étudiés ou utilisés pour la transplantation, une des techniques de réparation du cartilage. Cependant, leur culture est problématique puisque ces cellules ont tendance à perdre leur phénotype et à se dédifférencier. Le mode de culture le plus répandu est la culture en monocouche (en 2D, sur une surface), qui permet une croissance rapide, mais entraine une forte dédifférenciation. Une alternative est alors la culture tridimensionnelle (3D) sur un support, ou en suspension sans support sous forme agrégats cellulaires. Dans ces dernières conditions, le phénotype est maintenu quoique souvent de manière incomplète, mais la prolifération cellulaire est très faible. D'autre part, la taille excessive des constructions cellules/support ou des agrégats, atteignant généralement plusieurs millimètres d'épaisseur, peut entraver l'accessibilité des nutriments au centre de ces derniers. De ces différents types de culture, aucun n'est donc idéal, c'est-à-dire permettant à la fois la croissance et le maintien du phénotype dans des conditions de culture homogènes. De nombreuses recherches ont déjà été entreprises afin d'y parvenir et de nombreux progrès ont été réalisés. Dans ce travail,

nous avons proposé d'exploiter quelques possibilités, encore non examinées dans ce cadre.

Les travaux de recherche présentés dans cette thèse privilégient la culture de chondrocytes en agrégats cellulaires. Nous avons supposé que l'optimisation de la culture en suspension favoriserait une meilleure conservation du phénotype tout en permettant aux chondrocytes de bénéficier d'un environnement physiologique et homogène. Pour perfectionner ce système, nous avons également posé comme hypothèse qu'une amélioration du milieu de culture, notamment le développement d'un milieu sans sérum, permettrait de conserver plus efficacement l'état différencié des chondrocytes, mais aussi de limiter la taille des agrégats. Nous avons également supposé que l'utilisation d'un système de culture agité fournirait un meilleur contrôle de l'agrégation, ainsi qu'un transfert de matière amélioré.

Dans un premier temps, deux paramètres reliés au milieu de culture ont été testés. L'effet de la présence ou de l'absence de sérum ainsi que de la concentration en calcium (de 15µM à 1mM) ont été évalués à la fois sur l'agrégation et le phénotype des chondrocytes cultivés en suspension ou en monocouche. Les résultats de ces travaux ont fait l'objet d'un premier article paru dans Osteoarthritis and Cartilage en 2005. Il y est montré que i) l'absence de sérum améliore le maintien du phénotype et réduit l'agrégation ; ii) la concentration en calcium n'a pas d'incidence sur l'agrégation dans la plage testée ; iii) de basses concentrations en calcium favorisent le phénotype chondrocytique, notamment en monocouche. Il a également été déterminé qu'un milieu sans sérum et contenant 1mM de calcium favorisait la croissance et le caractère

chondrocytique des cellules en suspension et réduisait la taille des agrégats par rapport au milieu contenant du sérum. Ce milieu optimisé a ensuite été utilisé dans toutes les expériences subséquentes.

Ensuite, afin d'obtenir une meilleure distribution des nutriments, des gaz et des cellules dans l'ensemble du volume de culture, un système agité a été testé : le spinner. Ce dernier est notamment composé d'un arbre d'agitation muni de quatre pales perpendiculaires inclinées à 45° qui pompent le milieu du bas vers le haut et maintiennent les agrégats en suspension. En plus du milieu de culture préalablement optimisé (le milieu contrôle), deux autres milieux ont également été testés. L'un était composé du milieu contrôle additionné d'un surfactant connu pour protéger les cellules du cisaillement, le Pluronic F-68 (PF-68). Dans l'autre, en plus de l'ajout de PF-68, la concentration en facteurs de croissance (EGF, PDGF-BB et FGF-2) a été multipliée par dix afin de stimuler la croissance et la synthèse d'une matrice extra-cellulaire. Dans toutes les conditions, les cellules étaient viables, les agrégats étaient plus petits qu'en conditions statiques, le phénotype était maintenu et une matrice riche en collagène type II s'accumulait autour des cellules. Une croissance a été détectée mais dans les milieux contenant du PF-68 seulement (un doublement en 7 jours). Enfin, une meilleure maintenance phénotypique a été observée dans le milieu riche en facteurs de croissance (absence totale de collagène I, cellules rondes). Ces résultats ont été soumis dans la revue Tissue Engineering.

Comme le PF-68 permet la croissance des chondrocytes en suspension et un maintien du phénotype, il pourrait être utilisé pour l'expansion de cellules destinées à

être implantées dans un patient. Cependant, la manière dont cette molécule interagit avec les cellules était inconnue et de nombreuses études suggéraient qu'elle pouvait être incorporée dans la membrane plasmique et également intégrer l'espace intra-cellulaire. En supposant que ces chondrocytes doivent être implantés dans un patient, il devenait alors d'une importance cruciale d'élucider la nature des interactions cellule/ PF-68. Nous avons examiné cette question en marquant le PF-68 avec un fluorophore, afin d'être en mesure d'étudier sa distribution intra-cellulaire. La quantité de PF-68 intégrée dans les cellules a également été évaluée. Dans un article sous-presse dans le journal Biotechnology and Bioengineering en 2008, nous avons clairement démontré que le PF-68 entre dans les cellules et s'accumule dans la voie de l'endocytose. Cependant, cette accumulation n'est pas assez importante pour être problématique dans un cas de transplantation. Le PF-68 est donc un additif acceptable pour la culture de chondrocytes.

Enfin, pour pleinement caractériser ce système de culture et élucider les mécanismes moléculaires de l'agrégation cellulaire en suspension, nous avons étudié les interactions que les chondrocytes établissent avec leur environnement. Ces interactions sont connues pour influencer de nombreux phénomènes biologiques comme le développement, l'homéostasie, la dégradation du cartilage ou la survie cellulaire. Leur caractérisation est donc un pré-requis à la compréhension de ces phénomènes. Il a ainsi été démontré que l'interaction prédominante entraînant l'agrégation des chondrocytes en suspension implique une intégrine β1 se liant à une molécule de collagène contenant le motif GFOGER en triple hélice. Plus précisément,

l'intégrine $\alpha 10\beta 1$ et le collagène type II en sont très probablement les deux médiateurs. Par ailleurs, il a été montré que les chondrocytes dans les agrégats reproduisent une matrice péricellulaire très proche de celle observée dans le cartilage, maintenant les cellules dans un environnement physiologique représentatif des conditions *in situ*.

Nous avons donc réussi, lors de ces travaux à développer un mode de culture agité et homogène qui permet la croissance et la maintenance du phénotype chondrocytique. Les agrégats formés dans un tel système recréent un environnement péricellulaire similaire au cartilage et sont donc un outil très utile pour étudier la biologie des chondrocytes et leur réponse à divers stimuli. De plus, ces agrégats conçus *in vitro* étant composés de chondrocytes différenciés entourés d'une matrice cartilagineuse, ils présentent un intérêt thérapeutique et peuvent être utilisés comme transplant pour la réparation du cartilage.

ABSTRACT

Research on cartilage and its unique cell type, the chondrocyte, enables better understanding of the initiation and progression of joint diseases, such as arthritis and to develop new therapeutic strategies. Chondrocytes, which mediate both cartilage synthesis and degradation, can be isolated and cultured *in vitro*, to be studied or utilised for transplantation, one of the cartilage repair techniques. However, this culture is challenging as these cells tend to lose their phenotype and to dedifferentiate. The most common culture technique is monolayer culture (on a 2D surface), which allows a fast growth but leads to dedifferentiation. A possible alternative is three dimensional (3D) culture in a scaffold or in suspension as aggregates. In these conditions, phenotype is maintained, but often not fully and cell proliferation is weak. On the other hand, size of cell/scaffold constructs or aggregates, which are usually several millimetres thick, can limit nutrient access to their center. Among these culture systems, none is ideal, i.e. concomitantly enabling growth, phenotype maintenance and homogeneous culture conditions. Numerous studies have already been performed to reach this goal, and great progress has been achieved. In this work, we propose to explore some new avenues, not examined so far to further improve chondrocytes culture and understanding of chondrocyte biology.

The research in this thesis focuses on culture of chondrocytes in cell aggregates. The hypothesis underlying the thesis is the following: optimisation of the chondrocytes

culture in suspension could provide a system allowing phenotype maintenance, and enabling chondrocytes to be in a physiological and homogenous environment. With the goal of improving the performance of this culture system, we also hypothesized that an optimisation of the culture medium, more precisely the development of a serum free medium, would better stabilize the chondrocyte differentiated state, and limit aggregate size as well. We also hypothesized that an agitated culture system would improve aggregation control and enhance mass transfer in aggregates.

Initially, two parameters related to the culture medium were tested. The effects of the absence or presence of serum, as well as calcium concentration (from 15μM to 1mM) were studied both on chondrocytes aggregation and phenotype in suspension and in monolayer. Results of this work were the subject of a first paper published in Osteoarthritis and Cartilage in 2005. It was shown that i) absence of serum improves phenotype maintenance and reduces aggregation ii) calcium concentration exerts no effect on aggregation iii) low calcium concentrations sustain the chondrocyte phenotype, especially in monolayer. It was also determined that a serum-free medium containing 1mM calcium, when compared to a serum-containing medium, promotes growth, preserved chondrocytes phenotype in suspension, and reduced aggregates size. This optimized medium was then used in all subsequent experiments.

Afterward, to improve nutrients, gas and cells distribution in the culture volume, an agitated system was tested: the spinner bioreactor. The latter was supplied with a stirring shaft with four perpendicular 45 -angled paddles, dragging the medium upward and keeping aggregates in suspension. In addition to the previously optimised medium,

two others media were tested with this system. One was supplemented with a shear-protectant surfactant, Pluronic F-68 (PF-68), the other with PF-68 and a 10-fold higher growth factor concentration (EGF, PDGF-BB and FGF-2) to stimulate growth and extracellular matrix synthesis. In all tested conditions, cells are viable, aggregates are smaller in comparison with static conditions, the phenotype is maintained, and a type II collagen-rich matrix accumulates around cells. Growth was detected in PF-68 containing media (one doubling in seven days). Finally, a better phenotype maintenance was observed in presence of higher growth factors concentration (no type I collagen, round cells). These results were submitted to the journal Tissue Engineering.

As PF-68 enables chondrocyte growth and phenotype maintenance, it could be used to expand cells before implantation. However, the nature of cell-pluronic interactions is not known, and numerous studies suggest that PF-68 could be incorporated in the plasma membrane and even enter the cells. If the latter are to be implanted in a patient, it is a major issue to know if PF-68 penetrates the cells and in which amount. To examine this question we synthesised a fluorescent derivative of PF-68, to study its intracellular localisation. PF-68 uptake was also evaluated. In a paper accepted in Biotechnology and Bioengineering, in 2008, we clearly demonstrated that PF-68 can be found intracellularly and accumulates in the endocytic pathway. Nonetheless this accumulation is not sufficient to be a concern in a transplantation case. PF-68 is therefore a possible additive for chondrocytes culture.

Finally, to fully characterise this culture system, and shed light on molecular mechanisms of chondrocytes aggregation, we investigated chondrocyte interactions with their surrounding environment. These interactions are known to be implicated in various biologic phenomena as cartilage development, homeostasis, degradation or cell survival. Their characterisation is therefore a prerequisite for a better understanding of those phenomena. We demonstrated that the main interaction driving chondrocytes aggregation in suspension is composed of a β1 integrin binding a collagen molecule containing the triple helical GFOGER motif. More precisely, integrin α10β1 and collagen type II are the most probable mediators. Moreover it was established that chondrocytes in aggregates re-build a cartilage-like pericellular environment.

Therefore we managed in this thesis to develop an agitated and homogenous chondrocyte culture system enabling growth and phenotype maintenance. In such a system, aggregates exhibit a composition mimicking the cartilage pericellular environment, and are therefore a highly useful tool to study chondrocytes biology and response to multiple stimuli. Moreover, the *in vitro* engineered aggregates, as composed of differentiated chondrocytes in a cartilaginous matrix, have a therapeutic interest and could be used as transplants for cartilage repair.

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LISTE DES ABRÉVIATIONS

2D : deux dimensions

3D : trois dimensions

ACI : Autologous Chondrocyte Implantation.

ARN : Acide RiboNucléique

ARNm : ARN messager

BSA : Bovine Serum Albumin

CAM : Cell Adhesion Molecule

CaR : Calcium Receptor

CDC-Fluorescein : 5-(et 6-) Carboxy-2'7'DiChlorofluoresceine

cDNA : Complementary DNA

CHAD : CHondroADherine

CHO cells : Chinese Hamster Ovary cells

COMP : Cartilage Oligomeric Matrix Protein

DCC : DiCyclohexylCarbodiimide

DDR2 : Discoidin Domain Receptor 2

DHCB : DiHydroCytochalasin B

DMAP : DiMethylAminoPyridine

DMEM : Dulbecco's Modified Eagle's Medium

DMF : DiMethylFormamide

DMMB : DiMethyl MethyleneBlue

DNA : DeoxyriboNucleic Acid

dNTP : DeoxyNucleotide TriPhosphate

DTT : DiThioThreitol

ECM : ExtraCellular Matrix

EDTA : Ethylene Diamine TetraAcetic Acid

Ef1α : Elongation Factor 1α

EGF : Epidermal Growth Factor

EGTA : Ethylene Glycol TetraAcetic acid

FBS : Fetal Bovine Serum

FCS : Fetal Calf Serum

FIC : Freshly Isolated Chondrocytes

FITC : Fluorescein IsoThioCyanate

FGF-2 ou b-FGF : Basic Fibroblast Growth Factor

FU : Fluorescence Unit

GAG : GlycosAmoniGlycanes

GF : Growth Factor

GITC : Guanidium IsoThioCyanate

GLM : General Linear Model

GuCl : GUanidium hydroChLoride

HBSS : Hank's Buffered Salt Solution

HCl : acide HydroChLorique

HEK 293 : Human Embyonic Kidney 293

HEPES : Acide N-(2-HydroxyEthyl)-Piperazine N'-(-2 EthaneSulfonique)

ID_{50} : Inhibition Dose 50

IL-1β : Interleukine1β

ITS : Insuline, Transferrine, Sélénium.

LP : Link Protein

MEC : Matrice Extra-Cellulaire

MES : acide 2-[N-morpholino]ethanesulfonique

MW : Molecular Weight

mHBSS : Modified HBSS

MMLV : Moloney Murine Leukemia Virus

mRNA : messenger RNA

N-CAM : Neural Cell Adhesion Molecule

PGA : PolyGlycolique Acid

PBS : Phosphate Buffered Saline

PCR : Polymerase Chain Reaction

PDGF-BB : Platelet-derived Growth Factor BB

PEO : PolyEthylene Oxyde

PF-68 : Pluronic F-68

PL-61 : Pluronic L-61

PL-121 : Pluronic L-121

PLA : Poly (L-Lactic Acid).

PLGA : Poly (L-Lactic-co-Glycolic) Acid

PMF : Plasma Membrane Fluidity

PP-85 : Pluronic P-85

PPO : PolyPropylene Oxyde

PVDF : PolyViniliDene Fluoride

QRT-PCR : Quantitative Real-Time PCR

rhEGF : recombinant human EGF

rhFGF-2 : recombinant human FGF-2

rhPDGF-BB : recombinant human PDGF-BB

RNA : RiboNucleic Acid

RWV : Rotating-Wall Vessel

TNF-α : Tumor Necrosis Factor α

TSP : ThromboSpondin

SD : Standard Deviation

SDS : Sodium Dodecyl Sulphate

SCM : Serum-Containing Medium

SEM : Standard Error to the Mean/ Scanning Electron Microscopy

SFM : Serum-Free Medium

siRNA : Small Interfering RNA

INTRODUCTION

D'après un rapport national, "L'arthrite au Canada", édité par la Société d'arthrite (www.arthrite.ca) en 2003, l'arthrite est une des principales causes de douleur et d'incapacité physique au pays. Effectivement, en 2000, l'arthrite et les maladies rhumatismales touchaient près de 4 millions de Canadiens âgés de 15 ans ou plus, soit environ une personne sur 6. D'ici 2026, les prévisions annoncent que 6 millions de personnes de plus de 15 ans seront alors atteintes de cette affliction, soit 20% de la population. L'arthrite se place respectivement pour les femmes et les hommes aux 2ème et 3ème rangs des problèmes de santé chroniques les plus fréquents, juste après les allergies et au même niveau que les maux de dos.

Cette affliction atteint les articulations et se traduit par des douleurs, un gonflement et une raideur de ces dernières. Cela engendre également des incapacités et une gêne dans l'accomplissement des tâches quotidiennes, restreint les activités et provoque des invalidités qui ont un prix énorme pour la société. En 1998, le fardeau économique de l'arthrite sur la société canadienne se chiffrait à 4.4 milliards de dollars (Santé Canada, 2003). La majorité de ces coûts (76%) sont indirects et liés à des dépenses inhérentes à la perte de productivité, en raison d'incapacités de courtes et longues durées. Conscient de ces coûts et de son impact sur la qualité de vie des Canadiens, les efforts de recherche afin de lutter contre l'arthrite se sont intensifiés ces dernières années. La société d'arthrite qui œuvre au Canada depuis 1936 a permis

d'élaborer des plans stratégiques, de financer de nombreuses recherches et a notamment mis sur pied le Réseau canadien de l'arthrite (www.arthritisnetwork.ca) en 1997. Cet organisme a permis de développer un programme de recherche concerté et pluridisciplinaire pour lutter contre cette maladie.

La forme d'arthrite la plus courante (1 Canadien sur 10), l'arthrose, est caractérisée par une dégradation du cartilage, notamment dans les hanches, les genoux, les mains et la colonne vertébrale. Les causes de son apparition ne sont pas connues, mais certains facteurs favorisent son incidence comme l'obésité, des blessures antérieures, une sollicitation répétée de certaines articulations et bien sûr, l'âge. Il n'y a aucun moyen de guérir l'arthrite. Les traitements non chirurgicaux actuels (analgésiques, anti-inflammatoires, exercices, physiothérapie…) permettent d'atténuer la douleur et d'améliorer la mobilité, mais n'aboutissent pas à la guérison. Une approche chirurgicale, prescrite en dernier recourt, est aussi envisageable et consiste à remplacer l'articulation atteinte par une prothèse. Cependant, de par leur courte durée de vie (10-15 ans), ces prothèses sont à proscrire chez les patients trop jeunes.

Ainsi, non seulement les causes de l'arthrite sont jusqu'à ce jour inconnues mais en plus les thérapies existantes ne permettent pas la guérison, seulement un allègement transitoire des symptômes. Le cartilage est le tissu affecté par cette maladie, il se situe à l'extrémité des os dans les articulations et permet un mouvement de ces dernières sans friction. Il ne fait que quelques millimètres d'épaisseur chez l'adulte et a la particularité d'être dépourvu de nerfs et de vaisseaux sanguins ou lymphatiques. Par ailleurs, il est composé d'une matrice protéique dense et d'un seul type cellulaire, les chondrocytes.

Ces derniers sont responsables de la synthèse, de l'homéostasie, mais aussi de la dégradation du tissu. Comme ces cellules sont emprisonnées dans une matrice cartilagineuse, elles présentent une capacité de prolifération et de migration très réduite. Cette particularité, ainsi que le caractère avasculaire du cartilage, ont pour conséquence une capacité de guérison spontanée très faible. Cela reste cependant possible pour de petites lésions, chez des individus en développement. Cependant, de manière générale les lésions cartilagineuses, qu'elles soient d'origine traumatique ou dues à une dégradation globalisée du tissu, ne se réparent pas. Au contraire, la dégradation s'accentue sous l'effet des chondrocytes dont le métabolisme se dérégule lors d'une tentative de réparation du tissu.

Etant donné leur responsabilité dans le développement de l'arthrose et les causes encore inconnues de cette maladie, comprendre la biologie des chondrocytes est un enjeu majeur. Pour y parvenir, la culture de ces cellules en laboratoire est nécessaire. Néanmoins, pour être à même d'utiliser les résultats obtenus *in vitro* et les transposer aux phénomènes existant *in vivo*, il est nécessaire de développer des méthodes de culture préservant le plus possible le caractère chondrocytique de ces cellules et ceci dans des conditions similaires à l'environnement physiologique d'où elles proviennent. Dans ce cas, en réponse à un même stimulus, les comportements cellulaires devraient être plus fidèles à ceux qui seraient observés en conditions *in vivo*.

La culture de chondrocytes est également exploitée dans un but thérapeutique pour la transplantation autologue de cellules (ACI pour autologous chondrocyte implantation). Cette technique est basée sur la capacité des chondrocytes à synthétiser

une matrice cartilagineuse *in situ* qui viendrait remplir la lésion et donc réparer la surface articulaire. L'ACI comprend plusieurs étapes dont la première est l'isolation de chondrocytes à partir d'une biopsie de cartilage prélevée sur le patient lui-même. Ces cellules autologues sont ensuite amplifiées *in vitro,* puis, lorsque leur nombre est suffisant, elles sont réimplantées directement dans la lésion à réparer. Cependant, lors de cette étape d'amplification, les chondrocytes se transforment et perdent leur capacité à synthétiser une matrice cartilagineuse présentant les mêmes propriétés que le cartilage natif. Effectivement, le tissu de réparation observé post-opération est de type fibrocartilagineux et n'a pas d'aussi bonnes propriétés mécaniques que le cartilage environnant.

Que ce soit pour approfondir la compréhension de la biologie du cartilage ou dans un but thérapeutique, la culture de chondrocyte s'avère donc indispensable. Dans ces deux cas, le maintien du phénotype en culture est un enjeu majeur. C'est un paramètre clef du succès de la technique de l'ACI et c'est aussi nécessaire pour l'obtention d'un bon modèle *in vitro* permettant d'étudier ces cellules dans leur état différencié.

La problématique abordée dans cette thèse est donc celle de la culture de chondrocytes. Il existe plusieurs techniques de culture pour ces cellules, chacune présentant leurs avantages et inconvénients, mais aucune d'entre elles n'est réellement satisfaisante. La méthode la plus répandue, par ailleurs utilisée dans le cadre de l'ACI, est la culture en monocouche. Dans ce cas, les cellules s'étalent sur une surface bidimensionnelle et prolifèrent rapidement. Malheureusement, cette prolifération

s'accompagne d'un changement de phénotype qui se traduit par une perte du caractère chondrocytique des cellules. Elles deviennent fibroblastiques et leur capacité à synthétiser une matrice protéique cartilagineuse décroît. La principale raison de cette perte de phénotype semble être le caractère bidimensionnel de ce type de culture qui est distinct de l'environnement tridimensionnel des chondrocytes *in situ*.

Les chondrocytes peuvent également être ensemencés sur un support et être ainsi cultivés dans un environnement tridimensionnel. Dans un tel cas, le phénotype est majoritairement maintenu et les cellules synthétisent une matrice extracellulaire (MEC) de type cartilage qui s'accumule dans le support. Ces constructions cartilagineuses sont pertinentes pour l'étude de la physiologie des chondrocytes et la synthèse de MEC. Les cellules peuvent être ensemencées sur le support soit directement après leur extraction du cartilage, soit après une étape d'amplification en monocouche. Dans ce dernier cas, et c'est un atout majeur de la culture tridimensionnelle, la perte de phénotype est réversible. Cette capacité a par ailleurs été exploitée afin d'améliorer la technique d'ACI : les cellules préalablement amplifiées sont inoculées sur un support polymérique avant d'être réimplantées dans le patient. Le maintien ou le recouvrement du phénotype n'est cependant jamais total et la croissance cellulaire est très faible. D'autre part, un désavantage majeur consiste en un transfert de matière limité des nutriments dans les constructions cellules/support. Les matrices cartilagineuses résultantes sont alors de moindre qualité.

Les travaux présentés dans cette thèse se sont intéressés à un troisième mode de culture, apparaissant comme le plus prometteur : la culture en suspension.

Effectivement, il permet de conserver l'état différencié des chondrocytes et semble aussi favoriser la synthèse d'un tissu similaire au cartilage. Par ailleurs, dans ce cas, les cellules n'adhèrent pas à une surface en plastique ou à un support polymérique non physiologique, mais avec leur propre MEC. Cependant, ce mode de culture est peu exploité, mal caractérisé et présente certains désavantages : malgré un environnement 3D les cellules finissent par se dédifférencier et la taille excessive atteinte par les agrégats en suspension peut engendrer une limitation du transfert des nutriments au centre de ceux-ci. Les travaux présentés dans cette thèse ont pour objectif de remédier à ces inconvénients.

Cette thèse est organisée en sept chapitres. Après cette introduction, une présentation détaillée de la structure du cartilage, des techniques de réparation de ce dernier et des systèmes de culture de chondrocytes *in vitro*, incluant les bioréacteurs, constitue le chapitre 1. Le chapitre 2 décrit les hypothèses et l'organisation générale du document. Il est suivi des chapitres 3 à 6 qui présentent ensuite les articles publiés ou soumis dans le cadre de cette thèse. Le premier article décrit des travaux portant sur l'optimisation du milieu culture pour la culture de chondrocytes en suspension et en monocouche. Les deux articles suivants traitent du développement du système de culture agité en spinner pour la culture de chondrocytes en suspension sous forme d'agrégats et de l'effet d'un des additifs du milieu, le Pluronic F-68. Enfin, le dernier chapitre porte sur l'élucidation des mécanismes d'agrégation en suspension et sur la structure biochimique des agrégats. Finalement les chapitres 7 et 8 présentent une discussion et une conclusion générale.

CHAPITRE 1. REVUE DE LA LITTÉRATURE

1.1 Le cartilage

1.1.1 La structure et fonction du cartilage

Cette partie a été écrite selon les informations présentées dans les revues suivantes : Buckwalter et Mankin, 1997; Caplan, 1984; Huber et al., 2000.

Le cartilage articulaire (ou cartilage hyalin) est un tissu qui recouvre l'extrémité des os dans les articulations. Son rôle est de distribuer les charges dans ces dernières et d'assurer un mouvement articulaire presque sans friction. C'est un tissu qui n'est ni vascularisé, ni innervé et qui est également dépourvu de vaisseaux lymphatiques. Chez les jeunes adultes, le cartilage présente un aspect lisse, blanc et luisant, et ne fait que quelques millimètres d'épaisseur (Figure 1-1 A).

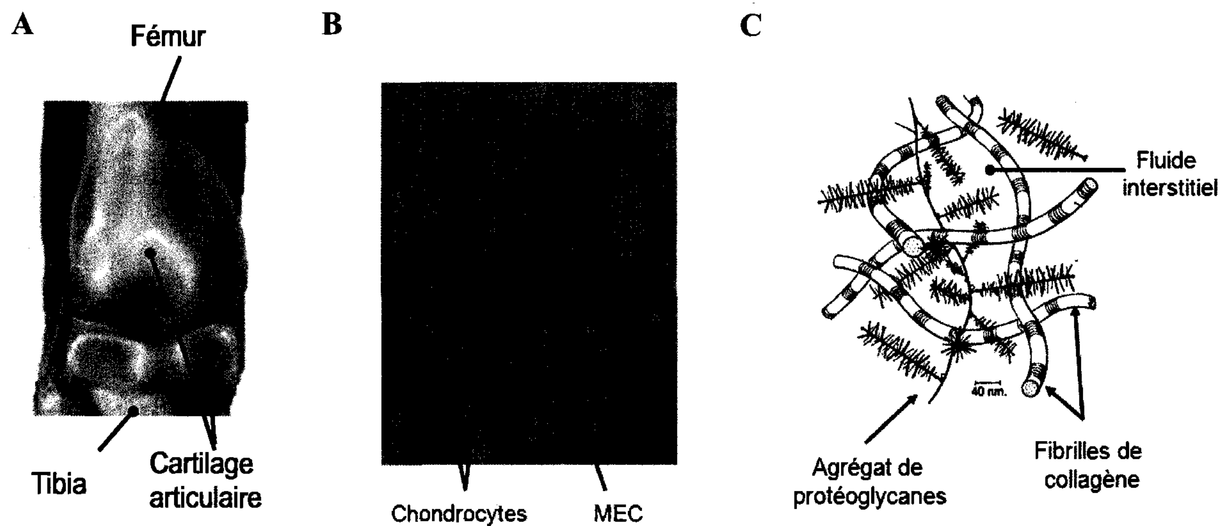

Figure 1-1 : Le cartilage. A. Cartilage à la surface des os longs de la jambe. **B.** Coupe histologique de cartilage colorée au bleu de toluidine, modifié de Hunziker et al., 2007. **C.** Schéma illustrant la composition de la MEC.

Le cartilage est composé d'un unique type cellulaire, les chondrocytes, qui ne représentent que 1% du volume du cartilage, et qui sont entourés d'une matrice extracellulaire (MEC, Figure 1-1 B). La MEC est constituée principalement d'eau, de fibres de collagènes et de gros agrégats de protéoglycanes (Figure 1-1 C). D'autres protéines comme les matrilines, les thrombospondines ou la fibronectine participent aussi à la structure de la MEC, mais s'y retrouvent en plus petites quantités (Neame et al., 1999, voir Tableau 1-1).

Tableau 1-1 : Composition du cartilage. Pourcentages tirés de Buckwalter et Mankin, 1997 et de Eyre et al., 2006. * en pourcentage du collagène total.

Composante	Proportion
Eau	80% du poids humide
Matrice	20% du poids humide
Collagène	60% du poids sec
Collagène II	75% (fœtal), >90% (adulte)*
Collagène IX	10% (fœtal), 1% adulte*
Collagène XI	10% (fœtal), 3% adulte*
Collagène VI	<1%*
Protéoglycanes	25-35% du poids sec
Autres	15-20% du poids sec

Principales composantes protéiques du cartilage

Les collagènes représentent 60% du poids sec du cartilage et en sont donc la principale composante. Les collagènes de type II, IX et XI s'associent pour former les fibrilles de collagène (Figure 1-2 A), dans lesquelles plusieurs molécules de collagène type II organisées en triple hélices s'associent autour de molécules de collagène type XI (Bruckner et van der Rest, 1994). Ces dernières auraient pour rôle la régulation du diamètre des fibrilles de collagène et sont enfouies à l'intérieur de celles-ci. Le collagène type IX, quant à lui, se retrouve à la surface des fibrilles et contribue à

l'organisation et à la stabilisation d'un réseau fibrillaire fonctionnel en permettant la formation de liaisons transversales entre les fibrilles (Eyre, 2002). La triple hélice de collagène type II, aussi appelée tropocollagène, est la forme la plus simple de collagène extracellulaire et mesure environ 1.5 nm de diamètre. Les fibrilles, quant à elles, mesurent entre 10 et 300 nm de diamètre (Mankin et Brandt, 1992) et présentent un motif strié avec une période de 67 nm reflétant l'emboitement en quinconce des tropocollagènes dans la fibrille (Figure 1-2 C). L'ensemble de ces fibrilles forme un maillage dense qui contribue à la rigidité du cartilage et dans lequel les autres constituants du cartilage s'insèrent.

D'autres types de collagènes sont également présents dans ce tissu, tel que les collagènes type VI et X. Le collagène type VI, non fibrillaire, est retrouvé principalement dans la matrice péricellulaire (i.e. jouxtant directement les cellules) et jouerait un rôle dans l'attachement des cellules à la matrice (Marcelino et McDevitt, 1995). Enfin, le collagène type X est également présent, mais seulement dans la zone calcifiée du cartilage (partie du cartilage la plus proche de l'os).

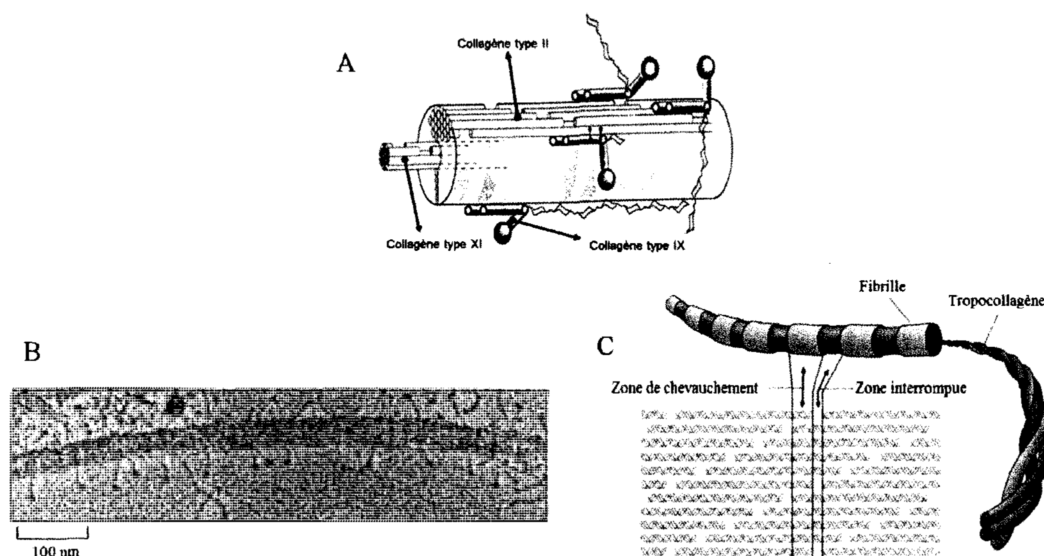

Figure 1-2 : Les fibrilles de collagène. A. Schéma détaillant la composition d'une fibrille de collagène de type II. De Bruckner et van der Rest, 1994. **B.** Micrographe électronique d'une fibrille de collagène II gainée de collagène IX. **C.** Schéma illustrant l'arrangement des tropocollagènes menant au motif strié caractérisant les fibres de collagène.

L'autre composante majeure de la MEC est les protéoglycanes. Les agrégats de protéoglycanes sont des macromolécules constituées de trois parties principales (Figure 1-3) : L'acide hyaluronique, l'agrécane et la protéine de liaison (ou link protein LP). L'agrécane est composé d'une chaîne protéique centrale, ramifiée de plusieurs chaînes de glycosaminoglycanes (GAG) qui sont majoritairement des chaînes de chondroïtine sulfate ou des chaînes de kératane sulfate, moins abondantes. Ces molécules d'agrécanes s'attachent à l'acide hyaluronique avec l'aide de la protéine de liaison, formant ainsi les agrégats de protéoglycanes. Ces derniers sont insérés dans le réseau

de fibrilles de collagène et confèrent au cartilage ses propriétés osmotiques et sa résistance en compression (Roughley, 2006). Une unité de protéoglycane mesure environ 180 à 210 nm de longueur (Mankin et Brandt, 1992).

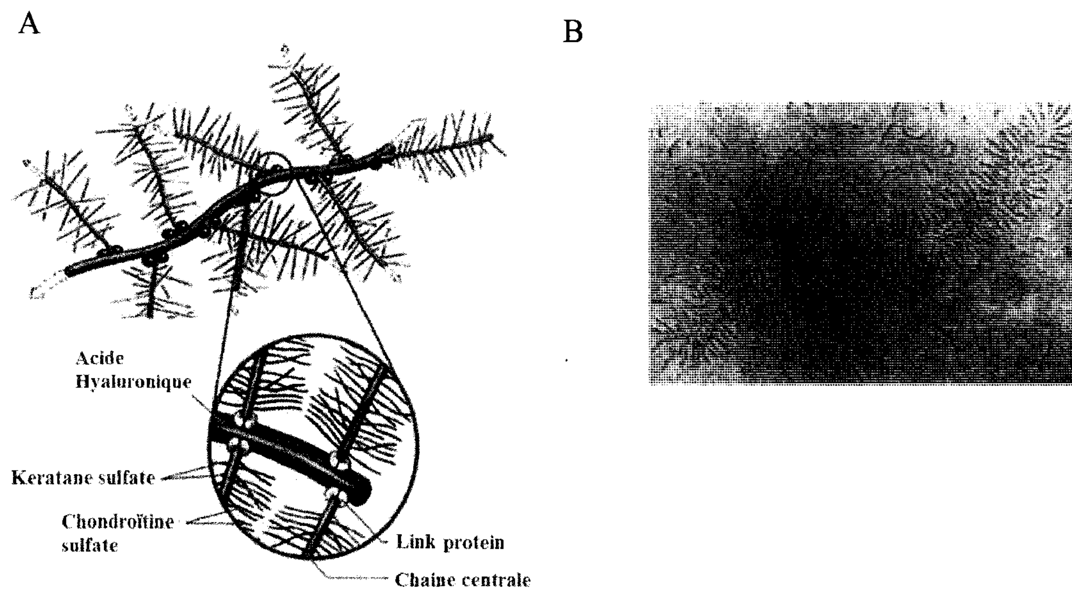

Figure 1-3 : Les protéoglycanes. A. Schéma illustrant la structure d'un agrégat de protéoglycanes, modifié de Schulz et Bader, 2007. **B.** Micrographe électronique d'un agrégat de protéoglycanes de cartilage articulaire bovin.

Les anisotropies du cartilage

La structure et la composition du cartilage articulaire varient selon sa profondeur. Ces variations incluent la forme et le volume des cellules, le diamètre et l'orientation des fibrilles de collagène, ainsi que la concentration en protéoglycanes. Selon ces paramètres, le cartilage peut être divisé en quatre zones distinctes : la zone superficielle

(I), la zone de transition (II), la zone profonde (III) et la zone calcifiée (IV) comme illustré à la figure 1-4.

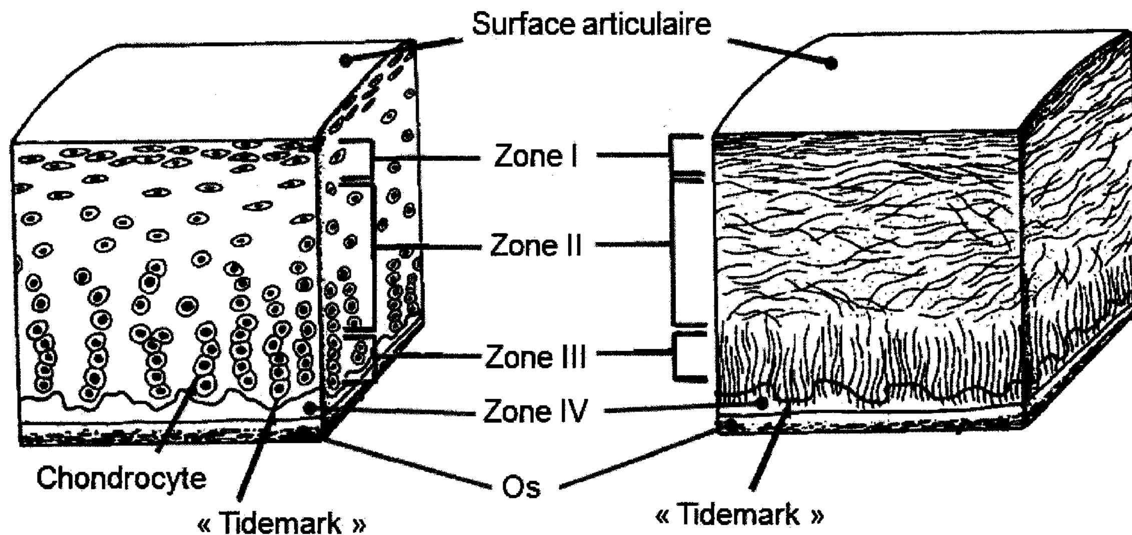

Figure 1-4 : Les anisotropies du cartilage. Image modifiée de Mow et al., 1989.

Des variations structurelles sont également observées dépendamment de la proximité de la MEC avec les chondrocytes. Directement autour de ces cellules se trouve la matrice péricellulaire, elle-même adjacente à la matrice territoriale. Plus éloignée, la matrice interterritoriale représente la majeure partie du volume du cartilage. La matrice péricellulaire est une fine couche adjacente à la membrane cellulaire qui entoure complètement le chondrocyte. Elle est enrichie en acide hyaluronique, en protéoglycanes sulfatés et en une variété de glycoprotéines matricielles comme la protéine de liaison, la fibronectine et la laminine. C'est aussi dans cette région que les collagènes type VI et IX sont concentrés. L'unité composée

d'un chondrocyte entouré de cette matrice péricellulaire est appelée un chondron (Poole, 1997). Morphologiquement, le chondron est caractérisé par un espace lacunaire (la matrice péricellulaire) dans lequel viennent se loger les chondrocytes. Cet espace est entouré d'une capsule composée de fines fibrilles qui semblent tisser une enceinte compacte autour de chaque cellule et séparer physiquement le chondrocyte et sa matrice péricellulaire de la matrice territoriale qui les entoure (Poole et al., 1987, Figure 1-5).

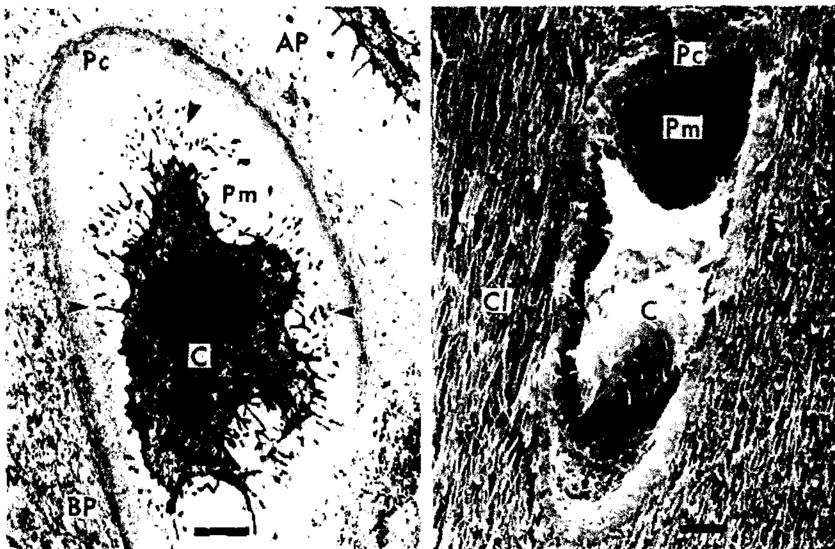

Figure 1-5 : Le chondron. Microscopie électronique à transmission (à gauche) ou à balayage (à droite) de chondrocytes dans le cartilage. Pc= Capsule pericellulaire, Pm= matrice péricellulaire, C=chondrocyte, Cl=fibres de collagènes. De Poole et al., 1987. Echelle : 1µm.

Les chondrocytes

Les cellules responsables de la synthèse et du maintien du cartilage sont les chondrocytes. Ils ne représentent qu'un faible volume du cartilage, mais leur rôle est prépondérant, puisque non seulement ils assurent la synthèse des molécules composants le cartilage, mais aussi l'organisation de ces molécules afin d'obtenir un tissu fonctionnel. Ces cellules prolifèrent rapidement et synthétisent de grandes quantités de matrice pendant la formation et la croissance d'un individu. Puis, avec la maturation du squelette, la division cellulaire diminue, ainsi que l'activité métabolique. Une fois la croissance terminée, les chondrocytes ne se divisent plus, mais contribuent alors au maintien de l'intégrité structurelle et fonctionnelle de la MEC par une balance entre activités anaboliques et cataboliques, afin de répondre à des altérations dans la composition macromoléculaire et l'organisation de la matrice. Ce processus peut être influencé par divers paramètres comme la concentration en certains facteurs de croissances ou cytokines, des charges mécaniques anormales s'appliquant sur l'articulation, le vieillissement du tissu ainsi que les blessures et chocs pouvant altérer la surface articulaire.

1.1.2 Les pathologies associées et les techniques de réparation du cartilage

Pathologies associées au cartilage

Un débalancement des activités synthétiques et de dégradation des chondrocytes couplé avec une synthèse et une incorporation défectueuses des nouvelles molécules dans la MEC peuvent entraîner la perte progressive de la structure normale du cartilage,

ce qui caractérise le syndrome clinique de l'arthrite. Les symptômes qui y sont associés sont une douleur de l'articulation, un mouvement réduit, des crépitements au mouvement, l'effusion et la déformation des articulations. Cette maladie se développe principalement en l'absence de cause connue, bien que l'âge soit un des facteurs favorisant son apparition. Dans le cas de l'arthrose, la maladie est tout d'abord caractérisée par une fibrillation et une dégradation de la surface articulaire qui se propage peu à peu en profondeur, avec pour résultat un amincissement du cartilage. Les mécanismes responsables de cette perte progressive sont mal compris, mais les chondrocytes y jouent un rôle prépondérant. Par exemple, ils peuvent synthétiser un grand nombre de protéases qui, ensemble, ont la capacité de dégrader la MEC et sont donc des acteurs clés de la dégradation du tissu. Ils sont aussi responsables de la progression de la maladie en produisant des niveaux accrus de cytokines inflammatoires telles que IL-1β et TNF-α. Ces cytokines, en retour, induisent chez ces mêmes cellules une diminution de la synthèse de protéines matricielles, une augmentation de la quantité de protéases sécrétées, ainsi que l'expression d'autres médiateurs pro-inflammatoires (Krasnokutsky et al., 2007 ; Goldring et Goldring, 2007 ; Goldring, 2000; Martin et Buckwalter, 2001). La réponse des chondrocytes durant le développement de la maladie peut être divisée en trois phases. Lors de la première, le métabolisme des cellules change et la concentration en agrécanes ainsi que le degré d'agrégation des protéoglycanes diminuent et la quantité d'eau augmente, ce qui réduit la rigidité du cartilage. Ensuite, les cellules répondent aux altérations de la matrice par une prolifération active ainsi que par des activités anaboliques et

cataboliques accrues dans une tentative de remodelage du tissu. Cependant, elles ne parviennent pas à le restaurer et le tissu continue à se dégrader (Buckwalter et Mankin, 1998).

D'autre part, des lésions traumatiques peuvent également endommager le cartilage. Elles sont la conséquence de fractures, de forts impacts ou de blessures des ligaments. Ces lésions ne guérissent généralement pas et peuvent progresser vers l'arthrose.

La réparation du cartilage

Cependant, lors de la formation de lésions traumatiques, si l'os et la moelle osseuse sont atteints (lésions osteochondrales), un caillot de fibrine provenant de l'os sous-chondral remplit la lésion et peut servir de base à une réparation localisée. Une fois le caillot formé, des cellules souches mésenchymales provenant de l'os sous-chondral commencent à pénétrer le caillot, qui va se transformer en un tissu vascularisé cicatriciel. Malheureusement, la composition de ce tissu de réparation est différente de celle du cartilage. Il est mécaniquement inférieur et s'intègre mal au cartilage natif et, bien qu'il soit maintenu un certain temps, il finit par dégénérer (Hunziker, 2002; Jackson et al., 2001; Shapiro et al., 1993). Cette réparation naturelle n'est donc pas durable.

De nombreuses techniques chirurgicales ont été développées afin de réparer ou régénérer ce tissu, mais aucune ne permet d'aboutir à une guérison complète. Certaines d'entre elles exploitent le phénomène naturel de réparation décrit ci-dessus et ont pour principe de creuser dans le cartilage jusqu'à atteindre l'os et la moelle osseuse, afin

entraîner un saignement (techniques d'abrasion, perçage, micro-fracture). Une réparation similaire à la réparation naturelle peut alors avoir lieu, mais tout comme cette dernière, la matrice nouvellement synthétisée n'a pas les caractéristiques du cartilage et les bénéfices ne sont que transitoires.

Le problème majeur associé à ces techniques est la rétractation du caillot formé à la suite du saignement et sa faible résidence dans la lésion. La stabilisation de ce caillot pourrait permettre une réparation accrue et mieux contrôlée du cartilage endommagé. Dans ce but, un nouveau biomatériau a été développé et étudié (Buschmann et al., 2007). Ce biomatériau est une solution physiologique de chitosan (un polysccharide naturel) et de glycérol phosphate formant un gel cytocompatible qui gélifie en quelques minutes, adhère au cartilage et à l'os et peut donc être utilisé pour remplir l'espace de la lésion (Hoemann et al., 2005b). Sa combinaison avec du sang autologue fraîchement prélevé permet de former une mixture hybride polymère-sang applicable à la surface des os ou du cartilage. L'utilisation d'un tel caillot stabilisé dans des lésions créées par micro-forage dans des lapins ou des ovins adultes a donné d'excellents résultats puisque l'établissement d'un cartilage de réparation à caractère hyalin, intégré à l'os sous chondral, a été observé (Chevrier et al., 2007; Hoemann et al., 2005a; Hoemann et al., 2007). Des expériences cliniques ont également donné des résultats très prometteurs (Buschmann et al., 2007; Shive et al., 2006).

D'autres techniques consistent à remplir la lésion avec un greffon périchondrial ou periostéal (tissus recouvrant le cartilage et les os respectivement, sauf au niveau des articulations) ou par transplantation ostéochondrale (greffe d'implant os-cartilage,

prélevé sur le patient lui-même ou sur un cadavre). Mais les effets bénéfiques sont temporaires (Hunziker, 2002 pour une revue).

Un autre domaine prometteur est celui du génie tissulaire consistant à reconstituer, *in vitro* ou par une combinaison *in vitro* et *in vivo,* un tissu cartilagineux. Ainsi, la transplantation de chondrocytes autologues (ACI pour Autologous Chondrocytes Implantation, Figure 1-6) a été expérimentée pour la première fois chez les humains par Brittberg et al., 1994. Puisque les chondrocytes sont le type cellulaire produisant le cartilage, le principe sous-tendant cette technique réside dans la capacité de ces cellules à synthétiser une MEC *in vivo* qui remplirait la lésion et reformerait un tissu cartilagineux fonctionnel. Communément, les chondrocytes sont isolés d'une biopsie de cartilage prélevée par arthroscopie dans une zone ne subissant pas de forces mécaniques. Les cellules en sont extraites par digestion enzymatique, puis amplifiées en monocouche. Quand une quantité suffisante de chondrocytes est obtenue, ces derniers sont alors directement réinjectées dans la lésion à traiter et y sont maintenus par un couvercle de périoste, suturé au cartilage environnant. Une réparation est effectivement observée ; cependant, le tissu de réparation ne correspond pas à du cartilage hyalin, mais à du fibrocartilage dont les propriétés mécaniques sont moindres. Les résultats cliniques sont satisfaisants, mais pas supérieurs à la technique de micro-fracture (Knutsen et al., 2004). Le problème majeur associé à cette technique est la perte de phénotype des chondrocytes lors de la prolifération cellulaire *in vitro*. Durant cette étape, les cellules perdent progressivement la capacité de synthétiser des

molécules cartilagineuses et ce sont des cellules à caractère fibroblastique qui sont alors ré-implantées dans le patient.

Lors de ces dernières années, l'ACI a évolué et les cellules sont tout d'abord ensemencées sur un biomatériau avant leur implantation. Ce support permet simultanément de combler la lésion articulaire et de fournir un support pour l'adhésion cellulaire dans un environnement tridimensionnel (3D), ce qui engendre une récupération partielle du phénotype chondrocytique (voir section 1.2). C'est la construction cellule-matériau qui va être implantée dans la lésion du patient. Deux approches sont possibles : soit le transplant est implanté directement après ensemencement des cellules (Cherubino et al., 2003; Marlovits et al., 2005), soit la construction cellules/support est cultivée un certain temps *in vitro*. Une matrice cartilagineuse peut alors se former avant l'implantation du transplant (Marcacci et al., 2007; Pavesio et al., 2003). De telles techniques ont déjà été appliquées chez les humains et les résultats semblent prometteurs. Cependant, un tel traitement n'a pas été comparé aux autres techniques déjà existantes et des études à plus long terme seraient nécessaires pour tester sa réelle efficacité.

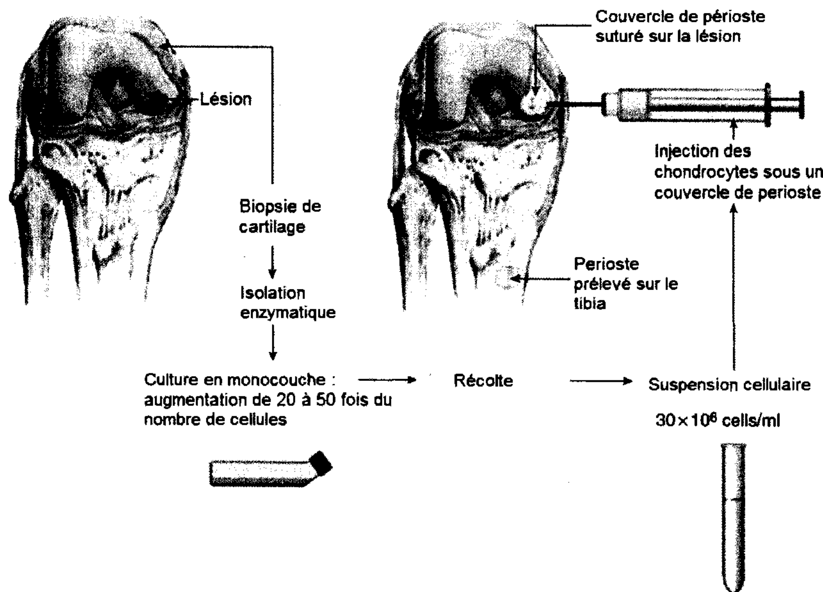

Figure 1-6 : Dessin schématique illustrant la technique de transplantation autologue de chondrocytes. Modifié de Jones et Peterson, 2006.

1.1.3 Les intérêts thérapeutiques de la culture de chondrocytes

Les chondrocytes étant les éléments responsables de la synthèse et de la dégradation du cartilage, une connaissance approfondie de leur biologie implique automatiquement une meilleure compréhension des mécanismes sous-tendant le développement, l'homéostasie ou la dégradation de ce tissu. Dans cette optique, la culture de chondrocytes est une méthode de choix. Cependant, pour être capable d'utiliser les résultats obtenus *in vitro* et les transposer aux phénomènes existant *in vivo*, il est nécessaire de développer des méthodes de culture préservant le plus possible le caractère chondrocytique de ces cellules, et le plus possible de similitudes avec le milieu physiologique d'où elles proviennent. Dans ce cas, les réactions cellulaires vont

être plus fidèles à la réaction qu'elles auraient développées *in vivo* en réponse à un même stimulus.

D'autre part, comme il a été vu précédemment, la culture de chondrocytes est utilisée à des fins de réparation du cartilage. Cependant, un problème persistant dans ce type de technique est associé à la dédifférenciation de ces cellules *in vitro* lors de l'étape de prolifération cellulaire. L'obtention de cellules exprimant un phénotype adéquat, permettant de re-synthétiser un tissu cartilagineux de qualité, est pour l'ACI un enjeu majeur, faisant l'objet d'un grand nombre d'études.

Que ce soit pour comprendre ou traiter les pathologies associées au cartilage, la culture de chondrocytes *in vitro* est donc un outil indispensable. Pour ce faire, les cellules sont extraites du tissu par digestion enzymatique, puis cultivées en laboratoire. Leur culture permet d'étudier une multitude de phénomènes comme la synthèse de matrice, la réponse à certains stress biochimiques ou mécaniques et les causes de la perte de phénotype. C'est un système dynamique puisque les cellules, une fois isolées du cartilage, sont complètement dépourvues de MEC et re-synthétisent activement la matrice en instaurant des interactions avec elle. Les diverses méthodes existantes pour cultiver les chondrocytes sont détaillées dans le chapitre suivant.

L'origine des cellules peut être variée. Une origine humaine est bien sûr favorable puisque les résultats vont être plus facilement transposables à l'homme. Cependant, des cellules d'origine animale peuvent être utilisées, puisque ces dernières sont beaucoup plus faciles d'accès.

1.2 Les systèmes de culture de chondrocytes

Principalement, les chondrocytes sont cultivés en monocouche (Figure 1-7A) et en 3D avec matrice (cellules encapsulées, figure 1-7B) ou sans matrice (culture en suspension, figure 1-7C).

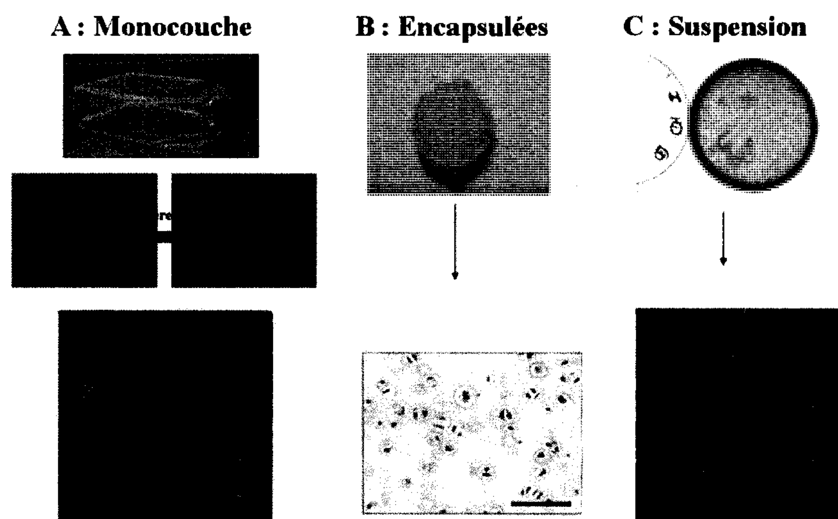

Figure 1-7 : Les différents types de culture de chondrocytes. A. Culture en monocouche. En début de culture, les cellules rondes adhèrent au plastique et s'étalent. Elles présentent alors un cytosquelette riche en fibres de stress (image du bas, colorée pour l'actine en vert et le noyau en bleu). **B.** Culture de cellules encapsulées en agarose. Vue de dessus du cylindre d'agarose et coupe transversale de la construction, colorée avec Fast Green, les cellules paraissent en vert clair. **C.** Culture en suspension. Les chondrocytes forment de gros agrégats dans lesquels les cellules restent rondes et sont séparées les unes des autres (image du bas, colorée pour l'actine en vert et le noyau en bleu). Echelles rouges : 50 µM. Echelle noire : 100µm. Images personnelles non publiées ou modifiées de Gigout et al., 2005 et Tran-Khanh et al., 2005.

1.2.1 La culture en monocouche

La technique de culture la plus répandue pour les chondrocytes est la culture en monocouche. Après extraction du cartilage, les cellules sont ensemencées en boites de Pétri et adhèrent sur la surface de ces dernières. Ce type de culture permet une croissance rapide et est notamment utilisé pour amplifier une population de chondrocytes pour l'ACI. Cependant, en monocouche, un changement graduel de la morphologie cellulaire est observé (Figure 1-7 A). Cette dernière, arrondie en début de culture, devient cuboïde et fibroblastique (Elima et Vuorio, 1989). L'expression du collagène type II, molécule connue pour être abondante dans le cartilage et pour être normalement exprimée par les chondrocytes, disparaît progressivement. Au contraire, celle de collagène de type I (caractéristique d'un phénotype fibroblastique) augmente. Par définition, les cellules se dédifférencient, c'est-à-dire qu'elles perdent progressivement leur phénotype initial. Le ratio collagène II / collagène I est très utilisé pour caractériser cet état de dédifférenciation. Par exemple, en terme d'abondance des ARNm, ce ratio peut varier de 215-480 à 0.1 -1 entre le début et la fin d'une culture, lorsque les cellules sont fortement dédifférenciées (Marlovits et al., 2004). Ce phénomène est aussi marqué par un changement du type de protéoglycanes synthétisés (Demoor-Fossard et al., 1998; Diaz-Romero et al., 2008), du type des collagènes exprimés (voir Tableau 1-2, Lefebvre et al., 1990; Zaucke et al., 2001) et du niveau d'expression de certains facteurs de transcription (Sox 9, Tew et Hardingham, 2006) ou des molécules d'adhésion cellulaire (Diaz-Romero et al., 2008; Takahashi et al., 2007).

Tableau 1-2 : Principaux marqueurs phénotypiques utilisés pour différencier les phénotypes chondrocytiques et fibroblastiques.

	Chondrocyte	Fibroblaste
Morphologie	Ronde	Allongée, cuboïde
Principaux types de collagènes exprimés	II, IX, XI	I, III, V
Principal type de GAG synthétisé	Aggrecan	Versican

Les causes de cette dédifférenciation ne sont pas complètement élucidées et sont probablement multiples. La plus importante serait le caractère bidimensionnel (2D) de la culture, induisant un étalement des chondrocytes sur la surface de culture et la formation de contacts focaux et de fibres de stress d'actine (Figure 1-8). Cette morphologie du cytosquelette est très différente de celle des cellules *in situ* ou cultivées en 3D (Figure 1-8, Tew et Hardingham, 2006), qui sont dépourvues de fibres de stress, restent rondes et dont la distribution du réseau d'actine se compare favorablement avec celle observée dans le cartilage (Langelier et al., 2000). Un certain nombre de travaux appuient cette hypothèse. Ainsi, la perturbation du réseau d'actine chez les chondrocytes cultivés en monocouche par la DHCB (dihydrocytochalasin B), prévient la formation de fibres de stress et entraîne une augmentation de la synthèse de protéoglycanes et de collagène type II (Benya et al., 1988). Ces phénomènes sont

accompagnés d'une baisse de la synthèse de collagène type I chez les chondrocytes cultivés en monocouche, limitant ainsi leur dédifférenciation. D'autres travaux ont également montré que l'inhibition de la formation de fibres de stress entraîne une sur-régulation de Sox9 (Tew et Hardingham, 2006), un facteur de transcription chondrogénique souvent associé avec une forte synthèse de collagène type II par rapport au type I (Li et al., 2004; Tew et Hardingham, 2006).

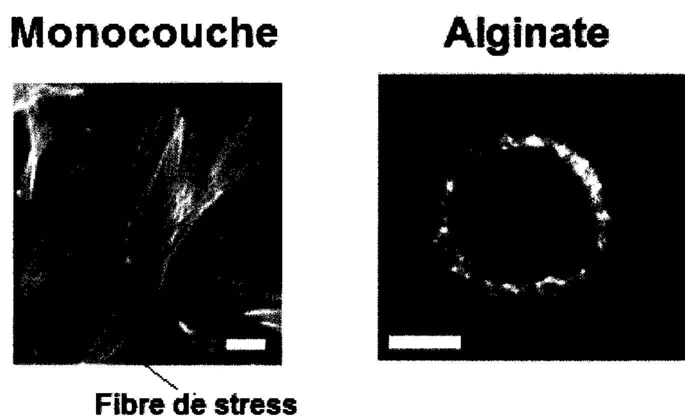

Figure 1-8 : Coloration de l'actine chez des chondrocytes cultivés en monocouche et en 3D. Echelle : 10µm. Modifié de Tew et Hardingham, 2006.

Parmi les autres éléments en partie responsables de la dédifférenciation, la présence de sérum dans le milieu de culture est reconnue pour favoriser la perte du phénotype chondrocytique (Mandl et al., 2004a; Mandl et al., 2002). Effectivement, le sérum contient une multitude de composantes, dont de nombreux facteurs de croissance dans des proportions variables et non déterminées, qui peuvent influencer le phénotype cellulaire. De manière similaire, la tension en oxygène est également un autre paramètre clé. La tension en oxygène couramment utilisée pour cultiver ces cellules est

de 21% (niveau d'oxygène dans l'air ambiant), alors qu'il a été démontré que la dédifférenciation est ralentie quand les chondrocytes sont cultivés à de plus faibles concentrations en oxygène (Domm et al., 2002; Grimshaw et Mason, 2001; Hansen et al., 2001; Murphy et Sambanis, 2001).

De nombreux efforts ont été accomplis afin de limiter le phénomène lors de la prolifération en monocouche. Cela inclut, par exemple, l'optimisation du milieu de culture (essai de différents mélanges de facteurs de croissance, Jakob et al., 2001), de la densité d'inoculation (culture à haute densité versus basse densité, Watt, 1988), l'adhésion des cellules sur une matrice limitant la formation de contacts focaux (Watt et Dudhia, 1988), ou la culture à basse tension d'oxygène (Hansen et al., 2001). Mais si la dédifférenciation peut être ralentie, elle n'est jamais complètement inhibée.

Que ce soit *in vivo* après implantation dans la lésion à traiter ou *in vitro* pour des études physiologiques, ce phénomène est problématique car ces cellules à caractère fibroblastique ne sont pas aptes à réagir ou à synthétiser une matrice cartilagineuse de la même manière que des chondrocytes pleinement différenciés.

1.2.2 La culture en matrice 3D

Une des alternatives à la culture en monocouche est la culture en 3D. Pour cela, les cellules peuvent être ensemencées sur ou dans un support. En 1992, Buschmann et al., 1992 ont montré que des chondrocytes articulaires bovins, encapsulés dans un gel d'agarose maintiennent une morphologie arrondie et synthétisent une matrice mécaniquement fonctionnelle et similaire au cartilage. D'autres études ont démontré

que, dans un environnement tridimensionnel, le phénotype chondrocytique est maintenu (Kimura et al., 1984; Tamponnet et al., 1992) et ceci, jusqu'à 8 mois après le début de la culture (Hauselmann et al., 1994).

Un autre aspect particulièrement intéressant de cette technique est la capacité des chondrocytes dédifférenciés à recouvrer partiellement leur phénotype en 3D (Aulthouse et al., 1989; Bonaventure et al., 1994). Cette particularité a été mise à profit pour améliorer la technique d'ACI. Au lieu d'implanter dans l'articulation des chondrocytes à caractère fibroblastique, les cellules préalablement amplifiées sont par la suite ensemencées dans/sur un support 3D qui sert d'échafaudage pour l'adhésion cellulaire, la redifférenciation vers un phénotype chondrocytique et la synthèse d'une MEC à caractère cartilagineux.

Que les chondrocytes soient ensemencés dans une matrice directement après leur extraction du cartilage ou après une étape de multiplication en monocouche, ils synthétisent une matrice riche en collagènes et en protéoglycanes qui s'accumulent autour des cellules. L'expression des collagènes de type II, VI, IX, et XI est favorisée, le profil des agrécanes synthétisés est similaire à celui du cartilage natif et une expression accrue de Sox 9 est détectée (Bonaventure et al., 1994; Dimicco et al., 2007; Girotto et al., 2003; Hauselmann et al., 1996; Liu et al., 1998; Petit et al., 1996). La présence de COMP (Cartilage Oligomeric Matrix Protein), protéine connue pour catalyser la fibrillogenèse du collagène, a également été observée dans la matrice péricellulaire des chondrocytes cultivés en alginate, indiquant qu'une matrice structurée

(i.e. avec des fibres matures) peut être synthétisée dans ces conditions (Zaucke et al., 2001).

Cependant, lorsque les cellules sont cultivées en 3D juste après isolation une certaine perte de phénotype peut tout de même être observée. D'autre part, si ce sont des chondrocytes dédifférenciés qui sont utilisés, le phénotype n'est jamais complètement récupéré. De nombreux travaux portent également sur l'optimisation, soit du maintien, soit de la récupération du phénotype chondrocytique en 3D. La qualité de la MEC synthétisée et son degré de similitude avec le cartilage natif font aussi l'objet d'un nombre important d'études. Les mêmes paramètres qu'en monocouche sont souvent pris en considération. Par exemple, l'étape de redifférenciation est habituellement réalisée en l'absence de sérum afin de favoriser le phénotype chondrocytique (van Osch et al., 2001); différents cocktails de facteurs de croissance peuvent être également utilisés (Jakob et al., 2001; Liu et al., 2007); par ailleurs, de faibles concentrations en oxygène ainsi que des forces mécaniques peuvent aussi stimuler la synthèse de matrice extra-cellulaire (Domm et al., 2002; Malda et al., 2004b; Wernike et al., 2008). Le choix de la matrice servant de support peut influencer le phénotype, la qualité et la quantité des molécules matricielles exprimées par les chondrocytes (Miot et al., 2005; Mouw et al., 2005).

La culture en 3D permet donc le maintien ou le recouvrement du phénotype chondrocytique et la synthèse d'une matrice cartilagineuse qui s'accumule autour des cellules. Ce type de culture présente un fort intérêt thérapeutique puisqu'il rend possible l'ingénierie de tissus cartilagineux *in vitro*. C'est aussi un outil d'étude

pertinent, notamment pour investiguer la formation et l'organisation des molécules matricielles nouvellement synthétisées. Cependant, dans de tels systèmes, les cellules ne se multiplient pas ou peu et des gradients de nutriments s'instaurent dans la construction entrainant une inhomogénéité du système (Malda et al., 2004a; Martin et Vermette, 2005). Enfin, le phénotype chondrocytique n'est jamais complètement maintenu ou recouvré puisque la synthèse de collagène I persiste (Bonaventure et al., 1994; Girotto et al., 2003; Petit et al., 1996; Zaucke et al., 2001).

1.2.3 La culture en suspension : en « culot » ou en agrégat

Un autre type de culture en 3D permettant également la conservation du phénotype est la culture en suspension en l'absence de support (Castagnola et al., 1988; Izumi et al., 2000; Tacchetti et al., 1989). Pour ce faire, les boites de Pétri sont recouvertes d'une couche d'agarose qui permet de prévenir l'adhésion cellulaire sur la surface de culture. Les chondrocytes s'agrègent alors rapidement (1-2h, Figure 1-7) pour généralement ne former qu'un seul gros agrégat qui peut atteindre plusieurs millimètres de diamètre. Tout comme pour la culture avec support, en agrégats les chondrocytes dédifférenciés se redifférencient; l'expression des ARNm de collagène type I diminue, alors que la synthèse des ARNm des collagènes type II et IX augmente (Castagnola et al., 1988). Le phénotype des cellules fraîchement extraites du cartilage est également maintenu (Izumi et al., 2000; Reginato et al., 1994).

D'autre part, la culture en « culot» (ou « pellet culture») est une variante de la culture en agrégat mais, dans ce cas, le rapprochement cellulaire est forcé par

centrifugation. Les chondrocytes, là encore, soit fraîchement isolés, soit préalablement cultivés en monocouche, sont concentrés par centrifugation. Le culot qui en résulte a donc une forte densité cellulaire qui persiste ensuite tout au long de la culture (Stewart et al., 2000).

Que cela soit en «culot» ou en agrégats, avec des cellules préalablement cultivées en monocouche ou non, ce type de culture permet de reconstituer *in vitro* un tissu partageant de multiples similitudes avec le cartilage natif, aussi bien au niveau de la distribution cellulaire que de la composition et de la structure de la MEC. Ces constructions sont riches en GAG et en collagène type II. L'expression du collagène type IX, de COMP, de Sox 9, de la protéine de liaison et des agrécanes y sont également détectés (Izumi et al., 2000; Kolettas et al., 1995; Tallheden et al., 2004; Zhang et al., 2004). De plus, certaines études structurales ont démontré que la matrice contient des fibrilles de collagène de mêmes diamètre et densité que celles trouvées dans le cartilage (Zhang et al., 2004, Figure 1-9).

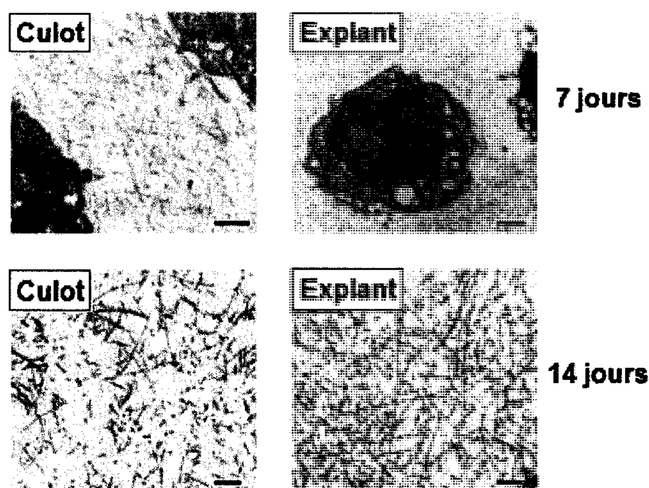

Figure 1-9 : Comparaison de la structure des culots de chondrocytes et d'un explant de cartilage par microscopie électronique, après 7 jours et 14 jours de culture. Echelle : 500nm. Dans les culots les cellules synthétisent une matrice riche en fibrilles de collagène qui est structurellement similaire à la MEC retrouvée dans le cartilage (explant). Modifié de Zhang et al., 2004.

Ce type de culture est particulièrement intéressant car les cellules sont dans un environnement tridimensionnel complexe synthétisé par les cellules elles-mêmes et qui est structurellement et biochimiquement similaire au cartilage. Or, la MEC délivre des signaux biologiques servant de médiateurs à de multiples phénomènes biologiques tels que le développement du cartilage (DeLise et al., 2000; Shakibaei, 1998; Woods et al., 2007), son homéostasie (Knudson et Loeser, 2002; Pulai et al., 2002), sa dégradation (Attur et al., 2000) ou encore la survie cellulaire (Cao et al., 1999). Ces signaux transitent par de nombreuses molécules transmembranaires telles que les intégrines qui

se lient, entre autre, aux collagènes et à la fibronectine, le CD44 qui reconnaît l'acide hyaluronique ou encore l'annexin V et DDR2 (Discoidin Domain Receptor 2) qui sont des récepteurs pour le collagène type II (Knudson et Loeser, 2002; Leitinger et Hohenester, 2007; Loeser, 2002; Reid et al., 2000). Ces molécules, selon la liaison qu'elles établissent avec un ligand, transmettent des messages différents auxquels les cellules réagissent. La prévalence des interactions cellule(s)-cellule(s) ou cellules-matrice se produisant naturellement chez les chondrocytes, contrairement aux interactions cellule-plastique ou cellule-biomatériau dans les autres types de culture, favoriserait donc probablement le maintien du phénotype et la synthèse d'une matrice cartilagineuse. Il est raisonnable de supposer que les chondrocytes dans les agrégats, puisqu'ils sont dans un environnement matriciel proche du cartilage, vont réagir de manière similaire aux chondrocytes *in vivo*. Cela fait de ce type de culture un outil de choix dans le cadre d'études physiologiques.

Une autre approche favorisant également la culture de chondrocytes en 3D dans un environnement cartilagineux réside dans la culture d'interface. Il a récemment été montré que les chondrocytes différenciés, situés à l'interface agarose/milieu de culture, ont l'habileté de proliférer rapidement tout en maintenant une morphologie chondrocytique (ronde) et en synthétisant une MEC cartilagineuse (Tran-Khanh et al., 2008).

Cependant, la synthèse de collagène type I est également observée dans ce type de culture, souvent à la périphérie de l'agrégat/culot (Anderer et Libera, 2002; Battistelli et al., 2005; Kolettas et al., 1995; Tallheden et al., 2004). La prolifération est

habituellement très limitée (Kolettas et al., 1995; Tare et al., 2005) et, tout comme dans le cas de la culture sur support, la taille de agrégats peut provoquer des limitations de transfert de matière, si celle-ci dépasse environ 500µm (Malda et al., 2004a, Sutherland et al., 1986).

1.3 La culture en bioréacteur pour l'ingénierie tissulaire

1.3.1 Les systèmes agités pour la culture 3D

La diffusion des nutriments est un paramètre critique et limitant pour la culture 3D en conditions statiques, puisque le transfert de matière dans la construction n'a lieu que par diffusion. Les conséquences observées de telles limitations sont, par exemple, l'apparition d'un centre nécrotique dans les sphéroïdes de plus de 1mm (Sutherland et al., 1986) ou une production amoindrie de matrice au centre de la construction (Figure 1-11, Martin et al., 1999; Vunjak-Novakovic et al., 2002). Ces limitations diffusionnelles sont considérablement réduites par l'utilisation de systèmes agités, comme les spinners (Figure 1-10 A). Ils permettent notamment un mélange efficace du milieu de culture et promeuvent le transfert de matière à l'interface gaz/liquide mais aussi à la périphérie des constructions, réduisant ainsi la couche limite à la surface de celles-ci. Par exemple, la culture de chondrocytes bovins dans une matrice de PGA (acide polyglycolique) en milieu agité induit une augmentation de la synthèse de GAG. La distribution de la matrice est également plus homogène dans la construction, par rapport aux cultures réalisées en conditions statiques (Figure 1-11, Gooch et al., 2001; Martin et al., 1999). Cependant, une capsule fibreuse est généralement observée à leur

surface. D'autre part, la synthèse totale de GAG est plus importante en conditions agitées, mais une plus faible proportion de ces derniers est retenue dans la construction. Cela est probablement imputable au flux turbulent généré par l'agitation, ce qui entraîne un fort cisaillement à la surface des constructions (Sucosky et al., 2004) et un relargage des GAG périphériques dans le milieu. Cette capsule fibreuse est donc particulièrement dépourvue de GAG, mais elle est aussi riche en collagène type I et est composée de cellules allongées (Bueno et al., 2005).

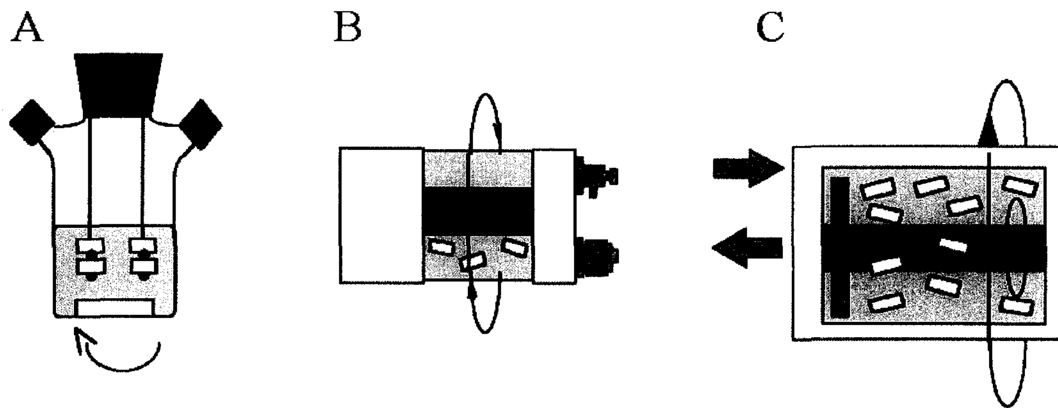

Figure 1-10 : Bioréacteurs pour la culture de chondrocytes. A. Spinner agité avec les constructions fixes, **B.** Enceinte rotative et **C.** Enceinte rotative perfusée avec des constructions en suspension dans le milieu. Modifié de Freed, 2000.

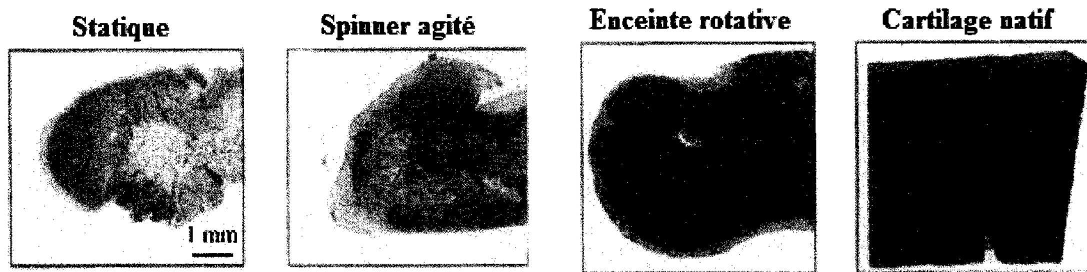

Figure 1-11 : Apparence histologique après coloration à la Safranine-O/Fast green de constructions chondrocytes/PGA, cultivées pendant six semaines en conditions statiques, en spinner ou en enceinte rotative. Une section transversale de cartilage est également présentée à titre comparatif. Les GAG apparaissent en rouge. Modifié de Vunjak-Novakovic et al., 1999.

Divers designs de réacteurs ont été testés (Figure 1-10), avec toujours pour enjeu l'obtention d'un compromis entre un transfert de matière et un mélange efficace et des forces de cisaillement limitées. Les réacteurs à parois rotatives (rotating-wall vessel, RWV, Vunjak-Novakovic et al., 1999), ou les bioréacteurs perfusant le milieu directement au travers des pores de la construction biomatériau/cellules (Davisson et al., 2002; Pazzano et al., 2000) donnent de meilleurs résultats que les spinners et permettent la synthèse de constructions cartilagineuses de qualité supérieure (Figure 1-11). Cependant, dans le cas du réacteur RWV, une capsule fibreuse est encore visible à la surface de la construction et dans le cas de la perfusion, la rétention de la matrice nouvellement synthétisée dans la construction est fortement défavorisée car entrainée par le flux de liquide (Davisson et al., 2002).

1.3.2 Potentiel d'une culture en suspension en bioréacteur

En ce qui concerne la culture de chondrocytes en suspension sans support, l'optimisation du design et des conditions d'opération pour trouver un compromis entre les forces de cisaillement et un transport efficace des nutriments est moins problématique. Étant donné que les agrégats sont généralement de plus petite taille que les constructions biomatériau/cellules, la diffusion des nutriments à toutes les cellules est facilitée et le cisaillement à la surface des agrégats est minimisé. De plus, un avantage majeur d'un système agité réside dans la possibilité de contrôler la taille des agrégats par le biais de l'agitation (Moreira et al., 1995; Sen et al., 2001; Sen et al., 2002). Dans un spinner agité, le flux est turbulent et caractérisé par la formation de 'eddies' qui sont des tourbillons cisaillant les cellules ou les constructions cellules/biomatériau. La turbulence est caractérisée par un nombre de Reynolds (Re) supérieur à 1000 et qui est donné par l'expression suivante (1-1) :

$$Re = \frac{N_i D_i^2 \rho}{\mu} \qquad (1\text{-}1)$$

Où N_i est la vitesse d'agitation (en s^{-1}), D_i le diamètre de l'agitateur (en m), ρ la densité du fluide à 37°C ($1.03.10^6$ g/m^3) et μ la viscosité (1g/m.s). Avec un spinner de 6.5 cm de diamètre et un agitateur de 4 cm (mesures classiques pour un réacteur de 100 mL), Re = 1648 à 60 rpm (i.e. N=1s^{-1}).

La taille de ces 'eddies' (η) peut alors être estimée selon l'échelle de Kolmogorov (formule 1-2,Papoutsakis, 1991).

$$\eta = (\nu^3/\varepsilon)^{1/4} \qquad (1\text{-}2)$$

avec ν la viscosité cinématique ($=\mu/\rho=1.29.10^{-6}m^2/s$) et ε le niveau d'énergie dissipée par unité de masse de fluide et qui peut être évaluée selon :

$$\varepsilon = \frac{N_P N_i^3 D_i^5}{V}. \qquad (1\text{-}3)$$

Np est un nombre de puissance adimensionnel qui dépend de la géométrie du système. Il est égal à 0.5 dans les réacteurs de type spinner (Cherry et Papoutsakis, 1988). V est le volume utile du réacteur en m^3.

Pour un volume de 100 mL et une vitesse de 60 rpm, les 'eddies' sont évalués à 254 µm. En général, les 'eddies' sont dommageables aux cellules quand ils sont de 2/3 à 1/2 fois le diamètre des agrégats, soit dans ce cas 380-500 µm. Les constructions cellules/biomatériau dépassent cette taille et donc subissent des forces de cisaillement à leur surface, alors que la taille des agrégats peut s'auto-réguler par l'agitation et se maintenir à des valeurs permettant d'éviter ce cisaillement. La culture en suspension en spinners semble donc plus avantageuse que la culture sur support.

La culture de chondrocytes en suspension en enceinte agitée n'est pourtant pas couramment utilisée et les derniers travaux utilisant un tel système remontent aux années 70. Des chondrocytes articulaires de lapin avaient été cultivés en suspension dans un spinner agité, en présence de 10 % de sérum dans un milieu sans calcium. L'expression de diverses molécules matricielles avait été étudiée et les auteurs avaient

démontré qu'en spinner les chondrocyte synthétisent du collagène type II et un profil de GAG comparable à celui du cartilage natif. Par contre, aucune croissance n'avait été observée en 4 jours (Deshmukh et Kline, 1976; Srivastava et al., 1974). Malgré le peu d'intérêt que semble susciter ce type de culture avec les chondrocytes, les spinners sont couramment utilisés avec d'autres types cellulaires, également cultivés sous forme d'agrégats, telles que les hepatocytes (Glicklis et al., 2004), les cellules souches epithéliales mammaires (Youn et al., 2005) ou les cellules souches neuronales (Kallos et al., 1999). Ces systèmes sont considérés comme étant plus simples à manipuler et plus économiques que la culture en monocouche ou sur support.

De multiples raisons laissent envisager que les bioréacteurs (spinners) seraient un outil de choix pour la culture d'agrégats de chondrocytes. Ils permettent i) de contrôler la taille des agrégats ii) de limiter les forces de cisaillement à la surface de la construction, iii) de faciliter le transfert de matière et d'assurer un environnement de culture homogène. Finalement, les réacteurs permettent de suivre en temps réel, de contrôler différents paramètres directement dans le milieu de culture (température, pH, concentration d'oxygène, concentration en nutriments...) et de générer des stimuli physiques physiologiquement appropriés tel que le cisaillement ou la pression hydrostatique, deux types de forces s'exerçant naturellement sur le cartilage pendant le mouvement des articulations (Darling et Athanasiou, 2003; Elder et al., 2006; Smith et al., 2000; Smith et al., 1996).

La culture de chondrocytes en agrégats permet la reconstitution d'un environnement cartilagineux et favorise le phénotype chondrocytique. Le principal

avantage de ce type de culture par rapport à la culture en monocouche ou en 3D sur un support polymérique réside dans l'établissement préférentiel d'interactions cellules-matrice physiologiques. La combinaison spinner-agrégats semble donc prometteuse. Elle permettrait d'obtenir des nodules composés de chondrocytes dans un environnement physiologique et homogène, maintenus dans un système versatile où de nombreux paramètres mécaniques ou chimiques sont aisément contrôlables. Un tel outil semble extrêmement avantageux pour étudier la biologie des chondrocytes et donc du cartilage mais peut aussi être une alternative aux techniques habituelles de génération d'implants cartilagineux dans une optique de réparation du cartilage.

CHAPITRE 2. OBJECTIFS ET ORGANISATION GÉNÉRALE DU DOCUMENT

La culture de chondrocytes présente donc un intérêt prépondérant dans la lutte contre l'arthrite puisqu'elle fait partie intégrante de certaines thérapies et permet d'étudier l'initiation et le développement de cette maladie. Cependant, aucune des techniques de culture n'est complètement adéquate. Effectivement, la culture en monocouche entraîne une dédifférenciation de ces cellules. La culture en 3D sur un support, quant à elle, permet le maintien du phénotype, mais a pour conséquence une faible croissance cellulaire et l'obtention de constructions cellules/support qui ne sont pas homogènes.

Par conséquent, une nouvelle approche permettant de maintenir entièrement le phénotype chondrocytique dans un environnement physiologique et homogène est requise pour mieux comprendre et soigner l'arthrite. Les travaux présentés dans cette thèse sont basés sur l'hypothèse que la culture de chondrocytes en suspension sous forme d'agrégats est la plus apte à remplir les conditions précédemment énoncées. L'objectif était donc d'utiliser ce mode de culture et de l'optimiser afin de pallier les problèmes qui lui sont inhérents. Pour ce faire, différents paramètres ont été pris en considération tels que la composition du milieu et l'environnement physique de culture (cultures statiques versus agitées).

Les travaux présentés dans le premier article de cette thèse, présentés dans le Chapitre 3 et publiés dans Osteoarthritis and Cartilage en 2005, se sont attachés à l'optimisation du milieu de culture. L'objectif en était de favoriser le phénotype chondrocytique, mais aussi de limiter la taille des agrégats se formant en suspension. Effectivement, une dimension excessive de ces derniers peut entraver l'accessibilité des nutriments à l'ensemble des cellules les composant, se traduisant par une faible accumulation de matrice, et une viabilité réduite en leur centre. Les deux paramètres principaux qui ont été testés sont la concentration en calcium et la présence ou non de sérum dans le milieu de culture. D'une part, puisque le calcium est nécessaire à une famille de molécules impliquées dans l'adhésion cellule-cellule, appelées cadhérines, réduire sa concentration pourrait réduire l'agrégation. D'autre part, de précédents travaux démontraient également que de faibles concentrations en calcium semblaient favoriser la synthèse de collagènes articulaires (II et XI, Koyano et al., 1996). Concernant le sérum, ce dernier contient des facteurs d'adhésion, comme la fibronectine, qui peuvent favoriser l'agrégation cellulaire (Tavella et al., 1994). Il a donc été posé comme hypothèse que i) de faibles concentrations en calcium (de 1mM à 15µM) réduiraient l'agrégation et favoriseraient le maintien du phénotype chondrocytique et ii) l'absence de sérum limiterait également l'agrégation.

Malgré les améliorations obtenues avec le milieu de culture optimisé, la taille des agrégats obtenus en culture en suspension restait encore un obstacle à l'obtention d'une culture homogène. Il a donc été décidé d'utiliser un système agité, le spinner, avec pour hypothèse que l'agitation permettrait d'améliorer la distribution des

nutriments et des cellules dans l'ensemble du volume de culture, mais aussi de contrôler la taille des agrégats, comme cela avait déjà été observé lors d'autres travaux (Morreira et al., 1995). Afin d'améliorer encore le système, le milieu de culture précédemment optimisé a été additionné d'un surfactant, le PF-68, connu pour protéger les cellules du cisaillement. Une autre formulation, contenant également du PF-68, mais avec en plus une concentration accrue de facteurs de croissance (20ng/mL au lieu de 2ng/mL de EGF, PDGF-BB, et FGF-2) a été expérimentée. Ces travaux sont présentés Chapitre 4 et ont été soumis dans Tissue Engineering.

L'addition du PF-68 dans la formulation du milieu s'est avérée très favorable, puisque cet additif semble favoriser la croissance. Cependant, certaines études ont soulevé la possibilité que ce polymère soit capable de s'insérer dans la membrane plasmique (Ramirez et Mutharasan, 1990; Zhang et al., 1992) et donc potentiellement d'entrer dans les cellules. Si cela s'avère exact, cela peut poser un problème dans les cas où i) les quantités de polymère entrant dans les cellules entraînent une baisse de sa concentration dans le milieu diminuant possiblement son effet protecteur ii) les cellules sont destinées à être implantées dans un patient, qui va alors recevoir une certaine quantité de PF-68. L'hypothèse d'une incorporation possible du PF-68 dans les cellules a été examinée dans un troisième article accepté dans Biotechnology and Bioengineering en 2008, composant le chapitre 5. L'objectif de cette partie était de vérifier si le PF-68 est un additif adéquat pour la culture de chondrocytes.

Enfin, dans le but de pleinement caractériser le système de culture de chondrocytes sous forme d'agrégats et de valider l'aspect physiologique de ce type de

culture, les interactions cellule-matrice ainsi que la structure et la composition de la MEC ont été étudiées. Nous avons supposé, pour les travaux exposés dans le dernier volet de cette thèse, dans le chapitre 6 et soumis dans Journal of Biological Chemistry, que les chondrocytes dans les agrégats reformaient des interactions cellules-matrice (et non cellule-cellule) et s'entouraient d'un matrice similaire à celle qui entoure les chondrocytes *in situ*.

CHAPITRE 3. ARTICLE - LOW CALCIUM LEVELS IN SERUM-FREE MEDIA MAINTAIN CHONDROCYTE PHENOTYPE IN MONOLAYER CULTURE AND REDUCE CHONDROCYTE AGGREGATION IN SUSPENSION CULTURE

3.1 Abstract

Objective: Extracellular calcium influences chondrocyte differentiation and synthesis of extra cellular matrix. Previously, calcium concentrations ranging from 0.1mM to 2mM have been used *in vitro* and these studies indicated that low calcium concentrations were generally favorable for chondrocyte culture. Our objective was to extend these findings to yet lower calcium concentrations and to comprehensively examine effects on morphology and phenotype in two cultures systems.

Methods: Serum-free media containing 1mM, 50 µM or 15 µM of calcium and a serum-containing medium were used to culture chondrocytes in suspension and in monolayer, at high and low inoculation density.

Results: In monolayer, at low and high density, removing serum and decreasing calcium concentration decreased cell spreading and lowered collagen type I expression whereas collagen type II expression remained stable. In suspension, cells aggregated

for all media tested, however aggregates were smaller and looser in the absence of serum.

Conclusion: The serum-free 50 μM and 1mM calcium media provide good alternatives to classical media for monolayer culture since both growth and chondrocyte phenotype were maintained. In suspension culture, the serum-free 1mM calcium medium also possesses the beneficial properties of limiting aggregate size while maintaining growth and phenotype.

Keywords: chondrocyte, calcium, cell aggregation, chondrocyte phenotype, suspension culture.

3.2 Introduction

In monolayer culture, primary articular chondrocytes dedifferentiate to a fibroblastic phenotype expressing collagen type I in preference to collagen type II[1, 2]. In contrast, culturing chondrocytes in 3 dimensional (3D) gels effectively maintains the chondrocyte phenotype, although at the expense of lowering cell division[3-6]. These latter phenotypically stable systems are, however, spatially inhomogeneous since cells are exposed to different microenvironments depending on their position within these 3D systems. Chondrocytes have also been cultured in suspension[7, 8] where chondroycte phenotype was promoted, however large cell aggregates formed quickly, also leading to the loss of culture homogeneity. Thus, the challenge remains to develop

homogeneous culture conditions for primary chondrocytes which allow growth, phenotypic stability and minimize cell aggregation.

Extracellular calcium has strong potentiating effects on cell adhesion and cell aggregation for diverse cell types *in vitro*. In monolayer culture, low calcium content reduces cell spreading in muscle[9], epithelial[10] and endothelial cells[11]. In suspension culture, lowering calcium to 100 μM reduces aggregation of HEK 293 cells[12]. Similarly, calcium removal by chelation with 5mM ethylene glycol-bis(β-aminoethylether)-N, N, N',N'-tetraacetic acid (EGTA) inhibited the condensation of limb bud cells, when calcium-dependent aggregation processes were active[13].

Extracellular calcium regulates matrix synthesis of chondrocytic cells. RNA levels for aggrecan and type II collagen in a chondrocyte cell line decreased with increasing initial medium Ca^{2+} concentration (ID_{50}~2mM for aggrecan and 4.1mM for type II collagen)[14]. Changes in calcium concentration in the range of 1-4 mM are also sensed by Ca^{2+} receptors (CaR), that belong to the G protein-coupled receptor superfamily[15]. These CaR are involved in the influence of extracellular calcium on differentiation where high calcium increased expression of osteopontin, osteonectin and osteocalcin in chondrogenic cells[16], and collagen type X in chondrocytes[17]. On the contrary, calcium concentrations below 0.5 mM promoted the production of articular collagens, type II and XI[18]. It has also been shown that using a medium without added calcium (but with 10% serum that contains calcium) has prevented rabbit articular chondrocytes from switching to a collagen type I-producing fibroblastic phenotype when grown in suspension culture[19, 20]. Finally, elemental analysis by electron

microscopy revealed low extra-cellular calcium levels (1/4 of the cytosolic concentration that ranges from of 0.1-10μM) in the proliferating zone of the cartilage growth plate[21]. Taken together, the above data from the literature strongly suggests that articular chondrocytes exist in a low calcium environment that may be necessary for their physiological function and phenotypic stability.

Although existing data clearly indicates that calcium has a regulatory role on chondrocytes and that low extracellular calcium may be beneficial to chondrocytes, the lowest calcium concentration reported to date in chondrocyte culture was 0.1 mM. We believed that yet lower extracellular calcium concentrations could bear certain advantages for the culture of primary chondrocytes. We specifically hypothesized that low calcium levels in medium would: (1) promote collagen type II expression over collagen type I expression; (2) promote a chondrocytic (round) cell morphology in monolayer culture and (3) reduce cell aggregation in suspension. Since serum contains about 4mM of calcium[22], a cell culture medium with calcium concentrations below 0.1 mM required the use of a serum-free medium (SFM). Therefore a serum-free medium with calcium concentrations of 1mM, 50μM, and 15μM was compared to a standard serum-containing (10%) formulation that had 2 mM calcium. The influence of these different calcium levels, as well as the presence or absence of serum, was investigated using two culture systems: standard monolayer culture as well as suspension culture each at low and high cell-seeding densities.

3.3 Materials and methods

3.3.1 Culture media

All reagents were from Sigma-Aldrich Canada, Oakville, Ontario, Canada, unless indicated. Cells were cultured in either a serum-containing medium (SCM) or a SFM. The SCM was chosen to represent commonly used SCM composed of DMEM low glucose (Life technologies, Burlington, Ontario, Canada) supplemented with 0.4 mM proline, non-essential amino-acids 1X (containing 8.9 mg/L alanine, 15 mg/L asparagine, 13.3 mg/L aspartic acid, 14.5 mg/L glutamic acid, 7.5 mg/L glycine, 11.5 mg/L proline and 10.5 mg/L serine), 22 mM sodium bicarbonate, 12.5 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), penicillin/streptomycin 1X (containing 100 U/mL penicillin and 0.1 mg/mL streptomycin)[23]. Medium pH was adjusted to 7.2 and sterile filtered prior to addition of 10% fetal bovine serum (FBS) and 30 μg/mL ascorbate, the latter added fresh just prior to medium change. The SFM was chosen based on commonly used SFM compositions using a 1/1 (v/v) mix of calcium-free HAM's F12 (US Biological, Swampscott, MO, USA) and calcium-free DMEM low glucose (US Biological, Swampscott, MO, USA) supplemented with 0.4 mM proline, 1.5 mM glutamine, 22mM sodium bicarbonate, non-essential amino acids 1X, 12.5 mM HEPES, penicillin-streptomycin 1X, ITS + (10 μg/mL insulin, 5.5 μg/mL transferrin, 0.05% w/v bovine serum albumin (BSA), 1.7 mM linoleic acid, 0.5 μg/mL sodium selenite), $5x10^{-5}$M β-mercaptoethanol and 10^{-8} M dexamethasone[24]. Medium pH was adjusted to 7.2 and sterile filtered. The following recombinant growth factors

(from R&D Systems, Minneapolis, USA) were added to SFM each at 2ng/mL : epidermal growth factor (in sterile 0.1%BSA in 10mM acetic acid), platelet derived growth factor -BB (in sterile 0.1% BSA in 4mM HCl), fibroblast growth factor-2 (in sterile 0.1% BSA, 1mM DTT in PBS without Mg^{2+} and Ca^{2+})[24]. Ascorbate, 30 µg/mL, was added fresh just prior to changing SFM. To this SFM was added a supplemental amount of $CaCl_2$ corresponding to 1mM, 50µM, 5µM, 1µM, or 0µM. Calcium content in media was then verified by atomic absorption spectrometry with an Analyst 200 from PerkinElmer (Boston, MA, USA) with $CaCO_3$ as a standard (from PerkinElmer). Lanthane chloride (1000ppm) was added to samples before analysis. This analysis revealed that these media contained in fact, 0.95mM, 50.25µM, 18.25µM, 14.5µM and 13.75µM of calcium respectively. Thus calcium levels below 10µM could not be achieved since calcium was present in the additives. Since our results obtained with cultures in 18.25µM, 14.5µM and 13.75µM of calcium were indistinguishable, we only present results obtained with 0.95 mM, 50 µM and 14.5 µM of calcium, indicated by the rounded concentrations of 1 mM, 50 µM and 15 µM.

3.3.2 Cell isolation

Cells were isolated from the femoropatellar groove of a 1-2 month-old calf knee, obtained from a local butcher within 24h of slaughter. Briefly, cartilage was sequentially digested, first for 90 min by protease Type XIV (Sigma-Aldrich Canada, Oakville, Ontario) 56 U/mL at 37°C in DMEM high glucose supplemented with 22mM sodium bicarbonate and 1X penicillin/streptomycin, and then for 3 h by collagenase

CLS2 (Worthington, Freehold, NJ, USA) 752 U/mL at 37°C in DMEM high glucose supplemented with 22 mM sodium bicarbonate, penicillin/streptomycin and 5 % FBS. Released cells were then filtered through a 200 µm mesh (using an autoclaved 200 µm screen mounted on a screen cup, Sigma-Aldrich Canada, Oakville, Ontario), centrifuged (190 g, 10 min at 4C) and filtered again through two serial 20 µm filters using a Swinnex filter holder (Fisher Scientific, Town of Mount-Royal, Quebec, Canada) containing a 20 µm Spectra/Mesh Nylon Macroporous Filter (Spectrum Laboratories, Rancho Dominguez, CA, USA). Cells were then washed three times in SFM without calcium, counted using a hemocytometer and seeded at low or high density in the different culture media and systems described below.

3.3.3 Cell culture

Cells were cultured either in monolayer or in suspension. In monolayer, cells were suspended in 5mL of culture media and seeded in 60 mm Petri dishes (internal diameter 54 mm, area of 23 cm^2). For suspension culture, Petri dishes were previously coated with 2% SeaPlaque low-melting-temperature agarose (Mandel, St. Laurent, Quebec, Canada) in a 1:1 (v/v) mixture of calcium-free HAM's F12 and calcium-free DMEM low glucose or in DMEM low glucose, supplemented with 22mM sodium bicarbonate, depending on which medium was to be subsequently used. In the SCM, and in 15µM, and 1mM calcium-containing SFM, cells were seeded at both low density (10^4 cells/cm^2 hence $2.3x10^5$ cells/dish) and at high density (10^5 cells/ cm^2 hence 2.3 $x10^6$ cells/dish) for both monolayer and suspension cultures. To reduce the number of

conditions to a manageable level, only low density cultures included the 50μM calcium-containing SFM condition, in both monolayer and suspension cultures. Half of the media volume was changed every 2 days. In suspension culture, this was achieved by centrifuging cells (190g, 10min at 4C) and removing 2.5mL of the supernatant followed by addition of 2.5mL of fresh media, that was then resuspended and transferred to the former Petri dish. Each culture condition was done in triplicate and for each result shown, at least one additional preliminary experiment confirmed the general trends of these results, supporting their reproducibility. At day 10, for two of the triplicates, cells were harvested with 1 or 0.5 mL Trizol® (Life technologies, Burlington, Ontario, Canada), depending on cell density, and flash-frozen in liquid nitrogen before storage at -80C. The remaining sample of each triplicate was fixed and permeabilized for immunocytochemistry as described below.

3.3.4 Microscopic observations

Microscopic observation was performed each day with an inverted microscope (Axiovert S100TV, Carl Zeiss Canada, North York, Ontario, Canada), in Kohler transmission mode. Images were acquired with a digital camera and Northern Eclipse Software (Empix Imaging, Mississauga, Ontario, Canada).

3.3.5 Aggregation percentage and aggregate size

To assess cell aggregation in suspension culture, 15 digital photos were taken using random sampling with a 4X objective on days 1, 2, 5, 7 and 9. The fraction of

cells in aggregates (% aggregation) and the number of cells per aggregate (aggregate size) were evaluated following the methods described by Martin et al.[25]. Briefly, images were calibrated with a hemocytometer, then thresholded and objects were analysed. Each object was fit to an ellipse and evaluated for its size and then identified as a single cell or as a cell aggregate. The number of cells in each aggregate (called aggregate size) was then evaluated by comparing the volume to the volume of a single cell. The percentage of cells in aggregates and single cells were then calculated. Aggregation percentage and average aggregate size were calculated as follows:

$$\text{Aggregation (\%)} = \frac{\text{Aggregated cells}}{\text{Total cells}} \times 100 \tag{3-1}$$

$$\text{Average aggregate size} = \frac{\text{Aggregated cells}}{\text{Number of aggregates}} \tag{3-2}$$

Since aggregates rapidly grew with time and became difficult to analyse when their size exceeded the frame of the digital photo, only results from day 1 are presented. As medium was replaced every 2 days by centrifugation, pipetting and centrifugation of aggregates could have disturbed the aggregation phenomena. Centrifugation did not seem to increase aggregation since aggregates were in the bottom 2.5mL (out of 5mL total) of the tube that was retained, but were still in solution and did not form a pellet. Pipetting did slightly disturb agregates, however, all samples were submitted to the same treatment and are comparable.

3.3.6 Cell viability

Cell viability was determined with 0.5 µM calcein AM and 1 µM ethidium homodimer-1 (LIVE/DEAD viability/cytoxicity kit, Molecular Probes, Eugene, OR, USA) in phosphate buffer saline (PBS). Samples were incubated for 30 min at 37ºC after which green (live) or red (dead) cells were visualized with an inverted fluorescence microscope for cells in monolayer or with a confocal microscope for cells in suspension (Axiovert S100TV or Axioplan LSM 510 META, Carl Zeiss Canada, North York, Ontario, Canada).

3.3.7 Cell proliferation

Cell number was estimated at day 10 by DNA quantitation with PicoGreen® (Molecular Probes, Eugene, OR, USA). Aliquots (10µL) of Trizol® extracts were diluted 1:10 in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.5) to obtain a 100µL final volume. 200µL of PicoGreen® reagent (200µL), previously diluted 200 times in TE, was added to each 100µL sample in a 96well microplate immediately prior to reading with a Molecular Devices Gemini II fluorescence plate reader (Sunnyvale, CA, USA) at 480nm excitation and 520 nm emission, with a 515 nm cutoff filter. Results were compared to a standard curve made with known cell numbers, with cells treated in an identical manner as the above samples. Fold doubling was calculated via

$$\text{Fold doubling} = \frac{\ln (X_t/X_0)}{ln2} \quad (3\text{-}3)$$

With X_0= initial cell (DNA) content and X_t= final cell (DNA) content at 10 days.

3.3.8 Glycosaminoglycan release to media

The dimethylmethylene blue (DMMB) assay was used[26] to quantify glycosaminoglycan (GAG) in the culture media. Media of 10 μL or 50 μL were mixed with 40 μL or 10 μL PBE (100 mM Na_2HPO_4, 5 mM EDTA pH= 6.5) respectively. DMMB reagent (200μL of 46 μM DMMB from Polyscience, Warrington, PA, USA, in 40 mM NaCl, 40 mM glycine, pH 3.0) was then added and absorbance at 525nm was read and compared to that of chondroitin sulfate C standards (Sigma-Aldrich Canada, Oakville, Ontario) containing the same amount of PBE and culture medium as the samples.

3.3.9 mRNA isolation, reverse Transcription and RealTime-PCR

Total RNA was isolated in 1 or 0.5 mL Trizol®, following the manufacturer's protocol (Life technologies, Burlington, Ontario, Canada). RNA was then quantified with the Quant-IT® RNA assay kit (Molecular Probes, Eugene, OR, USA) also according to the manufacturer's recommendations. Reverse transcription was performed with 0.05 μg/μL oligodT and 20 U/μL moloney murine leukemia virus reverse transcriptase, in the presence of 500 μM dNTP, 0.625 U/μL RNAse inhibitor and buffer 5X supplied with the enzyme (all reagents from Pharmacia-Amersham, Baie d'Urfée, Quebec, Canada). First 5μL of RNA was denaturated for 15min at 75°C, then the oligodT was added and allowed to anneal to RNA for 3 min on ice. The other

reagents (details above) were then added and the reaction proceeded for 1h at 37°C. Reverse transcriptase was inhibited by heating for 5min at 94°C, and the reverse transcription products diluted 5× in water.

Real-Time polymerase chain reaction (PCR) occurred in the RotorGene 3000 (Corbett Research, Mortlake, Australia) using SybrGreen to quantify cDNAs produced from collagen type I and collagen type II mRNA. Primers (purchased from BioCorp Montreal, Quebec, Canada) for bovine type II collagen (PUBMED accession number X02420) were 5'-GAA CCC AGA ACC AAC ACA ATC C-3' (forward) and 5'-TCT GCC CAG TTC AGG TCT CTT AGA GA-3' (reverse) while those for bovine type I collagen (PUBMED accession number S64596) were 5'-TGG CCC AGA AGA ACT GGT-3' (forward) and 5'-AGG AAG GTC AGC TGG ATG-3' (reverse). The optimized PCR mix consisted of 2 or 4 μL of cDNA, 1X reaction buffer, 200μM dNTP, 200nM of each primer, 2.5mM $MgCl_2$, 0.3X SybrGreen (from 10,000X, Molecular Probes, Eugene, OR, USA) and 0.05 U/μL JumpStart Taq Polymerase (Sigma-Aldrich Canada, Oakville, Ontario). The PCR began with a 5 min denaturation step at 94°C, followed by 7 cycles with a touchdown between 60°C and 53°C (20sec. at 95°C; 20sec. at 60°C with touchdown in 1°C intervals to 53°C; 30sec. at 72°C, 10sec at 82°C) and then 40 cycles of classic PCR (20sec. at 95°C; 20sec. at 57°C; 30sec. at 72°C; 10 sec. at 82 °C). A fluorescence reading was performed for each cycle at 72°C and at 82 °C, where the latter was chosen to eliminate signal from primer-dimers[27]. The run ended by an increase in temperature from 72°C to 99°C to obtain the melting curve. Absolute mRNA abundance was found using PCR kinetics described by

$$Nf = Ni(1+E)^n \tag{3-4}$$

where Nf = final copy number $\propto$ final fluorescence, Ni = initial copy number $\propto$ initial fluorescence, E = Efficiency and n = cycle number. By setting the cycle number equal to the threshold cycle number, $n = C_T$, we find:

$$N_i \propto Theoretical\ initial\ fluorescence = \frac{Threshold\ fluorescence}{(E+1)^{C_t}} \tag{3-5}$$

The *Threshold_fluorescence* was set to 0.01 fluorescence units (FU) and the Efficiency (E) was calculated by the software from Eq. (4) and amplicon specific fluorescence readings at 82°C[27]. We then calculated mRNA abundance relative to total_RNA found for each sample from Quant-IT (in µg/mL) according to:

$$Abundance\ (FU/(\mu g/mL)) = \frac{Theorerical\ initial\ fluorescence}{total\ RNA} \tag{3-6}$$

3.3.10 Immunostaining and confocal laser scanning microscopy

All steps were performed at room temperature unless otherwise mentioned. Cells in monolayer or in suspension were first washed in modified hank's balanced salt solution (mHBSS) (HBSS from Life technologies, Burlington, Ontario, Canada, supplemented with 2 mM $MgCl_2$, 2 mM EGTA, 5 mM MES (2-[N-morpholino]ethanesulfonic acid), 4 mM $NaHCO_3$, pH 6.5), and then fixed/permeabilized in 0.5 % w/v glutaraldehyde and 0.3 % w/v triton X100 for 10 min, treated against autofluorescence with $NaBH_4$ (2.5mg/mL, 2 x 10min on ice) and digested with 200 U/mL chondroitinase ABC and 400 U/mL keratanase (Seikagaku America Inc., East Falmouth, MA, USA) in tris buffered saline-BSA (100mM Tris,

100mM NaCl, 0.01% BSA, pH 7.4) for 16h at 37°C. For monolayers, the above incubations took place directly in the Petri dish while cells in suspension (mostly aggregates) were transferred onto a nylon membrane filter (0.45µm pore size Whatman, Clifton, NJ, USA) mounted in a centrifuge tube and the above solutions were added onto the filter retaining the cells, and then removed by centrifugation (800g, 1min). Samples were then stained for actin with Alexa-488 phalloidin (Molecular Probes, Eugene, OR, USA), diluted 1:1000 in mHBSS for 1h and for the nucleus with 0.5µg/µL Hoechst 33258 (Molecular Probes, Eugene, OR, USA) in mHBSS for 10 min. Each step was followed by three washes of 5 min in mHBSS. Finally, samples were treated against quenching with 11,700 U/mL beef catalase and 133.3 U/mL glucose oxydase (both from Fluka, Buchs, Switzerland) in mHBSS, 30 min and mounted in 16.67% (w/v) Mowiol 4-88 (Fluka, Buchs, Switzerland), 33.3% (v/v) glycerol, 0.75% (w/v) n-propyl gallat (Sigma-Aldrich Canada, Oakville, Ontario) in mHBSS. Confocal-imaging was performed using an Apochromat 40X/NA 1.2 water immersion objective mounted on an Axioplan 2 microscope equipped with an LSM 510 META confocal laser scanning module (all from Carl Zeiss). Alexa 488 was excited with the 488nm line from an argon laser and Hoechst 33258 was illuminated with two–photon excitation wavelength using a mode-locked pulsed Ti-saphire laser (VerdiV10/Mira 900 from Coherent Inc., Santa Clara, CA, USA) operating at 780 nm. Images were recorded using a BP 510/520 IR bandpass filter for Alexa 488 a BP 435-485 IR bandpass filter for Hoechst 33258.

3.3.11 Statistical analysis

Statistical analysis was performed with STATISTICA 6.1 (StatSoft Inc., Tulsa, OK, USA). The effect of calcium and serum were determined by the general linear model (GLM), with culture system (suspension or monolayer) and / or inoculation density (high or low) and / or serum (absence or presence) as categorical predictors and with calcium as a continuous predictor.

3.4 Results

3.4.1 Serum in the medium promotes fibroblastic morphology, phenotype loss and cell aggregation

SCM showed similar cell growth compared to SFM. During a 10-day culture in SCM in monolayer with half of the medium volume changed every 2 days, chondrocytes divided 5.3 times and 2.2 times at low and high density, respectively (Figure 3-1 B and D) while in SFM-1mM Ca^{2+} chondrocytes divided 4.2 and 1.8 times at low and high density, respectively. In suspension the tendency was similar as growth was slightly better in the SCM (Figure 3-1 A and C). Growth differences between monolayer and suspension culture were not statistically different, while growth was significantly higher at low density compared to high density cultures ($p<0.05$).

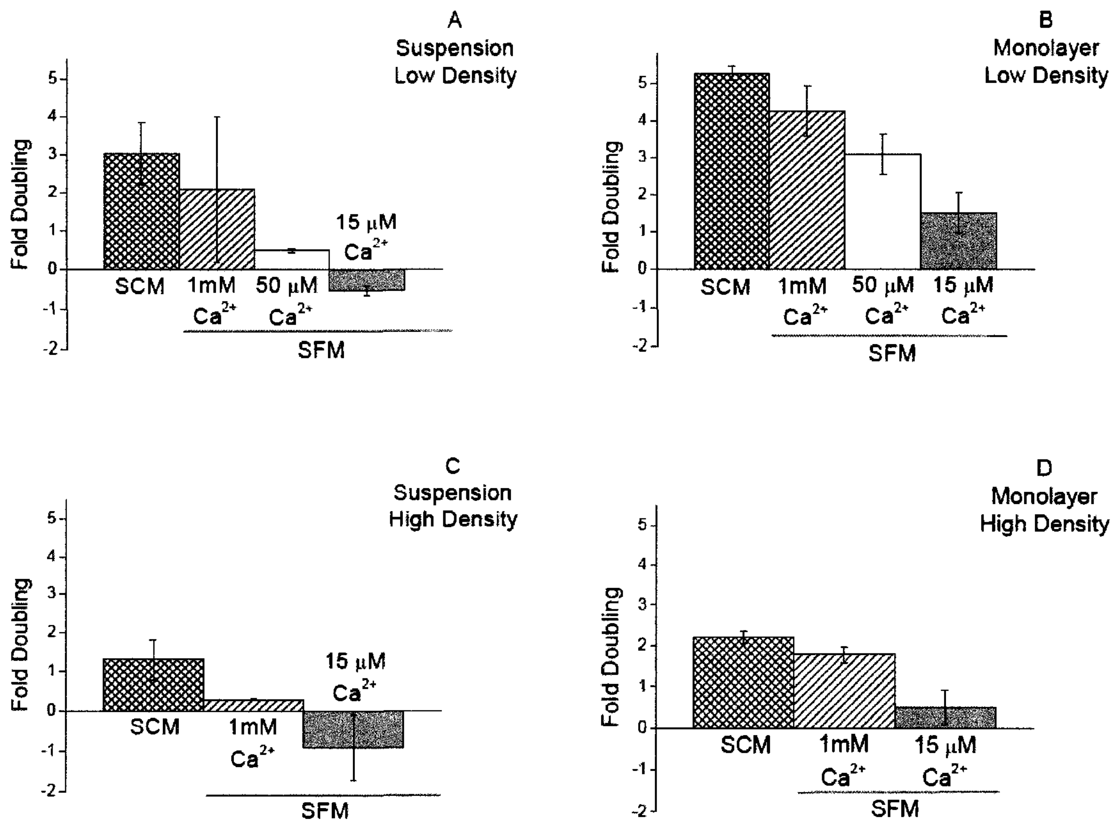

Figure 3-1 : Fold cell doubling after 10 days, determined by comparing inoculation density and cell density after 10 days of culture, by DNA quantitation. Cells were cultured at low density in suspension (**A**) or in monolayer (**B**) and at high density in suspension (**C**) or in monolayer (**D**). Culture media used included an SCM or an SFM supplemented with different calcium contents indicated on the abcissa. Negative results indicate net cell mortality. Results are mean of n=2 with error bars representing duplicates.

SCM promoted a fibroblastic morphology. In the SCM, cells cultured at low density were elongated and spindle-shaped (Figure 3-2 A) with only a few round cells remaining. In the SFM-1mM Ca^{2+}, cells were less elongated, and exhibited a "cobblestone"-morphology (Figure 3-2 B). On the contrary, when cultured at high density, more cells were round, whether serum was present or absent (Figure 3-2 E, F, G). Staining of the actin cytoskeleton revealed that, at low density with or without serum numerous stress fibers appeared (Figure 3-3 A and B) whereas at high density, stress fibers were mainly present with serum (Figure 3-3 E and F).

SCM promoted cell aggregation. In suspension, chondrocytes rapidly aggregated. After 9 days, aggregates contained several thousand cells (Figure 3-4). In the SCM, at low or high density, aggregates were large and dense exhibiting a smooth well-defined contour. On the contrary, aggregates in the SFM-1mM Ca^{2+} were looser with a more granulated contour (Figure 3-4 B, C, D versus Figure 3-4 A). At low density after 9 days in SCM 99.4% of the cells were in aggregates as compared to 93.7% in the SFM-1mM Ca^{2+} (Table 3-1). Average aggregate size was of 462 versus 44 cells, respectively in SCM and SFM-1mM Ca^{2+} at low density (Table 3-1). Analysis of the aggregates by confocal microscopy showed that, in SCM, at low or high density, the cells seemed to be well-separated (Figure 3-5 A and E) with few cells in direct contact. Also cells that were peripherally located in aggregates appeared elongated in SCM (Figure 3-5 A, arrow). In the SFM, on the contrary, cells were tightly packed, showed evidence of cell-cell contacts and were without elongated cells at the periphery (Figure 3-5 B and F).

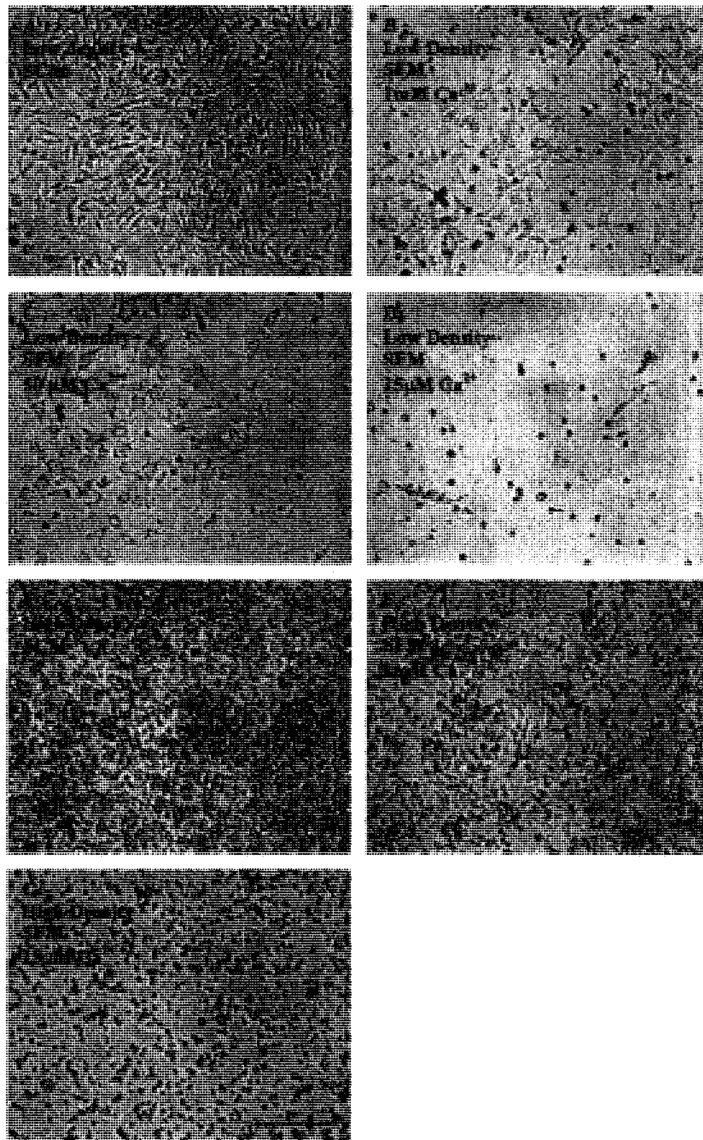

Figure 3-2 : Morphology of chondrocytes in monolayer at day 7. Cells were inoculated at low density (**A, B, C, D**) or high density (**E, F, G**) either in an SCM (**A, E**) or in an SFM containing 1mM (**B, F**), 50μM (**C**), 15μM (**D, G**) of calcium. Plating density, presence of serum and calcium content affect cell morphology. Scale bar = 250μm.

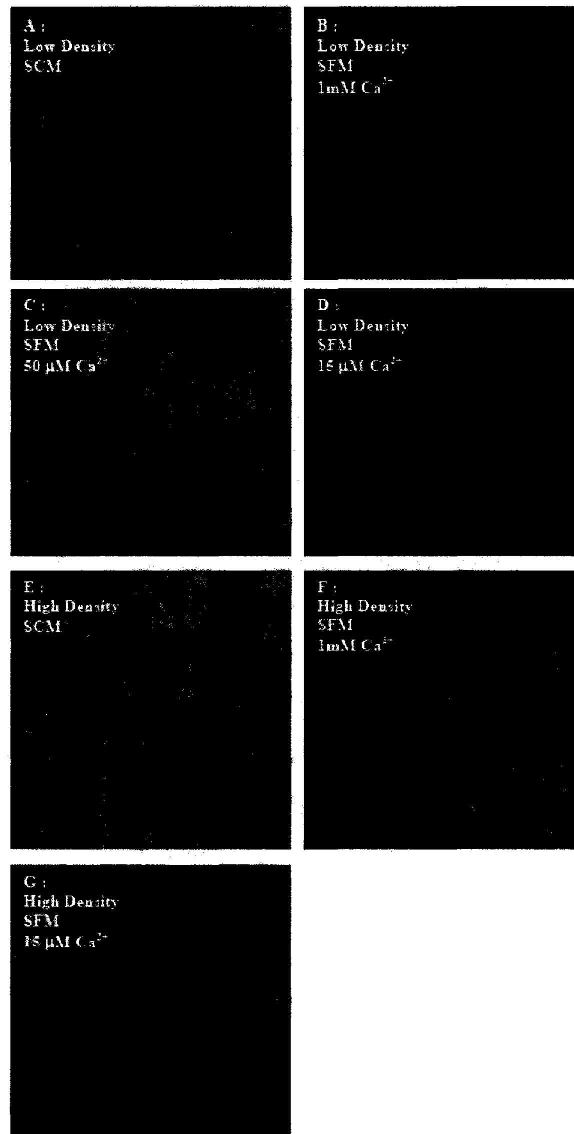

Figure 3-3 : Chondrocytes in monolayer inoculated at low or high density were stained for actin (green) and the nucleus (blue) at day 10. With SCM, SFM-1 mM and 50µM Ca^{2+}, most cells displayed stress fibers. In contrast, at 15 µM Ca^{2+}, cells presented far fewer stress fibers. Scale bar = 50µm.

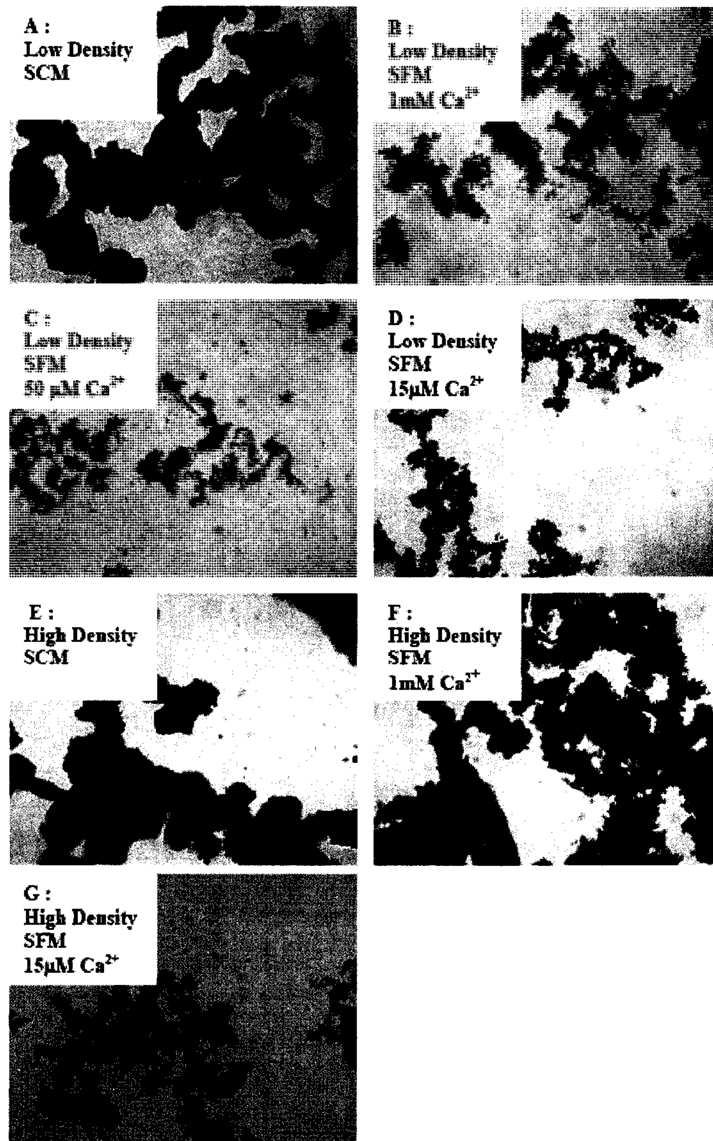

Figure 3-4 : Cell aggregation in suspension after 9 days. Cells were inoculated at low density (**A, B, C, D**) or at high density (**E, F, G**) in SCM (**A, E**) or in an SFM containing 1mM (**B, F**), 50μM (**C**) or 15μM (**D, G**) of calcium. In the SCM large and tightly bound aggregates were present. In the SFM medium, aggregates were smaller and more loosely bound. Scale bar = 500 μm.

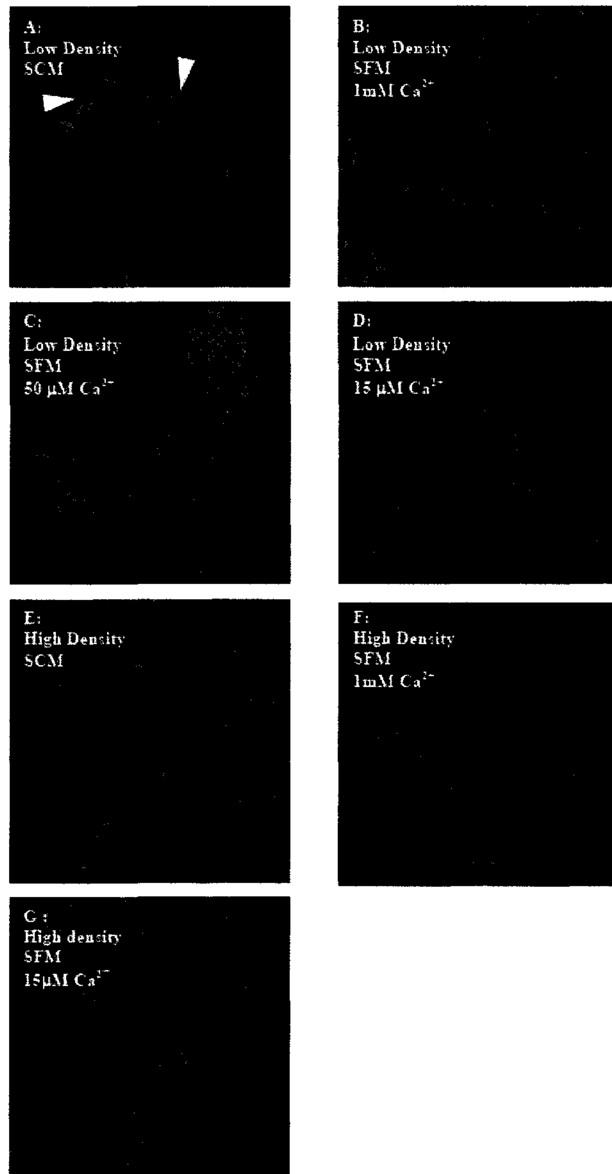

Figure 3-5 : Chondrocytes in suspension inoculated at low or high density on day 10 were stained for actin (green) and the nucleus (blue). In SCM aggregated cells were not tightly packed indicating the presence of intervening ECM. On the contrary in the SFM, cells were tightly packed most likely forming cell-cell contacts. No major differences appeared in the different SFM. Scale bar =50µM. Arrowheads in A show elongated cells at the aggregate periphery.

Tableau 3-1 : Aggregation and average aggregate size at low inoculation density. Aggregation percentage, average aggregate size, after 1 day of culture at low density in suspension. Aggregation is greater in the SCM, as the aggregation percentage is slightly higher and the average aggregate size is ten times greater than in SFMs. On the contrary, aggregation percentage and average aggregate size do not change for different calcium concentrations in SFM. Results are mean +/- standard deviation, n=3. *Aggregation (%) was significantly different in the presence and absence of serum ($p<0.05$) using GLM (see Material and Methods) with serum as categorical predictor.

	SFM-15μM	SFM-50μM	SFM-1mM	SCM
Aggregation (%)	96.3 ± 3.1	96.4 ± 2.0	93.7 ± 1.2*	99.4 ± 0.4*
Average aggregate size (cells)	41.7 ± 17.9	43.8 ± 26.4	44.0 ± 20.9	462 ± 287

SCM *increased collagen type I mRNA expression (p<0.05).* When cultured in monolayer, at high or low density in the SCM, collagen type I and type II mRNA abundance were similar (Figure 3-6 B and D). In low density monolayers collagen type II and I were 1.01 and 1.38 respectively (arbitrary FU normalized to total RNA), and at high density collagen types II and I were 1.23 and 0.87 respectively. In the SFM-1mM Ca^{2+}, the collagen type I expression was greatly inhibited and fell to 0.04 and 0.15 at low and high density versus 1.01 and 1.38 in SCM whereas collagen type II expression remained almost identical. Thus, SFM promoted maintenance of the chondrocyte

phenotype by suppressing collagen I expression. In suspension at both high and low density a similar preferential inhibition of collagen type I expression versus type II by SFM was seen (Figure 3-6 A and C). It can also be noted that collagen type I expression was in general lower in suspension versus monolayer.

Collagen type II and type I expression were also assessed in freshly isolated non-expanded chondrocytes and were found to be 8.45 and 0.018 respectively. This type II expression level was far greater than after 10 days in culture in any of our culture conditions, probably due to the response of chondrocytes to synthesize a pericellular matrix just post-isolation. Nonetheless the collagen type I to type II expression ratio can be used to compare expanded to non-expanded chondrocytes. By comparing this ratio it was found that samples cultured in suspension in the SFM-1mM Ca^{2+} have the lowest type I to type II ratio of 0.02 and 0.004 for low and high density respectively, that compares favourably with 0.0022 for non-expanded chondrocytes.

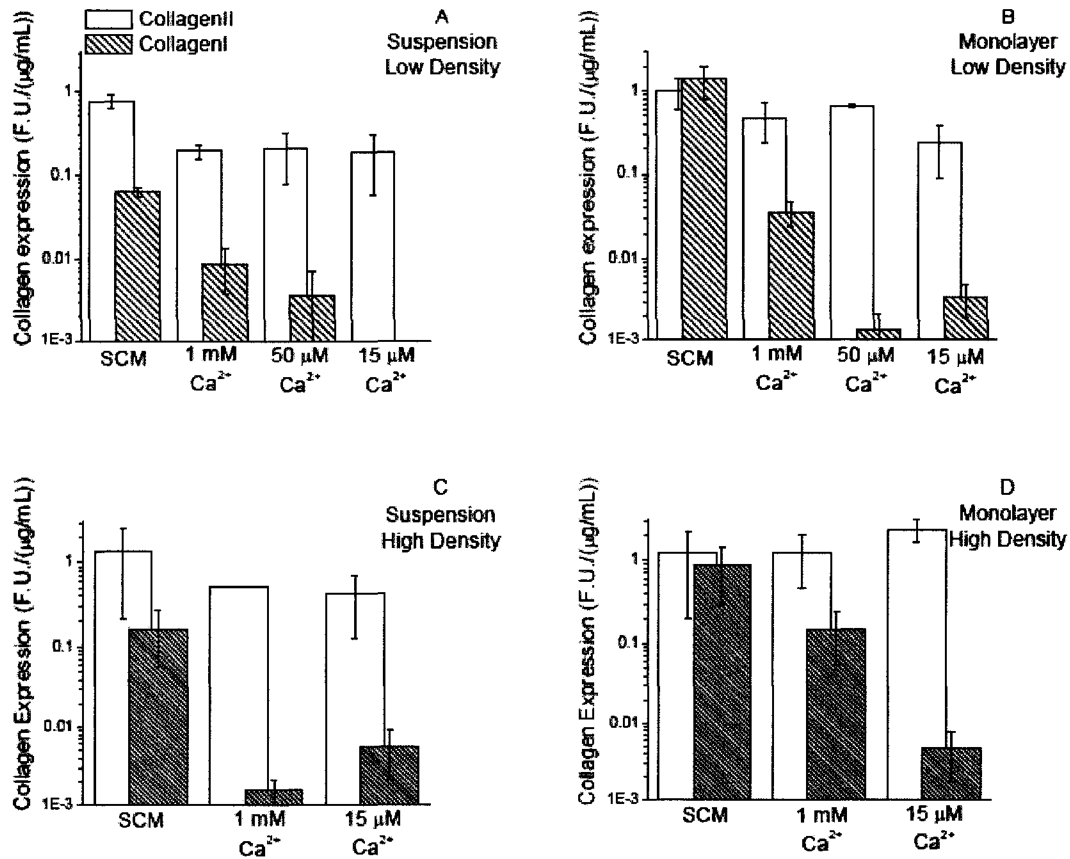

Figure 3-6 : mRNA abundance for collagen type II and collagen type I (Eq 3-6 in Methods) were evaluated by Quantitative Reverse Transcription-PCR. Cells were inoculated at low density **(A, B)** or high density **(C, D)** and cultured in suspension **(A, C)** or in monolayer **(B, D)** in SCM or in SFM supplemented with different calcium concentrations. The absence of serum greatly reduced collagen type I expression. Decreasing medium calcium also generally decreased collagen type I expression. On the contrary, collagen type II expression remained quite stable. Results are mean for n=2, with error bars representing duplicates.

3.4.2 Low extracellular calcium reduced chondrocyte proliferation and GAG production

In monolayer at low and high density, growth was about three times lower in the SFM-15μM Ca^{2+} than in the 1mM Ca^{2+} (Figure 3-1 B and D). This effect was even stronger in suspension, where at low density growth fell from 2.1 doublings/10 days in SFM-1mM Ca^{2+} to 0.5 in the SFM-50μM Ca^{2+} (Figure 3-1 A). At yet lower calcium concentration (15μM) in suspension there was no growth and even cell death (Figure 3-1 A). A similar inhibition of cell division was observed at high density in suspension as cells grew poorly in the SFM-1mM Ca^{2+} (0.3 doubling/10 days) and died in the SFM-15μM Ca^{2+} (Figure 3-1 C). At both low and high density proliferation was found to be statistically dependent on calcium concentration ($p<0.05$).

Lowering calcium concentration reduced GAG production. GAG production and release to media by chondrocytes was evaluated between days 8 and 10 (Table 3-2). In the SFM-1mM Ca^{2+}, when cells were cultured at high density GAG production was 30.8 +/- 1.8 and 26.7+/- 0.4 μg / 2days / million cells, in monolayer and suspension, respectively, and was significantly higher on a per cell basis than in SCM. Lowering calcium to 15μM reduced GAG production by 2.3 times at high density in monolayer and in suspension. At low cell density, GAG production was no longer detectable in the SFM-15μM Ca^{2+}.

Tableau 3-2 : GAG Production at low and high inoculation density, in both monolayer and suspension culture. GAG released to medium between days 8 and 10 for cultures inoculated at high and low density. For low density cultures, GAG in medium was not detectable below 1mM of calcium. GAG production per cell was higher at high density in SFM at 1mM calcium compared to SCM, however lower calcium concentrations than 1mM in SFM appeared to reduce GAG production per cell. Results are mean +/- standard deviation, n=3.

*GAG production was significantly different in the absence or presence of serum ($p<0.05$, with serum, culture system and density as categorical predictor).

‡ GAG production was significantly different depending on calcium concentration ($p<0.05$, with culture system as categorical predictor and calcium as continuous predictor)

GAG production μg/(2days×10^6 cells)	High Density			Low Density		
	SFM-15μM	SFM-1mM	SCM	SFM-15μM	SFM-1mM	SCM
Monolayer	5.8 ± 2.3‡	30.8 ± 1.8*‡	21.0 ± 5.6*	ND	6.7 ± 5.2*	15.1 ± 5.1*
Suspension	8.0 ± 4.8‡	26.7 ± 0.4*‡	24.6 ± 9.2*	ND	4.3 ± 5.3*	28.3 ± 19.9

3.4.3 Low extra cellular calcium does not impede cell aggregation in suspension culture

For the entire range of 1mM down to 15μM Ca^{2+}in SFM at low cell density, more than 90% of the cells were present in aggregates on day 1 (Table 3-1) and average aggregate sizes were between 30 and 45 cells. Actin staining showed that in SFM-1mM Ca^{2+} aggregated cells were round and tightly packed (Figure 3-5 B and F). Images suggested cell-cell contacts as actin cytoskeleton of adjacent cells seemed to be interconnected. Cell aggregates displayed a similar global morphology for the entire range of 1mM down to 15μM Ca^{2+}, at low or high inoculation density (Figure 3-5 B, C, D, F and G).

3.4.4 Low extra cellular calcium inhibited cell spreading in monolayer

In the presence of 1mM Ca^{2+}, cells adhered to the Petri dish and spread (Figure 3-2 B and F). Round cells were also present, and significantly more so at high density (Figure 3-2 E). In 50μM Ca^{2+} a similar spread morphology was observed (Figure 3-2 C), however cells were less elongated and were more rectangular than at 1mM Ca^{2+}. In 15μM Ca^{2+} this morphological shift was amplified, at both low and high cell density, where many cells were round, attached to the dish but for the most part did not spread (Figure 3-2 D and G). When observing the actin cytoskeleton, in SFM-1mM Ca^{2+}, numerous stress fibers were seen to expand in several directions (Figure 3-3 B and F). When calcium concentration was further reduced to 15μM, cells also exhibited stress fibers but to a lesser extent and in one direction only (Figure 3-3 D and G). Moreover,

many cells did not develop stress fibers and remained round with a diffuse actin distribution.

3.4.5 Low extracellular calcium reduced collagen type I expression but not collagen type II expression

Collagen type I expression depended significantly on medium calcium concentration at low density ($p<0.05$) (Figure 3-6). For example, in monolayer, lowering the calcium concentration from 1mM to 50μM had a drastic effect on the collagen type I expression which fell from 0.035 to 0.0013 whereas collagen type II expression remained relatively stable at low density (Figures 3-6 B). A similar tendency was observed in suspension at low density (Figure 3-6 A) and in monolayer at high density (Figure 3-6 D). Thus, reduced calcium levels decreased collagen type I expression to bring the type I to type II ratio closer to that obtained for non-expanded chondrocytes.

3.5 Discussion

One challenge in chondrocyte culture is to obtain high levels of growth without loss of cell phenotype, both to retain phenotype for physiological studies as well as to maximize chondrogenic potential when primary chondrocytes are used. In our study, we examined the effect of low calcium concentrations on chondrocyte growth, phenotype and morphology when cultured at low and high density, in both suspension and in monolayer culture. We hypothesized that low calcium levels in medium would

promote collagen type II expression over type I and promote a chondrocytic (round) cell morphology in monolayer culture. These hypotheses were confirmed by our results (Figures 3-2, -3 and -6). We also hypothesized that reduced calcium concentration would inhibit cell aggregation in suspension. This hypothesis was not supported by our data, however, removing serum from the media did have the unexpected effect of reducing aggregate size (Table 3-1 and Figure 3-4 and -5).

Lowering calcium concentrations promoted chondrocyte phenotype. Collagen type I expression was decreased in the presence of low calcium concentration whereas collagen type II expression was unaffected (Figure 3-6). This reduced dedifferentiation by lowering calcium could be partly due to lower growth as dedifferentiation and proliferation are related. However, our results indicate that slow growth is not the only parameter maintaining phenotype. For example, in the SFM-50μM Ca^{2+} at low density, growth was higher than at high density in the SCM and SFM-1mM Ca^{2+} but phenotype maintenance was better (ratio was 0.002 vs 1.08 and 0.11). Deshmukh et al., 1976 also found that collagen type II became predominant over collagen type I when calcium supplementation in culture medium (DMEM with no calcium but containing 10% FCS) was decreased from 1mM to 10μM. However, since collagen type II and I synthesis was expressed as a relative proportion than absolute levels, it was not clear from this study if collagen type II was stimulated or collagen type I expression was inhibited at low calcium levels. Chang *et al.*[14] saw an inhibition of collagen type II expression for concentrations higher than 4.2 mM $CaCl_2$, for concentrations ranging from 0.4 to > 6mM, but observed no effect below 0.4mM, also in accordance with our observations.

On the contrary, Koyano *et al.*[18] found that collagen type II expression was higher at low (< 0.9 mM) and high (> 4mM) calcium concentrations but lower at intermediate concentration (1.8mM) for fetal bovine chondrocytes where calcium concentrations ranged from 0.1 to 10mM. These latter contradictory results may arise from use of fetal cells, in which case existing literature and our data concord that low calcium concentrations enhance or stabilize collagen type II expression. Our results are the first, however, to demonstrate a clear reduction in collagen type I expression due to reduced calcium concentrations.

Imaging of the chondrocyte actin network allowed us to relate reduced cell spreading in low calcium media to a rounded morphology and promotion of the chondrocyte phenotype. Previously, inhibition of spreading and promotion of a rounded morphology has been achieved by 3D culture[28, 29] or by the use of dihydrocytochalasin B[30, 31] or by high density culture in monolayer[32, 33]. Moreover Mallein-Gerein *et al.*[31] also showed that type II collagen synthesis was coincident with faint actin architecture (i.e. non-spreading cells) and type I collagen with the presence of large actin filaments (i.e. spread cells) consistent with our observations (Figure 3-3). Different calcium concentrations could also be sensed by calcium receptors (CaR), which are known to be expressed by chondrocytes[15]. Since we found that low calcium decreased collagen type I expression in suspension as well (Figures 3-6 A and C), it would seem that both adhesion-dependent and direct sensing mechanisms could be operating. Calcium is, thus, a critical parameter that must be controlled in order to attain desired growth and differentiation properties for chondrocytes.

Aggregate size was reduced when serum was removed although the fraction of cells in aggregates was similar in SFM versus SCM (Figure 3-4, Table 3-1). In suspension, aggregates were significantly larger with serum (Table 3-1) and also contained important intercellular matrix (Figure 3-5 A and E). On the contrary, cells in SFM were tightly packed and established cell-cell contacts in suspension (Figure 3-5 B, C, D, F and G). Related observations have been made with chick embryo dedifferentiated pre-chondrogenic cells[7], where removing serum inhibited cell aggregation seen after 6 h of suspension culture. The addition of fibronectin restored cell aggregation. These authors proposed that aggregate formation is mediated by fibronectin, and then reinforced by cell-cell contacts via N-CAM (neural-cell adhesion molecule) and N-cadherin after which synthesis of ECM by cells fills the intercellular space[34]. In our study, the absence of fibronectin in SFM may have slowed down the aggregation process but did not inhibit it. Nonetheless smaller aggregates found in SFM are beneficial in facilitating nutrient transfer and creating a more homogeneous culture environment.

Aggregate size was not affected by extracellular calcium. This result was surprising as chondrocytes are known to express N-cadherins (calcium-dependant adhesion molecules) which play a major role in chondrocyte condensation[7, 34-36]. We expected that lowering calcium would have disrupted cadherin function resulting in lower levels of aggregation. Moreover, in a preliminary experiment we found that addition of EGTA to chelate all the calcium did reduce aggregation (data not shown). However our minimum calcium concentration of 15μM appears to be sufficient to

allow chondrocyte condensation and this process is probably not solely calcium-dependent[13].

One limitation of our study is the use of chondrocytes of only one age (young) and one species (bovine). For example, it has been shown that fetal chondrocytes are more responsive to calcium changes than adult chondrocytes[18]. Also, our phenotype analysis relied on absolute mRNA rather than protein levels of collagen type I and II. However, since this phenotype analysis based on mRNA levels correlated well with expected morphological changes in chondrocytes, we speculate that mRNA levels reflect well the actual phenotype.

3.6 Conclusion

Our study revealed that removing serum and controlling calcium concentration allow for better control of chondrocyte phenotype and aggregation behavior in monolayer and suspension culture. We found superior alternatives to currently used culture media, depending on the desired culture properties. If proliferation is required, monolayer culture inoculated at low density provides the highest growth rate and the use of SFM containing 1mM down to 50 μM of calcium enables significant cell doubling (four, versus five doubling in SCM over 10 days) and a greatly stabilized cell phenotype (ten times less collagen type I mRNA in SFM versus SCM). For physiological studies, suspension culture at high or low density more closely approximates the *in situ* microenvironment of chondrocytes and removal of serum allows for reasonable growth with negligeable collagen I expression and smaller, more

loosely bound cell aggregates that more closely approximate a homogeneous culture system. These results may be useful to proliferate chondrocytes with minimal loss of phenotype and to perform basic studies on chondrocytes in a homogeneous suspension culture environment.

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CHAPITRE 4. ARTICLE-CHONDROCYTES CULTURED IN STIRRED SUSPENSION WITH SERUM-FREE MEDIUM CONTAINING PLURONIC-68 AGGREGATE AND PROLIFERATE WHILE MAINTAINING THEIR DIFFERENTIATED PHENOTYPE

4.1 Abstract

The study of chondrocyte biology requires culture conditions that maintain cell phenotype. The latter is rapidly lost in monolayer but is maintained in 3-dimensionnal scaffolds, which however experience limited cell proliferation and limited mass-transport. In this study, we cultured chondrocytes in aggregates in stirred spinner flask suspension cultures to control aggregate size and promote mass transport. A previously optimised serum-free medium, containing growth factors (GF) EGF, PDGF-BB, and bFGF all at 2 ng/mL, was used as a control medium (CTR). In addition, two modified media were tested, one containing Pluronic F-68 (medium PF-68), and the other containing PF-68 and a 10-fold increase in GF concentration (20 ng/mL, medium PF-68/10×GF). Chondrocytes formed limited size aggregates within 24 h, exhibited a high viability (> 95%) and cell concentration doubled in seven days in presence of PF-68. Low or no collagen I expression was found for all 3 media, while collagen II accumulated between cells as revealed by a dense immunostaining. Integrin α10, a marker of differentiated chondrocytes and chondrogenic cells, was also found to be

highly expressed. The best performance of chondrocyte aggregate cultures in suspension, in terms of phenotype maintenance, cell morphology, and high cell proliferation was obtained with the PF-68/10×GF medium.

4.2 Introduction

In vitro culture of cell aggregates allows generation of structures resembling tissues, with regard to the cell shape, cellular environment, and cell-cell or cell-matrix interactions (1). There is increasing interest for such 3-dimensionnal (3D) aggregate cultures using various cell types, since they are a useful adjunct to traditional culture systems and animal experiments (2). For instance, *in vitro* 3D cultures have been used to test biomolecules, and to study cell proliferation, differentiation, extracellular matrix (ECM) organisation and cell interactions with the pericellular environment (3). Interest in aggregate or pellet culture of chondrocytes has arisen recently as these 3D culture techniques promote phenotype maintenance of chondrocytes. For example, chondrocytes grown in aggregates or pellets exhibited a rounded cell morphology, maintenance of type II collagen expression, and a cartilage like matrix composition and ultrastructure (4-9). This approach which enables dispersed cells to re-aggregate and to generate a tissue-like construct is a promising tissue engineering approach which does not require any additional biomaterial or scaffold. However, one major issue when culturing chondrocytes in suspension, is that cells condense to produce large aggregates (10, 11), leading to nutrient diffusion limitations and loss of culture homogeneity. An efficient approach to minimize cell aggregation and aggregates size, is the use of a

serum-free medium (9). In addition, the use of a stirred bioreactor system can be advantageous since shear stress from stirring can further control aggregate size (12).

Mechanically agitated bioreactors offer a controlled environment, enabling higher mass transfer rates and a more homogeneous environment in comparison to static culture systems. Several bioreactor designs have been developed for 3D culture of chondrocytes where cells are usually embedded in various types of scaffolds to produce cartilagenous grafts for *in vitro* physiological studies or for transplantation (13, 14). These mechanically agitated culture systems, in comparison to static cultures, permit the growth of larger and better organized engineered cartilage, since hydrodynamic and mixing in bioreactors are expected to improve nutrient diffusion and homogeneity, and also to mechanically stimulate cells (14). Therefore, in the current study we hypothesized that the culture of chondrocytes within the 3D physiological environment of cell aggregates in serum-free medium previously optimized for chondrocyte suspension culture (9), and inside a dynamic, continuously stirred bioreactor would simultaneously permit phenotype maintenance and chondrocyte proliferation with a controlled aggregate size. To generate a stirred environment, spinner flasks were used as bioreactors with controlled stirring speed, temperature and gas phase composition. Since high shear stress can be detrimental to cells in spinner flasks, the shear protectant Pluronic F-68 (PF-68) was added to some cultures. PF-68 is a shear protective agent known to limit cell death in mechanically agitated gas-sparged bioreactors (15) and is particularly useful in serum-free culture medium (16, 17). We have previously shown that PF-68 is taken up by chondrocytes but has no other evident effects on chondrocyte

behaviour in static or orbitally agitated suspension systems (18). Chondrocyte cultures were performed in the optimised serum free control medium CTR medium with and without 0.05% (w/v) of PF-68 (PF-68 medium). In addition a third culture condition was examined where the PF-68 supplemented medium contained tenfold higher concentration of growth factors.

4.3 Material and Methods

4.3.1 Culture media

Serum free medium (SFM). The SFM is a 1/1 (v/v) mix of calcium-free HAM's F12 and calcium-free DMEM low glucose (both from US Biological) supplemented with 1mM $CaCl_2$, 0.4 mM proline, 1.5 mM glutamine, 22mM sodium bicarbonate, non-essential amino acids 1X, penicillin-streptomycin 1X. *Supplemented SFM used as control medium (CTR)* is SFM with ITS+1 (Sigma-Aldrich Canada), EGF, PDGF-BB, bFGF all 2 ng/mL (R&D Systems), 10^{-8} M dexamethasone, $5x10^{-5}$ M β-mercaptoethanol, and 30 μg/mL ascorbate, added fresh. *CTR with PF-68 (PF-68)* is *CTR* defined above with 0.05% (w/v) Pluronic F-68. *CTR with PF-68/10×GF (PF-68/10×GF)* is the same as *PF-68* but with growth factor concentrations increased tenfold to 20 ng/mL. *Serum containing medium (SCM)* is composed of DMEM High Glucose (Invitrogen) supplemented with 0.4 mM proline, 2 mM glutamine, 44mM sodium bicarbonate, non-essential amino acids 1X, penicillin-streptomycin 1X, 10 % FBS and 30 μg/mL ascorbate was added fresh.

4.3.2 Cell isolation and culture

Cells were isolated from the femoropatellar groove of a 1-2 month calf knee, obtained from a local butcher within 24h of slaughter. Briefly, cartilage was sequentially digested, first for 90 minutes by protease Type XIV (Sigma-Aldrich Canada) 56 U/mL in DMEM High Glucose with 44mM sodium bicarbonate and penicillin-streptomycin 1X at 37°C, then for 3 hours by collagenase CLS2 (Worthington) 752 U/mL at 37°C in the same medium with 5 % FBS. Released cells were filtered through a 380 μm mesh (Sigma-Aldrich Canada), followed by 100 and 20 μm nylon filters (Spectrum Laboratories). Cells were then washed three times in *SFM*, and seeded at 200,000 cells/mL in three spinner flasks (Bellco) one each containing the CTR or PF-68 or PF-68/10×GF medium, in a total medium volume of 100mL. Each spinner was equipped with a custom-made glass stirring shaft consisting of four 45° angled paddles, which rotated clockwise at 60 rpm. All surfaces of the spinner, which were in contact with the cell suspension, were made of glass and were siliconized with Sigmacote (Sigma-Aldrich Canada) to prevent cell adhesion, prior to culture. Temperature and pH control were achieved by placing the spinner, with loosened arm caps to enable gas exchange, in a 37°C incubator with 5% CO_2. To ensure reproducibility, the reported data from these 3 spinner cultures were compared to two previous spinner culture experiments performed under the same conditions with the PF-68 medium. Spinner cultures were also compared to standard monolayer culture,

performed in triplicate, in 60 mm Petri dishes inoculated with 800,000 cells in SCM. Fresh ascorbate at 30μg/mL was added daily for both spinner and monolayer cultures.

4.3.3 Culture sampling

Daily sampling was achieved by harvesting 1mL of suspension culture medium from each spinner, for microscopic observation with an inverted microscope (Axiovert S100TV, Carl Zeiss), in Kohler transmission mode. Images were acquired with a digital camera and Northern Eclipse Software (Empix Imaging) and aggregate size was evaluated with the *Line Measure* software tool by randomly selecting a minimum of 16 aggregates in several fields of view and measuring the largest lateral dimension. The suspension was then divided in two 500μL volumes and centrifuged at 300g for 5minutes; the supernatant was frozen and stored until further analysis, and the pellets were processed for cell viability and quantification of aggregation (see below). After 7 days of culture, the entire remaining volume of the spinner culture (about 90mL) was divided into four parts, with particular care to evenly distribute cells in each part. One part was lysed in 500 μL of GITC (4 M GITC, 50 mM Tris, 1 mM EDTA) for DNA analyses (see ***Cell count in spinner flasks*** below), a second in GuCl (4 M GuCl, 50 mM Tris, 1 mM EDTA) for glycosaminoglycan analysis, and a third part in lysis buffer for RNA extraction for ***QRT-PCR*** (see below). The remaining fourth part was used for immunostaining (see below).

In monolayer culture, after 7 days with no passaging, culture medium from 3 independent replicate dishes was sampled by taking 1 mL for glucose, glutamine,

lactate, ammonia, and glycosaminoglycan analyses. Afterward all three dishes were harvested with Trypsin/EDTA to estimate cell concentration and viability. Three other dishes were lysed in lysis buffer for RNA extraction for ***QRT-PCR*** (see below).

4.3.4 Cell viability and estimation of aggregation in spinner flasks

As described above, the 1 mL sample from each daily harvest was divided equally and centrifuged. The first pellet was resuspended and used to count single cells only using a haemocytometer. The second pellet was digested by 752U/mL collagenase CLS2 (Worthington) to release cells from aggregates, before cell counting. Viability was assessed by the Trypan blue exclusion dye on both pellets. An estimate of aggregation was calculated as (digested pellet cell count – undigested single cell count) x 100 /(digested pellet cell count). This method was not used to calculate cell concentration in the spinner as the sampling was not consistent when decantating aggregates in the spinner and pipettes.

4.3.5 Cell count in spinner flasks

Cell number was estimated at day seven by DNA quantification with Hoechst 33258 (Molecular Probes) on GITC extracts as described previously (19). Results were compared to a standard curve made with known cell numbers. An extra standard curve was also realised in presence of PF-68. No interference of PF-68 on Hoechst 33258 fluorescence was observed.

4.3.6 Glucose, glutamine, lactate, ammonia, glycosaminoglycan and pluronic assays

Glucose, glutamine, lactate and ammonia concentrations in the culture media were determined using enzymatic assays (glucose assay kit GAHK-20, glutamine assay kit GLN-2, ammonia assay kit AA0100, all from Sigma-Aldrich Canada; lactate assay kit 735-11 from Trinity Biotech). The dimethylmethylene blue (DMMB) assay was used to quantify glycosaminoglycan in the culture media or in GuCl extracts as previously described (19) using standard curves of chondroitin sulfate C (Sigma-Aldrich Canada) in the presence and absence of Pluronic 0.05% (w/v). PF-68 was found not to interfere with these assays. As cell concentration was measured only at days 0 and 7, specific nutrient consumption and metabolite production rates were calculated using these two data points. PF-68 content in the medium was quantified as previously described (20) where thiocyanate-pluronic complex were formed, intensively washed and finally resuspended in acetone prior to absorbance reading.

4.3.7 Quantitative Reverse Transcriptase PCR (QRT-PCR)

Total RNA was isolated with the RPN kit (Sigma-Aldrich Canada). Reverse transcription and composition of the PCR mix are described elsewhere (9). Primers are depicted in Table 4-1. Quantitative PCR in the RotorGene 6000 (Corbett Research) began with a 5 min denaturation step at 94°C, followed by 7 cycles with a touchdown between 65°C and 59°C and then 40 cycles of classic PCR with a melting temperature of 59°C. A fluorescence reading was performed for each cycle at 72°C. The run ended by an increase in temperature from 72°C to 99°C to obtain the melting curve. Relative

mRNA abundance was calculated following the Liu and Saint method (21) with Ef1α as the housekeeping gene to calculate relative mRNA expression.

Tableau 4-1 : Primer pairs used for PCR.

Gene	PUBMED accession number	Sequence
Bovine type II collagen	X02420	5'-GAA CCC AGA ACC AAC ACA ATC C-3' (forward) 5'-TCT GCC CAG TTC AGG TCT CTT AGA GA-3' (reverse)
Bovine type I collagen	S64596	5'-TGG CCC AGA AGA ACT GGT-3' (forward) 5'-AGG AAG GTC AGC TGG ATG-3' (reverse)
Bovine Type VI collagen	XM_582924	5'-AGT CTG GAG GCA GAA GTC CA-3' (forward) 5'-AGT GCG ACC ACA AGA GTC CT-3' (reverse)
Bovine Type IX collagen	XM_582924	5'-ATG GCT GCG AGT CTG AAG C-3' (forward) 5'-TTT GGG ACC TCT TCC TGG G-3'(reverse)
Bovine Fibronectin	K00800	5'-ACT GCC CAC TCC TAC AAC CA-3' (forward) 5'-CAA AGG CAT GAA GCA CTC AA-3' (reverse)
Bovine NCAM	BT020673	5' CCG GAG ATG CCA AAG ATA AA 3 (forward) 5'-CGA TGT TGG CGT TGT AGA TG 3' (reverse)
Bovine integrin sub-unit β1	U10865	5'-TCA AAT CCA GCC ACA GCA GC-3' (forward) 5'-CCG TGT CAC ATT CCA CCA AC-3' (reverse)
Bovine N-Cadherin	X53615	5'- GGA CAT CGG GGA CTT CAT TAA TG -3' (forward) 5'- TGG TTT GCA GCC TCT GCC AAA G -3'(reverse)
Bovine integrin sub-unit α1	XM_616068	5'-CAA GCA TGA CTT TCG GGA TT-3'(forward) 5'-GCC CTT TTC TGT GGT GGA TA-3'(reverse)
Bovine integrin sub-unit α 2	L25886	5'-TTT CCG AGC CTT AAG CGA AA-3'(forward) 5'-CAG TGC CAT ATT TCG GCT TCG CAA CTG AT-3'(reverse)
Bovine integrin sub-unit α 5	XM_872198	5'-TTT GCT GTG AAC CAG AGT CG-3'(forward) 5'-TAA GAG AGA CCT GGG CCT GA-3'(reverse)
Bovine integrin sub-unit α 10	XM_582982	5'- AGG CCT CAC CTC AGA CAA GA -3'(forward) 5'- ACC CTC GTC CTT TCC AAA CT -3'(reverse)
Bovine integrin sub-unit α 11	XM_602058	5'- AAC TGC ACC AAG CTC AAC CT-3'(forward) 5'- CCA TGT AGG TCT GGCACC TT-3'(reverse)
TSP5/COMP	X74326	5'- ATG CGG ACA AGG TGG TAG AC-3'(forward) 5'- TCT CCA TAC CCT GGT TGA GC-3'(reverse)
Bovine EF1 α	AJ238405	5'-AGC TGA AGG AGA AGA TTG ATC-3'(forward) 5'-GGC AGA CTT GGT GAC CTT G-3'(reverse)

4.3.8 Immunochemistry

Chondrocyte aggregates were fixed in 0.4% paraformaldehyde, blocked with 10% v/v Goat Serum, and stained for the antigens described Table 4-2 with the primary antibody incubated overnight at 4°C and secondary antibody incubated 2h at room temperature. All samples were counterstained for Actin with Phalloidin-Alexa 488 (1/40, Molecular Probes). The nucleus was stained with Hoechst 33258 (0.5μg/mL, Molecular Probes). Samples were mounted in 16.7% (w/v) Mowiol 4-88 (Fluka), 33.3% (v/v) glycerol, 0.75% (w/v) n-propyl gallat in PBS. Confocal microscopy was performed using an LSM 510 META (Carl Zeiss).

Tableau 4-2 : Antibodies used for immunostaining.

Antigen	Antibody
Type II Collagen	Clone II-IIB3 (1/10, Developmental Studies Hybridoma Bank)
Fibronectin	Biogenesis 4470-3589 (1/500)
Type I Collagen	Clone Col 1 (1/100, Sigma)
Integrin β1	Clone 4B4 (1/200, Beckmann Coulter)

4.3.9 Statistical analysis

All measurements from spinner cultures were realized in triplicates from the same culture volume. Triplicate monolayer cultures were sampled independently. For spinner cultures, standard error (SEM) were calculated for endpoint measurements only

(such as DNA and GAG assays), for which no other time points were sampled. For QRT-PCR, SEM obtained for monolayer cultures and freshly isolated cells allowed measurement error to be evaluated.

4.4 Results

In this work, three spinner cultures were performed with three slightly different culture media. The serum-free medium previously described in Gigout et al., 2005 (9), and supplemented with a shear-protectant PF-68 (PF-68), was used here to reproduce previous results obtained with chondrocytes from a different cell isolation. Since results were similar between these two cell isolations, only those for the isolation comparing the 3 distinct culture media are reported which included the control medium in the absence of PF-68 (CTR) and the PF-68 containing medium with a higher growth factor concentration (PF-68/10×GF medium).

4.4.1 Chondrocytes aggregate and proliferate in spinner flasks with pluronic F-68 in serum free medium

Chondrocytes aggregated and generated dense structures of several μm in mean diameter in spinner flask suspension cultures (Figure 4-1). The aggregates in the control culture (CTR) had an average size of 171±44 μm (n=16) and were smaller ($p<0.005$) than in the PF-68 and PF-68/10×GF cultures, where average aggregate sizes were 257±75μm (n=17) and 373±138μm (n=18), respectively (Figure 4-1).

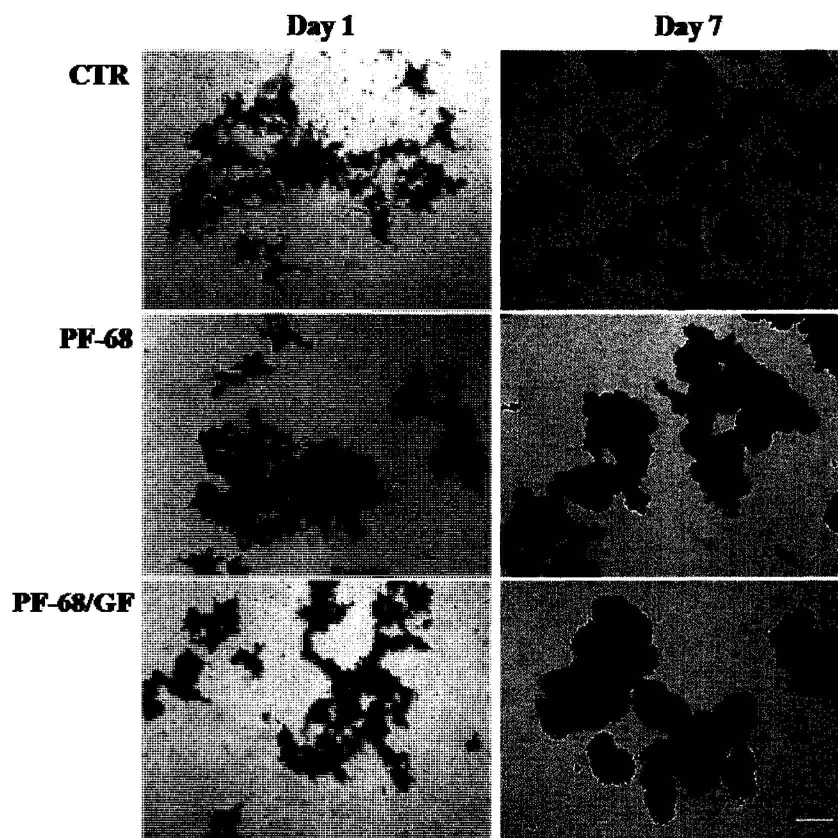


Figure 4-1 : Chondrocyte aggregates after 1 and 7 days in spinner culture in serum free medium (SFM) as control (CTR), in SFM with 0.05% (w/v) PF-68 (PF-68) and in SFM with 0.05% (w/v) PF-68 and 10 × higher concentration of growth factors than SFM (20ng/ml vs 2ng/ml) (PF-68/10×GF). Representative images are from 1mL samples harvested from each suspension culture. Scale bar = 500 μm.

Cell aggregation was rapid in all suspension cultures, as aggregation percentages reached 72.3, 86.3 and 85.5% after 24h in the CTR, PF-68 and PF-68/10×GF cultures respectively, and remained high throughout the culture (Figure 4-2 A). Cell viability was found to be higher than 95 % in all spinners. However, single cells, not in the aggregates, exhibited a lower viability (Figure 4-2 B). Cell proliferation was observed in the two spinners containing PF-68, where cell concentration almost doubled after seven days of culture (Figure 4-2 C). Growth in monolayer was about 2.5 times faster (Doubling time in Table 4-3).

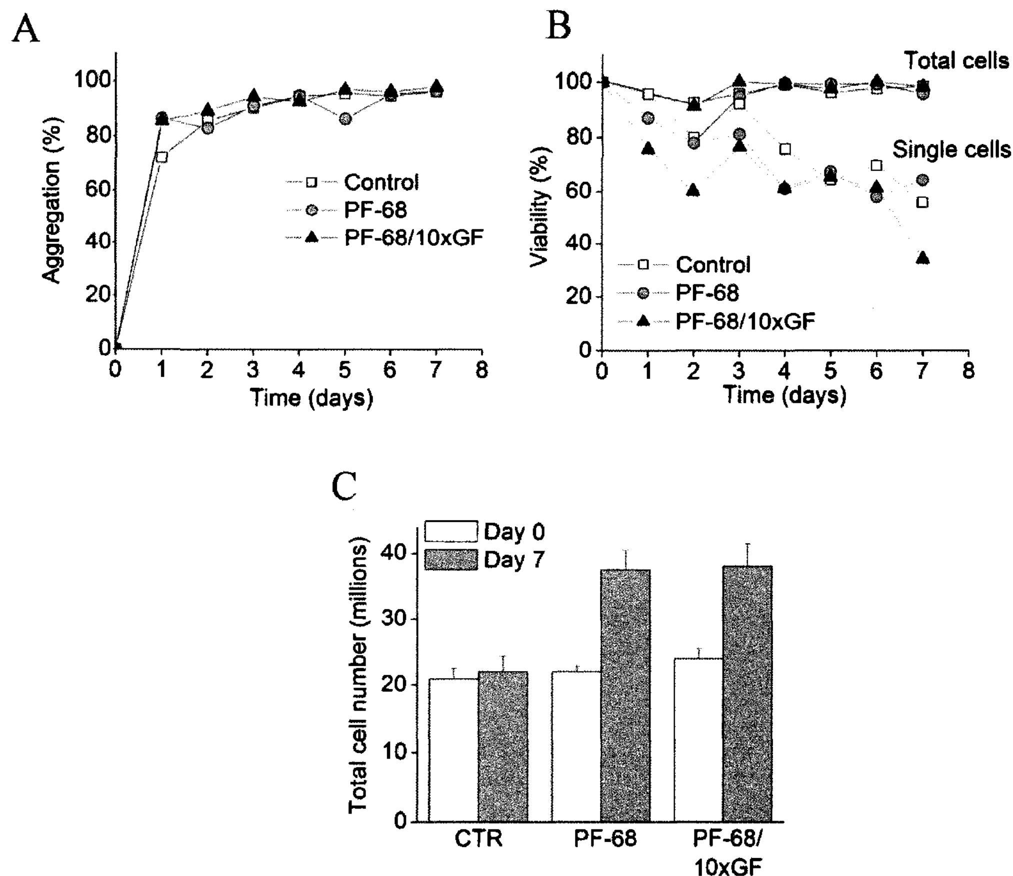


Figure 4-2 : (A) Cell aggregation was evaluated daily in each of the three spinner culture media by comparing single cell number to that released from aggregates by enzyme dissociation. **(B)Viability of single (non-aggregated) and total cells (single cells and in aggregates)** was estimated by trypan blue exclusion of un-dissociated or enzymatically dissociated cell suspension. **(C) Total cell number** was measured at day 0 by cell counting and at day 7 by DNA quantification followed by conversion into cell number using a standard curve with known cell concentrations. Results are mean ±SEM of three different measurements.

4.4.2 Chondrocytes are metabolically active in spinner culture

Evolution of metabolite concentrations with time was very similar and almost superposed for the three different culture media (Figure 4-3). Rates of consumption and production of glucose, glutamine, lactate and ammonia per million cells, were also quite similar for the three conditions (Table 4-3), although always slightly higher in CTR. When compared to cells in monolayer, glutamine consumption and ammonia production rates resembled those calculated for spinner cultures. However, monolayer cells exhibited higher glucose consumption and lactate production rates (about 2-fold higher) compared to cells in suspension.

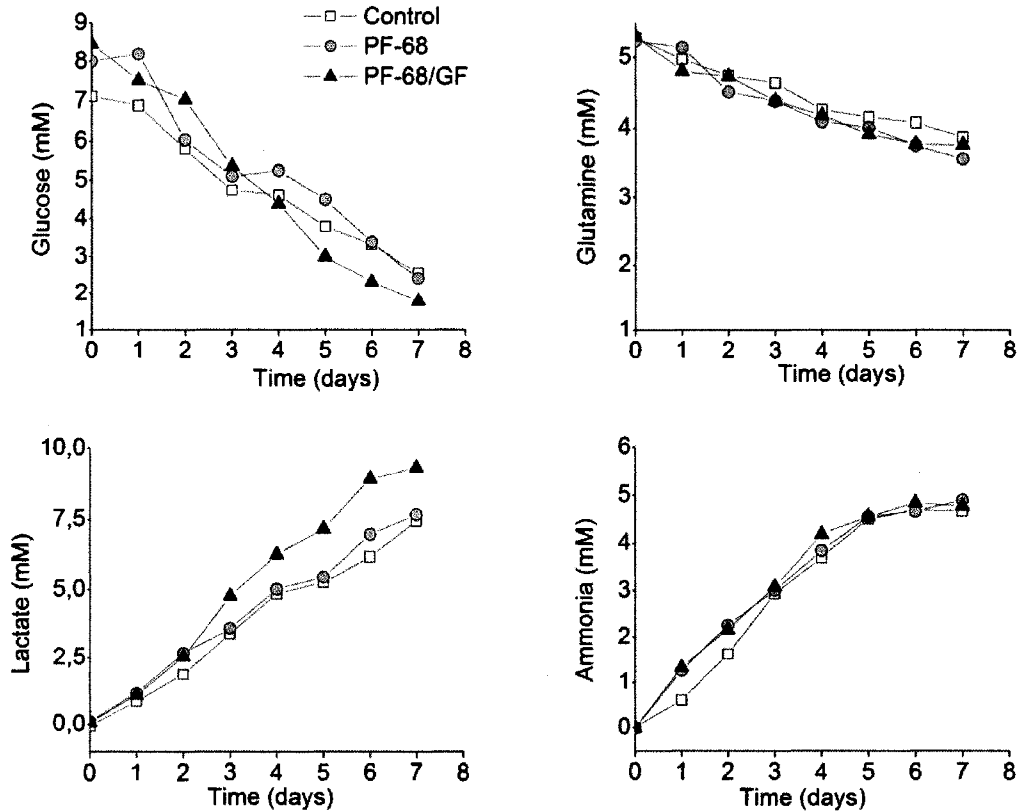


Figure 4-3 : Glucose, glutamine, lactate, ammonia concentrations versus culture time, in spinner flasks with the three different serum–free media.

Tableau 4-3 : Metabolic parameters for chondrocytes in aggregates in suspension spinner cultures with three different serum-free media (CTR, PF-68, PF68/10×GF described in Materials and Methods) and in standard monolayer culture with serum. Standard errors (SEM) are calculated from 3 different measurements for each single spinner culture, and from 3 independent monolayer cultures. *rates in μmol/million cells/7 days, † rate in μg/million cells/7 days, ‡ +∞ means collagen I was not detected.

	CTR	**PF-68**	**PF68/10×GF**	**Monolayer**
Doubling Time (days)	+∞	9.1	10.5	3.9
Glucose Consumption*	21.3±0.4	18.9 ± 0.4	21.6±0.5	52.1±1.0
Glutamine Consumption*	6.6±0.1	5.7±0.1	4.9±0.1	11.6±0.2
Lactate Production*	34.6±0.7	25.7±0.5	30.0±0.8	90.9±1.8
Ammonia Production*	21.8±0.4	6.4±0.3	15.4±0.4	32.3±0.6
GAG total production † *	216±4	118±2	152±4	1584±31
Collagen II/ I (FIC = +∞)	240±69	180±26	+∞ ‡	0.56±0.05

4.4.3 Glycosaminoglycan production is affected by PF-68 and GF concentrations

GAG concentration continuously accumulated in all culture media with time (Figure 4-4 A), to reach 2.54, 2.64 and 2.26 mg/spinner after 7 days in CTR, PF-68 and PF-68/10×GF respectively. Total GAG contents (released in the medium plus in cell aggregates) were 4.64, 3.51 and 4.7 mg/spinner, in CTR, PF-68 and PF-68/10×GF cultures. Total GAG production rate per million cells (Table 4-3) was more than two

times lower in PF-68-containing cultures, compared to the control, primarily due to a doubling of cell number with PF-68. Finally total GAG production was about 10-fold higher in monolayer (Table 4-3) due to large amounts being released to the medium.

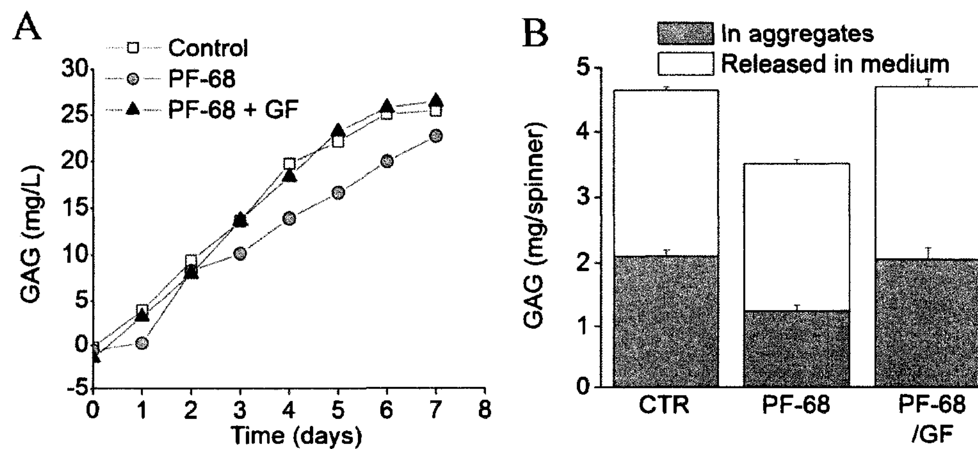


Figure 4-4 : (A) Released glycosaminoglycan (GAG) concentration measured daily in the culture medium of the three spinners and **(B) total GAG synthesis (GAG released in the medium plus GAG retained in aggregates)** by cells cultured in the three different media. Results are mean ±SEM of three different measurements.

4.4.4 The chondrocytic phenotype is maintained in all three media in spinner cultures

Expression of extracellular matrix molecules was analysed by QRT-PCR (Fig 4-5A). Collagen II, the hallmark of the chondrocytic phenotype, was found to be expressed in all culture conditions. Collagen I, on the contrary, a marker of chondrocyte dedifferentiation, was found to be highly expressed in cells cultured in monolayer. It was barely detected in the CTR and PF-68 spinner cultures and was not

detected at all in PF-68/10×GF or in FIC. The ratio of collagen II to I was consequently much higher in suspension cultures compared to monolayer culture (Table 4-3). By immunostaining, Collagen I was not detected in the cell aggregates generated in spinner (not shown), but collagen II was positively stained (Figure 4-5 B) and was found to fill the space between cells. Collagen VI and IX, as well as COMP, which all participate in matrix organisation in intact cartilage, were also detected by QRT-PCR in cells cultured in spinner, for the three different media. In addition, fibronectin was expressed in all conditions and immunostaining (not shown) revealed its presence in small amounts, mainly at the periphery of the aggregates.

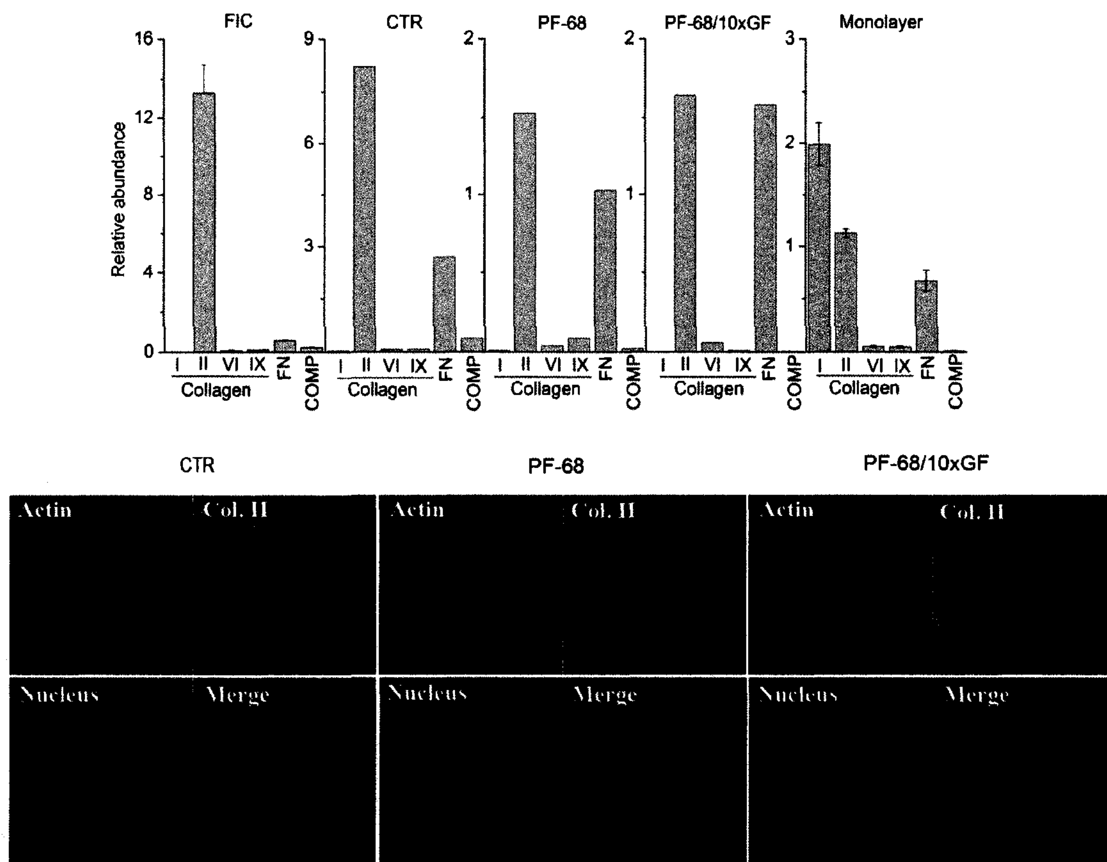
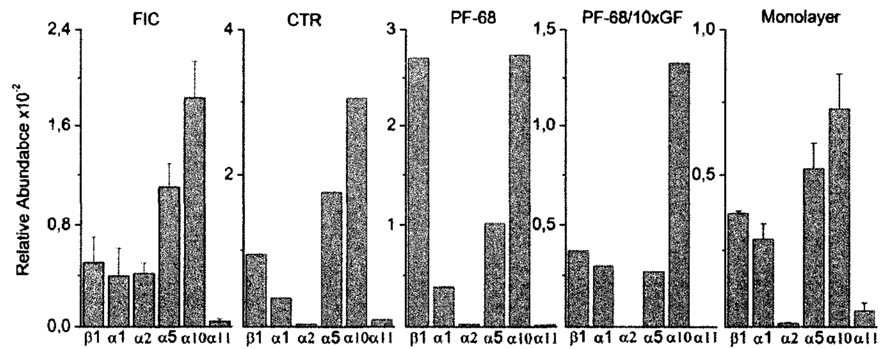


Figure 4-5 : (A) mRNA expression of Collagen Type I, II, VI and IX, of fibronectin and COMP by QRT-PCR, at day 7, by chondrocytes in spinner cultured in 3 different serum-free media, by freshly isolated chondroytes (FIC) and by chondrocytes cultured 7 days in monolayer. **(B) Immunostaining for collagen type II (in orange) counterstained for actin (in green) and the nucleus (in blue), of aggregates sampled in the three different spinner cultures** at day 7, and imaged by confocal microscopy.

4.4.5 Chondrocytes cultured in spinner culture express the chondrogenic Integrin α10

Expression of Integrin subunits β1, α1, -2, -5, -10 and -11 were analysed by QRT-PCR (Figure 4-6 A). Integrin β1 expression was revealed in all cultures by mRNA QRT-PCR analysis and by immunostaining (Figure 4-6 B), which exhibited a strong pericellular staining consistent with β1-containing integrins being involved in cell-matrix interactions. These images also reveal differences in cell morphology for the different culture media. Chondrocytes cultured in CTR and PF-68 media were more elongated than those in PF-68/10×GF which maintained the most spherical morphology (Figure 4-6 B). Furthermore, a higher cell density was observed in aggregates cultured in presence of PF-68, an observation that affirms the higher cell numbers on day 7 for the spinner cultures containing PF-68 (Figure 4-2 C). Integrin subunits α2 and α11 were expressed at very low levels in all culture, while integrins α5 and α10 expression patterns were similar, with integrin α10 exhibiting a higher relative abundance and was therefore the most highly expressed α subunit in chondrocytes cultured in spinner flasks (Figure 4-6 A). Finally, direct cell-cell adhesion molecules such as N-Cadherin and NCAM were also analysed and only detected in very low amounts (mRNA abundance in the order of 10^{-4}, not shown).



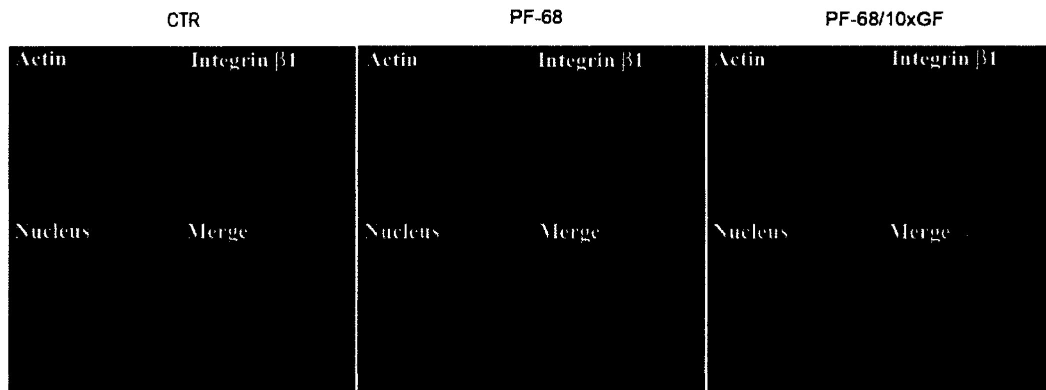


Figure 4-6 : (A) mRNA expression analysis of Integrin β1, α1, α2, α5, α10 and α11 by QRT-PCR, at day 7, by chondrocytes cultured in the 3 different media in spinner, by freshly isolated chondrocytes (FIC) and chondrocytes cultured 7 days in monolayer. **(B) Immunostaining for integrin β1 (in orange) counterstained for actin (in green) and the nucleus (in blue), of aggregates sampled at day 7 in the three different spinner cultures**, and imaged by confocal microscopy.

4.4.6 PF-68 is uptaken by the cells

We have recently reported (18) that PF-68 enters and accumulates in chondrocytes. After 7 days in spinner cultures, PF-68 concentration decreased by 0.009 and 0.007 % (w/v) in PF-68 and PF-68/10×GF cultures (Figure 4-7). This corresponds to a PF-68 cell uptake of 184 μg and 240 μg PF-68/10^6 cells in PF-68 and PF-68/GF, respectively. These levels were ~4 times higher than a previously measured value of 56 μg/10^6 cells, after 3 days in suspension culture over agarose coated dishes, in static or orbitally shaken cultures (18). The longer culture time and the possible interaction or entrapment of PF-68 molecules within the developing ECM could explain the increased PF-68 uptake observed in the current study, in addition to the differences in the culture conditions.

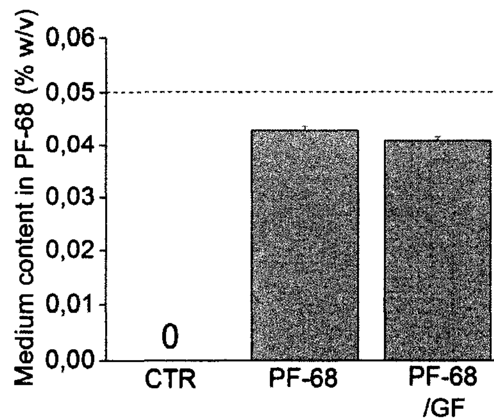


Figure 4-7 : PF-68 concentration in the three different media, after 7 days of spinner culture. Both medium containing PF-68 had an initial concentration of 0.05% (w/v), represented by the dotted line. Results are mean ±SEM of three different measurements.

4.5 Discussion

4.5.1 PF-68 in spinner culture enables simultaneous cell proliferation and maintenance of chondrocyte phenotype

Cells rapidly aggregated in the three culture media (CTR, PF-68 and PF-68/10×GF) to form structures of 200-300 μm in size (Figure 4-1), that were metabolically active (Figure 4-3) with high viability (>95%) (Figure 4-2), and high collagen type II to I ratio (Figure 4-5) compared to monolayer culture. The high ratio of collagen II:I mRNA demonstrated maintenance of the chondrocyte phenotype and was confirmed by a lack of immunostaining for collagen I in aggregates, whereas collagen II gave strong immunostaining spanning the intracellular space. The chondrocyte phenotype was particularly well preserved in the PF-68/10×GF culture where no

collagen I mRNA expression could be detected and the cells showed a round morphology (Figure 4-5 B). The results obtained in the three spinners are unique when compared to other studies using bioreactors where chondrocytes were cultured in a biomaterial or scaffold to synthesize cartilaginous constructs (see Table 4-4). Collagen I expression, a marker of early events of the dedifferentiation (22), was often detected in cartilaginous constructs, meaning that chondrocytes dedifferentiate even when cultured in these 3D systems (23-25), possibly due to the presence of 10% serum (26, 27). In the current work, the use of a serum-free medium containing PF-68 and a high concentration of three specific growth factors (PF-68/10×GF), in combination with suspension culture conditions, maintained a completely differentiated chondrocyte phenotype.

Cell proliferation was only observed in media containing PF-68 where the total estimated cell number almost doubled (1.9 fold) in 7 days. This result is unequalled when compared to other studies (Table 4-4), since after 7 days in SFM, the obtained cell expansion was similar to that seen by others after 3-6 weeks in scaffold-based cultures containing 10% serum. PF-68 is not usually directly associated with an increase in cell growth rate, although this has been occasionally reported (28, 29). In the present work, we demonstrated that PF-68 at 0.05% (w/v) in combination with a calcium containing SFM stimulated chondrocyte proliferation simultaneous with maintenance of chondrocyte phenotype.

4.5.2 Chondrocytes in spinner culture express a cartilaginous ECM

GAG synthesis per million cells was found to be higher in CTR culture and about 35-45% of the total GAG was observed in aggregates (see Table 4-4). The GAG content obtained in spinner cultures is low compared to that of scaffold cultures where GAG content ranged from 110 to 800 μg/million cells (Table 4-4), in part due to the scaffold retaining synthesized GAG. Culture duration, in this work, was also relatively short compared to the 3-6 weeks in the literature. Significant GAG release to the medium has been previously observed in spinner cultures with as high as 92 % of total GAG released to the medium under stirred conditions (30).

Tableau 4-4 : Summary of results obtained in the current work compared to previous studies using various bioreactors for 3D chondrocyte culture. All studies were performed with articular chondrocytes from calves a few months old, excepted in Mahmoudifar and Doran 2005 (51) (human fetal epiphyseal chondrocytes, passaged twice before seeding) and in Chen et al., 2004 (30) (articular cartilage from 7 day old rats). [a] all other studies, except the current work, were performed with cells embedded in a scaffold. [b] GAG amount in construct unless otherwise mentioned. [c] Calculated with 1 chondrocyte = 10^{-10}g (49). PGA : polyglycolic acid. PLA : poly (L-lactic acid). PLGA : poly (L-lactic-co-glycolic) acid. ND: not determined.

Culture conditions[a]	Cell proliferation	GAG (μg/million cells)[b]	Culture duration	Phenotype	Reference
Suspension cells rapidly form aggregates in spinners cultured in serum-free medium, with no medium change.	No proliferation in CTR while 1.9 fold in both PF-68 and PF-68/10×GF	210, 93, 124 (total GAG) 95, 33, 54 (GAG in aggregates) in CTR, PF-68, PF-68/10× GF, respectively.	7 days	Collagen II immunodetected and analysed by RT-PCR, no collagen type I detected	Current work
10% FBS, in PGA, in rotating bioreactor, with medium changes.	No proliferation	559[c]	40 days	Collagen II synthesized, type I not evaluated	(49)
10% FBS, in PLA, in a concentric cylinder bioreactor, with medium changes.	2.3 fold	310	28 days	Collagen II synthesized, type I not evaluated	(50)
10% FBS, in PGA, with periodic medium flow reversal and medium changes	1.6 fold	370	5 weeks	Both collagens I and II present	(51)
10% FBS, in PGA, in a rotating vessel, with medium changes	3 fold	800	6 weeks	ND	(52)
10% FBS, in PGA, in a rotating vessel, with medium changes	2.7 fold	550	5 weeks	ND	(53)
10% FBS, in PLGA, in a rotating shaft bioreactor, with continuous medium infusion, and medium changes	2.3 fold	110μg (in construct) 4mg (total GAG) 92 % of total GAG released in the medium	4 weeks	Collagen II synthesized, type I not evaluated I	(30)
10% FBS, in PGA or Hyaff-11®, in a rotating vessel, with medium changes	3.4 fold	637	1 month	Both collagens I and II present but type II gave a more intense staining	(24)
9% FBS, in PGA, in a wavy walled bioreactor, with medium changes	4.8 fold	220	28 days	Both collagens I and II present	(25)

Concerning collagen expression, chondrocytes in spinner expressed mainly collagen II, followed by collagen IX and VI maintaining relative proportions similar to those *in situ* (31). In addition, COMP (cartilage oligomeric matrix protein or thrombospondin V), that is known to catalyze collagen fibril assembly (32), was also detected by PCR in spinner cultures as was fibronectin. Compatible results were obtained by Stewart *et al.* 2000 (5), who detected collagen II and fibronectin expression in chondrocytes aggregates with low levels of collagen I. Collagen II, IX and VI, as well as fibronectin, and GAG are all components of the pericellular and territorial matrix of chondrocytes, sometimes called the chondron (33, 34), and their presence in aggregates supports the notion that aggregate culture presents a pericellular environment that is highly similar to *in situ* physiological conditions.

4.5.3 Chondrocyte aggregates in spinner culture express chondrogenic surface markers

Since cell-matrix interactions play a predominant role in cell survival, proliferation and phenotype maintenance, and are mainly mediated by integrins (35), the expression of integrins was assessed. The $\beta1$ integrin subunit was detected by QRT-PCR and gave a strong pericellular staining for all culture conditions, indicating that cells interacted with the surrounding matrix through $\beta1$ integrin. The $\alpha10$ subunit, which is believed to be the major receptor for collagen II in chondrocytes (36-38), showed the highest expression levels among all analysed α subunits. Integrin $\alpha10$ is also known to be highly expressed in differentiated chondrocytes and chondrogenic

cells (39-41). The integrin $\alpha5$ subunit, usually highly expressed by chondrocytes, and which binds fibronectin when associated with $\beta1$ (42, 43), was also detected in all spinner aggregates. Furthermore, direct cell-cell adhesion molecules such as N-Cadherin and NCAM were only detected in very low amounts, which was expected since differentiated chondrocytes in cartilage do not express those molecules (44-46).

4.5.4 Performance of the three tested media and study limitations

A consistent cell and aggregate behaviour was observed in the three culture media enabling the following general conclusions to be drawn: i) chondrocytes aggregate quickly and remained viable over a 7-day period; ii) cell aggregates are smaller (170-370 μm) than these obtained in static conditions (usually several mm) (9) and have a size compatible with efficient nutrient transfer (47, 48); iii) chondrocytic phenotype is maintained since collagen II was found to be highly expressed in comparison to type I at the mRNA level and immunostaining of aggregates showed abundant collagen II and GAG with no detectable collagen I and finally; v) cell-matrix interactions in spinner aggregates contained integrins typical of chondrocytes in cartilage.

When compared with a previous experiment performed in duplicate with the PF-68 medium, we found similar results in terms of viability, aggregation rates and size, cellular growth and metabolism demonstrating repeatability with PF-68 containing medium. As this previous experiment was performed with cells isolated from a different animal, it also shows that results are reproducible from one isolation to the

other. Finally, PF-68 was found to be beneficial as it enabled cell proliferation and the PF-68/10×GF medium permitted a fully maintained phenotype with no detectable collagen I mRNA expression, correlating to a round cell morphology.

4.6 Conclusion

Spinner culture in a specifically designed medium enabled the culture of chondrocyte aggregates with high viability, cartilaginous matrix synthesis, maintenance of chondrocyte phenotype, and, when PF-68 is present, a significant cell proliferation over seven days. Cell aggregates were 170-370μm in size, allowing nutrient mass transfer, including dissolved gases, to all cells. Aggregating differentiated chondrocytes synthesized matrix molecules and integrin receptors which corresponded closely to those found in cartilage. We found the cell aggregate environment to closely mimic the *in vivo* pericellular environment of chondrocytes in terms of matrix molecules and integrin receptors, such that chondrocytes in aggregates should be a good model for *in situ* cell behaviour. The ability to proliferate phenotypically stable chondrocytes in addition to the close to physiological character of this culture system indicate that this culture system may be of practical use to attain greater numbers of differentiated chondrocytes and to examine basic aspects of cartilage biology.

4.7 Acknowledgement

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CHAPITRE 5. ARTICLE -THE FATE OF PLURONIC F-68 IN CHONDROCYTES AND CHO CELLS

5.1 Abstract

The surfactant Pluronic F-68 (PF-68) is widely used in large-scale mammalian cell culture to protect cells from shear stress that arises from agitation and gas sparging. Several studies suggested that PF-68 is incorporated into the cell plasma membrane and could enter the cells, but without providing any direct evidence. The current study has examined this question for two cell types, one of pharmaceutical interest (CHO cells) and the other of biomedical interest (chondrocytes or cartilage cells). A fluorescent derivative of PF-68 was synthesized to detect and localize internalized Pluronic with culture time. PF-68 uptake by the cells was quantified and characterized. We clearly demonstrate that PF-68 enters the cells, and possibly accumulates in the endocytic pathway. CHO cells showed an average uptake of 11.7 ± 6.7 (SEM) µg PF-68/10^6 cells while the uptake of chondrocytes was 56.0 ± 10.9 (SEM) µg PF-68/10^6 cells, independently of the initial PF-68 concentration (between 0.01 and 0.2% w/v) and of cell concentration (from 1.10^6 to 4.10^6 cells/mL). These uptake values were identical for both static and agitated culture conditions. Finally, we found that CHO cells are able to eliminate intracellular fluorescent PF-68 but chondrocytes are not. These results

show that the uptake of PF-68 by the cells can severely affect PF-68 concentration in the culture medium and thus shear protection effect.

Keywords : Pluronic-F68, mammalian cells, endocytosis, shear stress.

5.2 Introduction

Pluronics are water-soluble triblock copolymers of polyethyleneoxyde (PEO) and polypropylene oxide (PPO) and are part of an important class of non-ionic surfactants containing a hydrophobic centre (PPO) and two hydrophilic tails (PEO). Commercial Pluronics are characterized by their different lengths of PEO and PPO which alter their molecular weight and hydrophobicity. Each Pluronic fits for a specific application, from drug solubilization to bioprocess applications. For instance, Pluronic F-68 (PF-68, $(EO)_{75}$-$(PO)_{30}$-$(EO)_{75}$) is used as a shear protective agent which limits cell death in mechanically agitated gas-sparged bioreactors (Murhammer and Goochee, 1990) and is particularly useful in serum-free medium culture (Keane et al., 2003; Schroder et al., 2004). The protective effect of PF-68 has been demonstrated for suspension culture of mammalian cells (Jordan et al., 1994; Ramirez and Mutharasan, 1990; Zhang et al., 1992), insect cells (Goldblum et al., 1990; Murhammer and Goochee, 1990) and plant cells (Sowana et al., 2002). PF-68 is widely used for large-scale production of therapeutic proteins by animal cells, and it can also be helpful in biomedical applications where bioreactors are increasingly used to amplify a cell population. For

example, neural stem cells can now be expanded in bioreactors (Sen et al., 2001) as can T cells (Carswell and Papoutsakis, 2000), hematopoeitic cells (Sardonini and Wu, 1993; Zandstra et al., 1994), osteoblasts (Yu et al., 2004), astrocytes (Sa Santos et al., 2005) and chondrocytes (Malda et al., 2004).

Two mechanisms have been proposed in literature to explain the cell protection effect of PF-68. The first mechanism suggests that PF-68 affects culture medium characteristics. In gas-sparged bioreactors, animal cells are damaged from cell-bubble interactions leading to cell death when a bubble breaks at the liquid-gas interface (Handacorrigan et al., 1989). PF-68 has been shown to stabilize the foam layer, thereby preventing the cell's interaction with foam, to decrease bubbles velocity (Handacorrigan et al., 1989; Jordan et al., 1994; Michaels et al., 1991) and hydrodynamic forces accompanying bubble break-up (Dey et al., 1997) and to reduce cell-bubble interactions (Chattopadhyay et al., 1995; Meier et al., 1999; Michaels et al., 1995). Secondly it is proposed that PF-68 affects cell resistance to shear stress. For instance, Ramirez and Mutharasan, 1990 showed that PF-68 decreased plasma membrane fluidity (PMF) of hybridomas during a batch culture. Zhang et al., 1992 observed that TB/C3 hybridoma strength increased in the presence of 0.05% (w/v) PF-68 via the measurement of bursting membrane tension and compressibility modulus. Interestingly, Palomares et al., 2000 showed that the protective action of PF-68 continues even after removal of Pluronic from the culture medium, suggesting that PF-68 confers a persistent resistance to shear. PF-68 has even been shown to have biological effects in the absence of shear stress. Growth of fibroblasts and melanoma

cells cultured in Petri dishes was stimulated (Bentley et al., 1989), and fibroblast attachment to plastic surfaces increased (Hokett et al., 2000) in presence of PF-68. Finally it is also known that PF-68 is able to integrate artificial lipid bilayer due to its surfactant properties (Firestone and Seifert, 2005). Hence there is strong evidence that PF-68 interacts with the cell's plasma membrane.

Similarly, the broad use of other types of Pluronics for drug delivery (Pluronics P-85, L-61, F-127; or P-105, Batrakova et al., 1996; Kabanov et al., 2002; Rapoport et al., 2002) and gene delivery (Pluronics P-85, PL-61 and PF-127, Lemieux et al., 2000; Yang et al., 2005) also strongly suggests Pluronic trafficking through the cell membrane. It has also been shown that Pluronic copolymers can be absorbed into the cells, probably through an endocytic pathway (for Pluronics P-85, PL-35 PL-121 and PP-105, Batrakova et al., 2003; Rapoport et al., 2002). However, concerning PF-68, prior to the current study, it was still unknown if PF-68 polymer enters the cell. The current work therefore addresses the characterization of PF-68 internalization and processes. Two different cell types were studied, CHO cells adapted to suspension culture and chondrocytes (cartilage cells) which grow in aggregates when cultured in suspension. The former cell type represents the use of PF-68 in pharmaceutical bioprocesses and the latter, in the biomedical field.

5.3 Materials and methods

5.3.1 Synthesis of fluorescent PF-68

All reagents were from Sigma-Aldrich Canada, Ontario, Canada, unless indicated. The reaction was performed as described by Rapoport et al., 2002 with minor modifications. 22 µmol of PF-68, 44 µmol of 5-(et 6-) carboxy-2'7'dichlorofluoresceine (CDC-Fluorescein, Molecular Probes, OR, USA), 88 µmol of dicyclohexylcarbodiimide (DCC) and 90 µmol of dimethylaminopyridine (DMAP) were mixed in 15 mL of dimethylformamide (DMF). The reaction continued for 3 days at room temperature under agitation, and the reactants were protected from light. Then, the mixture was precipitated with 10 volumes of cold ether. The precipitate was collected by centrifugation (15,000 *g*, 10 min) and washed 5 times with cold ether. This step enabled to remove ether-soluble molecules (DCC, DMAP, DMF, and unreacted CDC-Fluorescein) whereas insoluble molecules (PF-68 and labeled PF-68) remained in the precipitate. Finally the mixture was dried overnight at room temperature in a chemical hood to obtain a red powder.

To characterize the reaction products, the final powder was diluted in 1.5 mL PBS, and the solution was purified by gel exclusion chromatography. 1.5 mL was applied on a Sephadex G25 column (HiTrapDesalting column, Amersham, NJ, USA, exclusion limit 3, 000 Da, void volume of 1.5 mL), and eluted with 10.5 mL of PBS. 24 fractions of 0.5 mL were collected. PF-68 (see below, PF-68 quantification) and CDC-Fluorescein (analyzed by absorbance at 530 nm, Dynatech MRX Microplate reader,

Dynextechnologies, VA, USA) concentrations were evaluated in each fractions. Free PF-68 and PF-68 bound to 1 or 2 CDC-Fluorescein should be eluted first because of their high molecular weight (Table 5-1).

Tableau 5-1 : Molecular weights of reagents used in PF-68 labeling, and theoretical molecular weights of the products.

MW of Reactants (g/mol)		MW of Products (g/mol)	
PF-68	8400	Pluronic F68-CDCFluorecein	8827
CDC-Fluorescein	445.21	Pluronic F68-2CDCFluorescein	9254
DCC	206.33		
DMAP	122.17		
DMF	73.09		

5.3.2 Fluorescence Spectra

Absorbance spectra of labeled PF-68 or CDC-Fluorescein in PBS were obtained by spectrophotometry (Beckmann DU-600, Beckman Coulter, Ontario, Canada), scanning from 300 to 800 nm (1 nm steps). Fluorescence emission spectra were acquired with a microplate spectrofluorometer (Spectramax^{TM} Gemini XS, Molecular Devices, CA, USA) where the maximum absorbance was chosen as the excitation wavelength (i.e 505 nm for CDC-Fluorescein and 525 nm for labeled PF-68). Emitted fluorescence was scanned from 515 to 600 nm (5 nm steps) for CDC-Fluorescein and from 530 to 650 (5 nm steps) for labeled PF-68.

5.3.3 Culture media

All reagents were from Sigma-Aldrich Canada, Ontario, Canada, unless indicated. *SCM for chondrocytes* (Serum-Containing Medium used for cell expansion) was composed of DMEM low glucose (Invitrogen, Ontario, Canada) supplemented with 0.4 mM proline, non-essential amino-acids 1X (containing 8.9 mg/L alanine, 15 mg/L asparagine, 13.3 mg/L aspartic acid, 14.5 mg/L glutamic acid, 7.5 mg/L glycine, 11.5 mg/L proline and 10.5 mg/L serine), 22 mM sodium bicarbonate, 12.5 mM HEPES, penicillin/streptomycin 1X (containing 100 U/mL penicillin and 0.1 mg/mL streptomycin), 10 % FBS and 30 μg/mL ascorbate, the latter added fresh. *SFM for chondrocytes* (Serum-Free Medium used for PF-68 experiments, see Gigout et al., 2005) was a 1/1 (v/v) mix of calcium-free HAM's F12 (US Biological, MO, USA) and calcium-free DMEM low glucose (US Biological, MO, USA) supplemented with 0.4 mM proline, 1.5 mM glutamine, 22 mM sodium bicarbonate, 1 mM $CaCl_2$ non-essential amino acids 1X, 12.5 mM HEPES, penicillin-streptomycin 1X, ITS^+ (10 μg/mL insulin, 5.5 μg/mL transferrin, 0.05% w/v bovine serum albumin, 1.7 mM linoleic acid, 0.5 μg/mL sodium selenite), $5x10^{-5}$ M β-mercaptoethanol and 10^{-8} M dexamethasone, rhEGF, rhPDGF-BB, rhFGF-2, all 2 ng/mL (from R&D Systems, MN, USA). Ascorbate, 30 μg/mL, was added fresh. *Medium for CHO cells* was composed of Biogro-CHO culture medium (Biogro Technologies Inc., Manitoba, Canada) supplemented with 7.5 mg/L phenol red, 3.5 mM glutamine and 2.7 mM glucose.

5.3.4 Chondrocytes isolation and culture

Cells were isolated from the femoropatellar groove of a 1-2 month calf knee, obtained from a local butcher within 24 h of slaughter. Briefly, cartilage was sequentially digested, first for 90 min by protease Type XIV (Sigma-Aldrich Canada, Ontario, Canada) 56 U/mL at 37°C in DMEM high glucose (Invitrogen, Ontario, Canada) supplemented with 22 mM sodium bicarbonate and 1X penicillin/streptomycin, and then for 3 h by collagenase CLS2 (Worthington, NJ, USA) 752 U/mL at 37°C in DMEM high glucose supplemented with 22 mM sodium bicarbonate, penicillin/streptomycin and 5 % FBS. Released cells were then filtered through a 200 μm mesh (using an autoclaved 200 μm screen mounted on a screen cup, Sigma-Aldrich Canada, Ontario, Canada), centrifuged (190 *g*, 10 min at 4°C) and filtered again through two serial 20 μm filters using a Swinnex filter holder (Fisher Scientific, Quebec, Canada) containing a 20 μm Spectra/Mesh Nylon Macroporous Filter (Spectrum Laboratories, CA, USA). Cells were then cultured in monolayer inoculated at 2×10^6 cells per 100 mm Petri dish with 10 mL of SCM. The SCM was used to sustain rapid cell proliferation in monolayer and to obtain enough cells for all experiments. Cells were harvested at confluence and passaged 1:4 by Trypsin/EDTA digestion (0.25 % Tryspin and 0.38 g/L EDTA 4 Na in Calcium/Magnesium free HBSS, Invitrogen Ontario, Canada). No more than 4 passages were done. All suspension cultures were performed using the SFM which was previously optimized

for suspension culture to enable phenotype maintenance and limit cell aggregation (Gigout et al., 2005).

5.3.5 CHO cells culture

CHO (Chinese Hamster Ovaries) cells (ATCC, CRL9606) adapted to serum-free suspension culture were kindly provided by Dr. Michael Butler (University of Manitoba). Cells were cultured in monolayer, harvested at confluence and passaged 1:4 by Trypsin/EDTA digestion.

5.3.6 Cell staining and confocal microscopy

10^6 cells/mL were incubated for different times (1.5 h, 18 h, 3 or 7 days) at different concentrations of labeled PF-68 (from 0.01 to 0.2 % w/v). To maintain the cells in suspension, BSA-coated 15 mm Petri dishes were used (incubation overnight with 1% BSA in PBS). For longer incubation times (3 and 7 days) and for chondrocytes only, Petri dishes were coated with 2 % SeaPlaque low-melting-temperature agarose (Mandel, Quebec, Canada) diluted in 1/1 (v/v) PBS and SFM without ITS^+, β-mercaptoethanol, dexamethasone, growth factors and ascorbate.

To stain endocytic compartments, cells were incubated for at least 18 h with Dextran-Alexa 647, 50 μg/mL (10 kDa, Molecular Probes, OR, USA) and Dextran was always left in the media during the entire incubation time. Nucleus staining was performed 1 h before observation, by adding Hoechst 33258 (Molecular Probes, OR, USA) at 4 μg/mL to the culture medium. All incubations were done in Biogro-CHO for

CHO cells, or in SFM for chondrocytes. For each condition, a negative control was systematically added, where labeled PF-68 was replaced by unlabeled PF-68. Extra controls were also performed with CDC-Fluorescein alone or a mix of CDC-Fluorescein and PF-68 in a ratio corresponding to the 100% labeled PF-68 experiments. No staining was detected for all negative controls.

Before observation, cells were centrifuged (300 *g*, 5min) and resuspended in the same medium without fluorochrome, at the same (unlabeled) Pluronic F-68 concentration, unless indicated. Confocal-imaging was performed using an Apochromat 63X/1.0 VIS-IR water immersion objective mounted on an Axioplan 2 microscope equipped with an LSM 510 META confocal laser scanning module (Zeiss, Ontario, Canada). Labeled PF-68 was excited with the 514 nm line from an argon laser, Dextran-Alexa 647 with a 633 nm Helium-Neon Laser and Hoechst 33258 was illuminated with two–photon excitation using a mode-locked pulsed Ti-saphire laser (VerdiV10/Mira 900 from Coherent Inc., CA, USA) operating at 780 nm. Images were recorded using a BP 535/590 IR bandpass filter for labeled PF-68, a BP 644-687 meta filter for Dextran-Alexa 647 and a BP 435-485 IR bandpass filter for Hoechst 33258.

5.3.7 PF-68 quantification

The method has been previously described (Ghebeh et al., 1998). Briefly, 100 μL of PF-68 standards (in PBS from 0.01 to 0.2% w/v) or samples, 100 μL of cobalt thiocyanate solution (0.3 g/L cobalt nitrate, 2 g/L of ammonium thiocyanate in ddH_2O) and 200 μL of ethyl acetate were mixed. The solution was vortexed and centrifuged at

11,500 *g* for 3 min. A blue pellet appeared, corresponding to the thiocyanate-Pluronic complex. The supernatant was then discarded and the pellet resuspended in 200 μL of ethyl acetate. The solution was centrifuged again at 11,500 g for 3 min. These washing steps were repeated 6 to 8 times until the supernatant appeared clear and colorless. After the last wash, the pellet was resuspended in 1 mL of acetone and the absorbance was read at 624 nm. To quantify PF-68 in the presence of cells, 200 μL of standards (from 0.005 to 0.4% w/v, in culture medium) or samples were mixed with 80 μL ethanol in addition to the 100 μL cobalt thiocyanate solution and 200μL ethyl acetate. Doubling the sample volume lowered the detection limit to 0.005 % (w/v). Quantification was found to be linear from 0.005 to 0.4 % w/v (R^2=0.999) when using 200 μL of standards diluted in the media used for chondrocytes or CHO cells. The calibration curve was repeated at different days, systematically before each experiment, and the same level of reproducibility has been observed (data not shown).

5.3.8 Pluronic uptake assays

CHO cells or chondrocytes were incubated in 300 μL of Biogro-CHO medium or SFM respectively, at different cell concentrations (from 1 to 4×10^6 cells/mL), for different times (from 1.5 h to 3 days) and with different PF-68 concentrations (from 0.01 to 0.2 % w/v), in polypropylene 96-well plate. Polypropylene was used because PF-68 absorbs to standard polystyrene plates, leading to inconsistent results. Moreover, the cells did not adhere to polypropylene and remained in suspension. Incubations were done at 37°C under 5% CO_2. Each experiment was performed in triplicate (n=3). For

each condition, a negative control omitting cells was used to detect any artifact due to PF-68 adsorption onto the tubes' walls, or evaporation. After the desired incubation time, cell suspensions were centrifuged 5 min at 300 *g*. PF-68 concentration was evaluated in 200 µL samples of the supernatant (see PF-68 quantification). To calculate PF-68 uptake by the cells, the results obtained for the control without cells were averaged and the final PF-68 concentration in presence of cells was subtracted, for each of the triplicates. Remaining media were discarded and cell pellets were resuspended in 1/1 (v/v) PBS and Trypan Blue (4 % w/v in PBS) for cell counting and viability estimation on a hemacytometer.

5.3.9 Statistical analysis

Statistical analysis was performed with STATISTICA 6.1 (StatSoft Inc., Tulsa, OK, USA). Significant effects ($p < 0.05$) of cells and PF-68 concentrations were tested following the general linear model routine with a univariate test of significance and a Tukey HSD post-hoc test, with PF-68 initial concentration or cell concentration as a continuous predictor and absence/presence of cell as a categorical predictor. To compare labeled and unlabeled PF-68 uptake, the categorical predictor was the type of PF-68 used (labeled or unlabeled).

5.4 Results

5.4.1 PF-68 labeling with fluorescein

PF-68 was conjugated with CDC-Fluorescein as described (Fig. 5-1 A) in Materials and Methods. Reaction products were analyzed by gel exclusion chromatography and the collected fractions were analyzed for their content in Pluronic and Fluorescein. Results showed that Pluronic and Fluorescein were found within the same early fractions (Figure 5-1 B). Since the only explanation for fluorescein to be eluted rapidly is to be bound to Pluronic, it was thus concluded that the majority of the fluorochrome was conjugated to Pluronic and that the labeling reaction was highly efficient. Finally, the four fractions with the highest Pluronic content were pooled (represented by closed symbols on Figure 5-1 B) and used in the following experiments.

Fluorescence spectra of CDC-Fluorescein and Fluorescein bound to Pluronic were characterized. Interestingly, the Fluorescein fluorescence spectrum was modified after conjugation to Pluronic, which is consistent with previous reports (Rapoport et al., 2002). Fluorescein exhibited a maximum excitation at 505 nm and a maximum emission at 535 nm (Figure 5-1 C), as specified by the manufacturer. However, Fluorescein-PF-68 emission and excitation maxima were shifted toward higher wavelengths to reach 525 nm and 550 nm, respectively (Figure 5-1D). Consequently, these latter wavelengths were used in the following study to localize labeled PF-68 in cells.

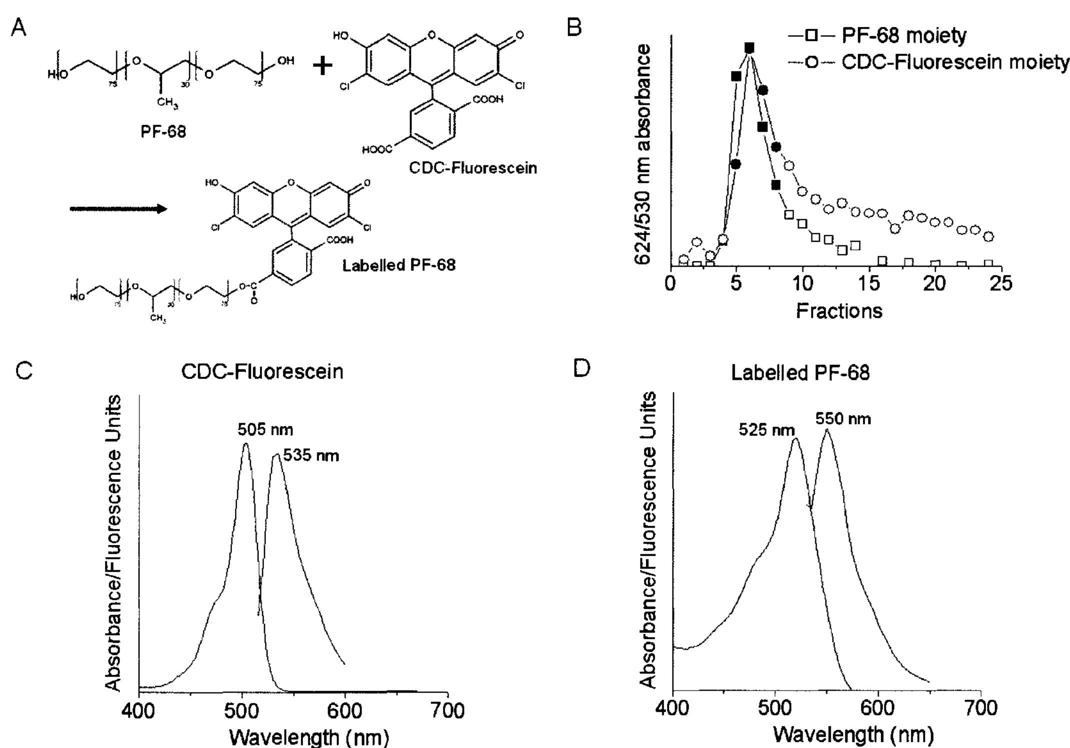

Figure 5-1 : (A) Reaction scheme of PF-68 derivatization with CDC-Fluorescein. (B) Reaction products were analyzed by exclusion chromatography. In each fraction of 0.5mL, PF-68 was assayed as described in Material and Methods and presence of CDC-Fluorescein was assessed by absorbance at 530 nm. In the first fractions, CDC-Fluorescein and PF-68 were detected, indicating that these two molecules are linked. Closed symbols represent fractions harvested for further experiments. **(C) Excitation and Emission spectra of free CDC-Fluorescein and (D) bound CDC-Fluorescein to PF-68.** Spectrum of linked CDC-Fluorescein is shifted toward higher wavelength. Excitation/Emission maxima were 505/535 and 525/550 for free CDC-Fluorescein and CDC-Fluorescein linked to PF-68 respectively.

5.4.2 PF-68 cell internalization and localization

Labeled PF-68 was used to determine whether PF-68 enters the cells and into which intracellular compartments. Most of the internalization assays were performed at 0.1 % (w/v) PF-68 which is within the usual range used in animal cell suspension cultures (from 0.05 to 0.2 % w/v, Chisti, 2000; Xu et al., 1995). Chondrocytes or CHO cells were incubated at 10^6 cells/mL, for 1.5 h or 18 h with 0.1 % (w/v) labeled PF-68 (Figure 5-2). In parallel for the same cells, endocytic compartments were stained with Dextran-Alexa 647 (red channel) and the nucleus with Hoechst 33258 (blue channel). A punctuate staining corresponding to labeled PF-68 was observed after 1.5 h at the chondrocytes cell membrane (Figure 5-2 A), and this staining was not co-localized with Dextran-Alexa 647. On the contrary, after 18 h, labeled PF-68 was observed to be intracellular and co-localized with Dextran-Alexa 647 (Figure 5-1 B). For CHO cells, labeled PF-68 has been readily observed to be intracellular after 1.5 h, co-localized with Dextran-Alexa 647 (Figure 5-2 C). Thereafter, the staining distribution did not evolve from 1.5 to 18 h but became more intense (Figure 5-2 D), meaning that cells have continued to accumulate labeled PF-68. Longer incubation times (3 and 7 days) with labeled PF–68 did not reveal any evolution of the staining pattern. Even after 7 days, labeled PF68 was still co-localized with Dextran-Alexa 647 in CHO cells and chondrocytes (data not shown). Then, to assess whether there was an effect of the culture medium content in PF-68 on its uptake behavior, cells were incubated for 18 h

at different labeled PF-68 concentrations from 0.01 to 0.2 % (w/v), but no differences were observed (data not shown).

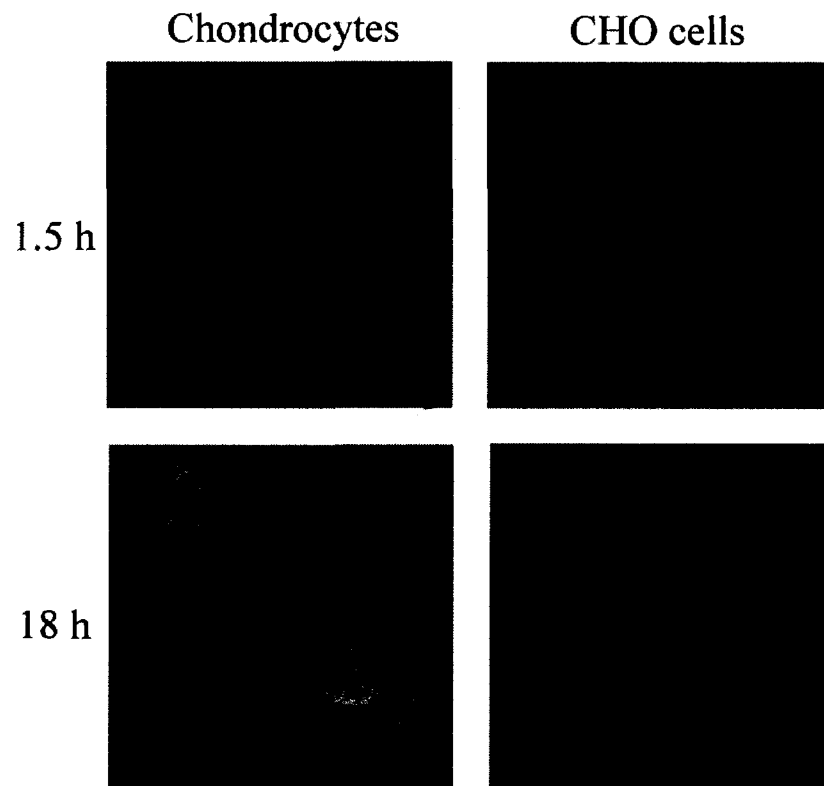

Figure 5-2 : Chondrocytes (A, B) or CHO cells (C, D) at 10^6 cells/mL were incubated with 0.1 % (w/v) of labeled PF-68 for 1.5 h (A, C) or 18 h (B, D). Labeled PF-68 appears in green. Endosomes/lysosomes were stained in red (Dextran-Alexa 647, incubated 18 h), and nucleus in blue (with Hoechst 33258, incubated 1 h). Scale bar = 10 μm.

5.4.3 Quantification of PF-68 uptake

Once it was confirmed that PF-68 enters the cells, PF-68 internalization was quantified. Commonly used PF-68 concentrations vary from 0.05 to 0.2 % (w/v) (Chisti, 2000; Xu et al., 1995) and thus this range of PF-68 concentration was used with CHO cells. However, as the PF-68 may affect cell phenotype, a critical parameter for chondrocytes, lower PF-68 concentrations were used with chondrocytes (from 0.01 to 0.1% w/v). All cells have been expanded in a PF-68 free medium and are thus devoid of Pluronic at the beginning of each experiment. First, to estimate the uptake of PF-68 by chondrocytes, cells were incubated at different concentrations (from 1×10^6 to 4×10^6 cells/mL) with 0.05% (w/v) of PF-68 (Figure 5-3 A). After 18 h, cells were removed by centrifugation and the remaining amount of PF-68 in the medium was measured. The uptake of PF-68 was proportional to cell concentration, reaching 39.3 % of the initial amount, in the presence of 4×10^6 cells/mL (Figure 5-3 A) and was significantly different from the control at all cell concentrations tested. The final cell content in PF-68 at 18 h was similar for all experiments (with 1×10^6, 2×10^6 and 4×10^6 cells/mL) with an average of 66.2 ± 5.8 (SEM) μg of PF-68/10^6 cells (Figure 5-3 C). The effect of distinct PF-68 concentrations on its uptake by chondrocytes was then characterized (Figure 5-3 B and D) by incubating 10^6 cells/mL with 0.01, 0.05 or 0.1 % (w/v) of PF-68 for 18 h. Cell uptake represented a decrease of 0.006 % (w/v) of the different initial PF-68 concentrations. For the lower polymer concentration tested (0.01 % w/v), it means that the cells have taken up 60 % of the initial medium content in Pluronic. This

illustrates the impact of cell uptake on Pluronic concentration in the culture medium when low initial concentrations have to be used. As showed previously, under all conditions tested, final PF-68 concentrations in the presence of cells were found to be significantly lower than the controls (without cells). Finally, it was observed that the uptake of chondrocytes was 57.8 ± 19.5 (SEM) μg Pluronic F-68/10^6 cells (mean of all results showed in Fig. 5-3 D) independently of the initial PF-68 concentration involved.

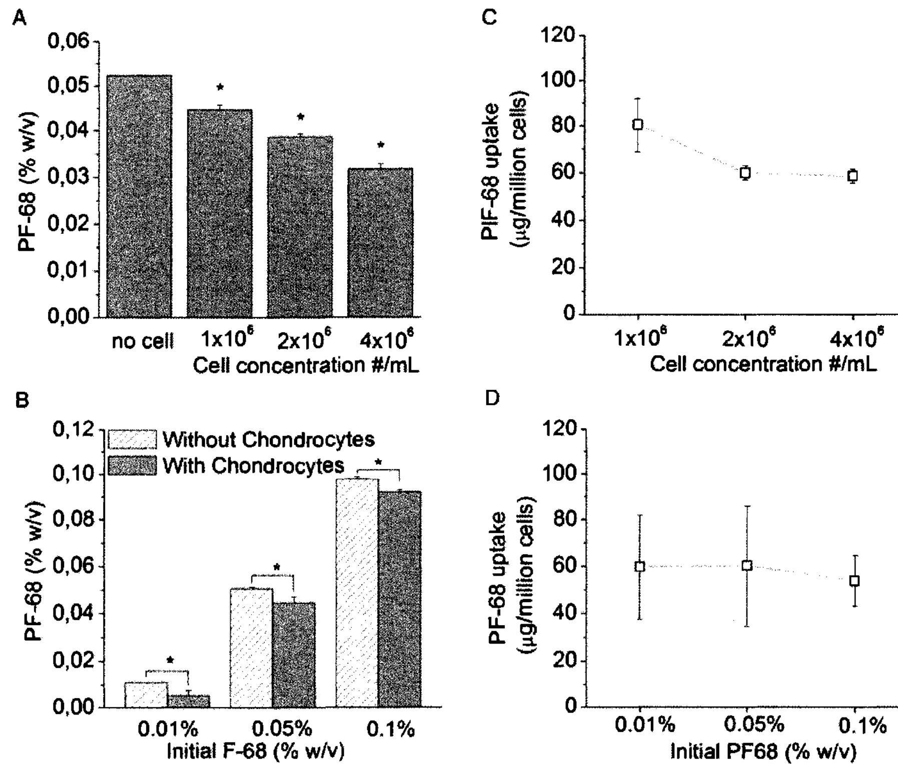

Figure 5-3 : (A) Chondrocytes at different concentrations were incubated with 0.05 % (w/v) PF-68 or (B) chondrocytes at 10^6 cells/mL were incubated with different PF-68 concentrations. After an incubation of 18 h at 37°C, final PF-68 concentration was measured in the medium. * Indicates samples statistically different from the control without cells (see Materials and Methods). **(C-D) PF-68 uptake/10^6 cells was calculated for the different conditions.** This uptake was found to be independent of cell densities and initial PF-68 concentration. Mean ± SEM (n=3) are shown.

The same experiments were performed with CHO cells, but at higher PF-68 concentrations. In contrast to chondrocytes, the presence of CHO cells only weakly affected PF-68 concentration in the medium. Hence statistical analysis revealed that the decrease in PF-68 concentration in presence of 1×10^6 and 2×10^6 cells/mL was not significantly different than for the control (Figure 5-4 A). However, the experiment with 4×10^6 cells/mL showed a significant decrease of 0.005 % (w/v) which represented 2.4 % of the initial Pluronic concentration, after 18 h (Figure 5-4 A). This decrease corresponded to an average cell uptake of 12.8 ± 11.6 (SEM) µg Pluronic F-68 /10^6 cells (mean of all results showed in Figure 5-4 C). Considering these results, it was decided to use a cell density of 4×10^6 cells/mL in the following experiments with CHO cells. As for chondrocytes, the effect of PF-68 concentration on its uptake was then characterized and PF-68 concentrations of 0.05, 0.1 and 0.2 % (w/v) were used (Figure 5-4 B). The uptake of PF-68 was similar in all the conditions tested, with a decrease in polymer concentration of 0.0041, 0.0044 and 0.0058 % (w/v) respectively after 18h, for initial concentrations of 0.05, 0.1 and 0.2 % (w/v) (Figure 5-4 B). These decreases were found to be significant in comparison to controls without cells. Finally, similarly to chondrocytes, CHO cells showed an uptake at a constant amount of polymer with 11.9 ± 7.9 (SEM) µg PF-68/10^6 cells for all experimental conditions tested (mean of all results showed in Figure 5-4 C and D, obtained with a cell concentration of $4x10^6$ cells/mL). Interestingly, CHO cells showed an uptake about 4 times less PF-68 than chondrocytes.

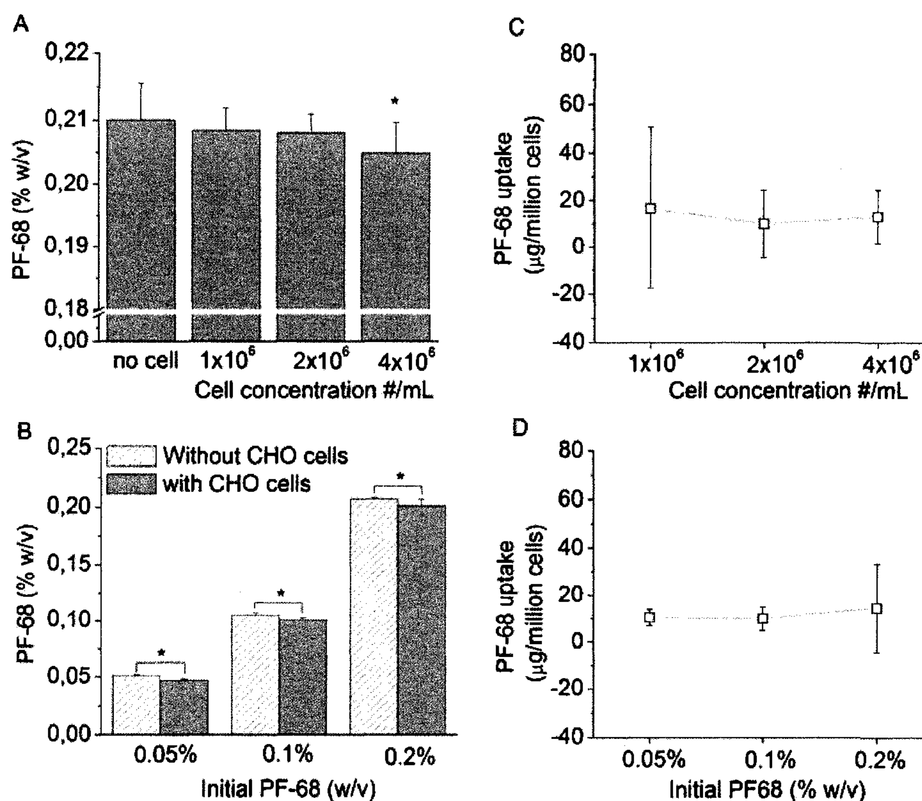

Figure 5-4 : (A) CHO cells at different concentrations were incubated with 0.2 % (w/v) PF-68 or (B) CHO cells at 4×10^6 cells/mL were incubated with different PF-68 concentrations. After an incubation of 18 h at 37°C, final PF-68 concentration was measured in the medium. * Indicates samples statistically different from the control without cells (see Materials and Methods). **(C-D) PF-68 uptake /10^6 cells was calculated for the different conditions.** This uptake was found to be independent of cell densities and initial PF-68 concentrations tested. Mean ± SEM (n=3) are shown. Negative values of uptake contained within the error bar range are due to uptake representing the difference in medium PF-68 concentration without cells versus with cells and variability in these values.

5.4.4 Effect of agitation on PF-68 uptake

The effect of agitation on PF-68 uptake was investigated by performing incubation assays on an orbital shaker at 80 rpm. Experiments were performed with CHO cells and chondrocytes at the same initial Pluronic concentrations as the previous experiments performed under static conditions (Table 5-2). Agitation did not modify the cells' uptake in PF-68, compared to static culture conditions. CHO cells uptake of 11.6 ± 5.0 (SEM) μg and 11.5 ± 6.7 (SEM) μg of PF-68/10^6 cells were observed in agitated and static conditions respectively, and values of 44.0 ± 7.3 (SEM) μg and 57.8 ± 19.5 (SEM) μg PF-68/10^6 cells were measured for chondrocytes in agitated and static conditions respectively. For CHO cells and chondrocytes, uptake values obtained in agitated and static conditions were not statistically different. In parallel, to assess whether agitation affects PF-68 distribution in cells, chondrocytes or CHO cells were incubated at 10^6 cells/mL with 0.1 % (w/v) of labeled PF- 68 on an orbital shaker at 80 rpm for 18 h. The cell staining distribution profile under agitated conditions were similar to those observed in static conditions (Figure 5-5).

Tableau 5-2 : Comparison of PF-68 uptake in agitated and static conditions. CHO cells and chondrocytes were cultured at 4×10^6 cells/mL and 1×10^6 cells/mL, respectively, with 0.05, 0.1 and 0.2 % (w/v) PF-68 for CHO cells and with 0.01, 0.05 and 0.1 % (w/v) PF-68 for chondrocytes. Samples were incubated at 37°C for 18h under orbital agitation (80 rpm) or under static conditions. PF-68 uptake in µg/106 cells at the 3 different PF-68 concentrations were averaged (n=9). Difference between agitated and static conditions, for both cell types, were not significantly different, while chondrocytes took up significantly more PF-68 per cell than for CHO cells.

CHO cells		Chondrocytes	
µg PF-68 uptaken / 10^6 cells ± SEM			
Agitated	Static	Agitated	Static
11.6±5.0	11.5± 6.7	44.0± 7.3	57.8± 19.5

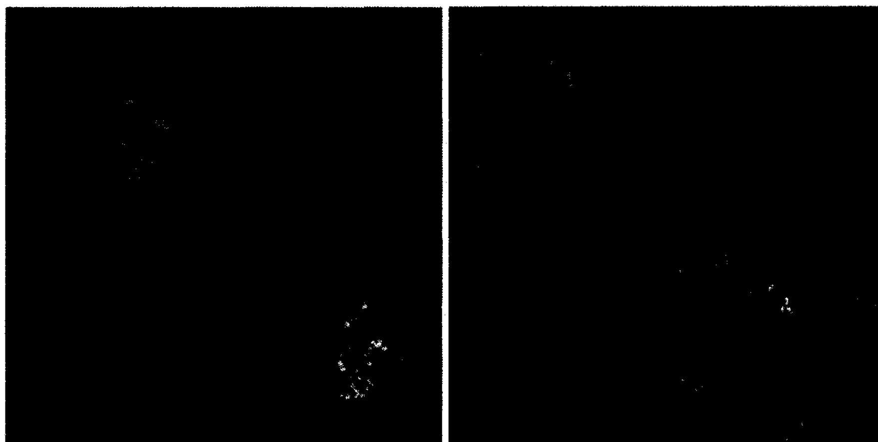

Figure 5-5 : (A) Chondrocytes or (B) CHO cells were incubated at 10^6 cells /mL with 0.1% (w/v) labeled PF-68 for 18 h under agitation. Green is PF-68, endosomes/lysosomes are red and the nucleus blue (see Figure 2 caption for details). Scale bar: 10μm.

5.4.5 PF-68 accumulation in cells

Remaining PF-68 concentration was measured in the culture medium after 1.5, 18 and 72 h in order to cover a whole cell passage. Chondrocytes (1×10^6 cells/mL) or CHO cells (4×10^6 cells/mL) were incubated with 0.05 or 0.1 % (w/v) of PF-68 respectively and evolution of PF-68 concentration was evaluated with time (Figure 5-6). The medium content in PF-68 decreased readily after 1.5 h in both chondrocytes and CHO cells cultures, reaching 93 % and 95 % of the initial value, respectively. Then, cultures with chondrocytes showed no further significant decrease in PF-68 medium content. During that period (0-72 h) there was no cell growth as previously reported (Gigout et al., 2005). CHO cells cultures behaved differently than

chondrocytes since PF-68 concentration decreased and showed no signs of stabilization for the time period assayed (0-72 h). The specific decrease in the medium content in PF-68 can be explained by cell growth from 4×10^6 to 5.5×10^6 cells/mL in 3 days. These results suggest that the cells have a maximum accumulation capacity which differs with cell origin.

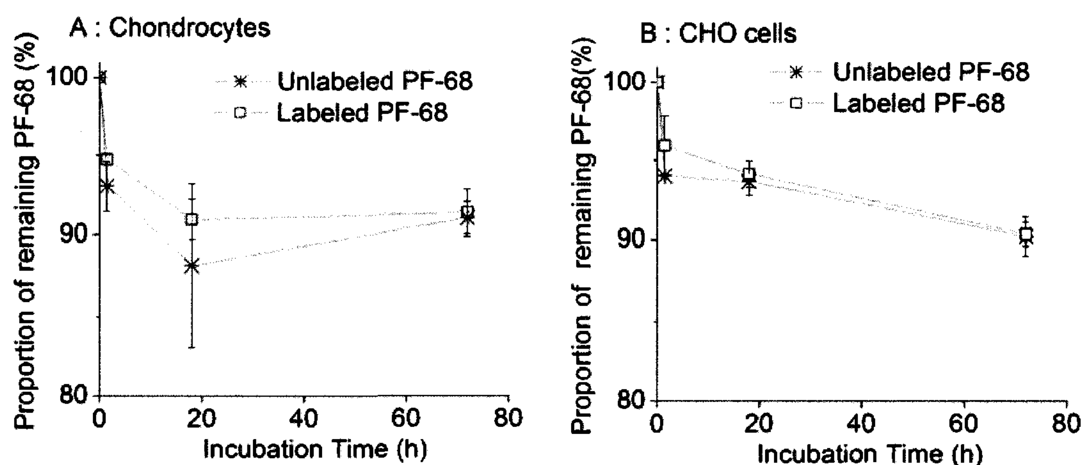

Figure 5-6 : (A) Chondrocytes at 10^6 cells/mL or (B) CHO cells at 4×10^6 cells/mL were incubated at 37°C with 0.05 % and 0.2 % (w/v) respectively of PF-68 or labeled PF-68. After 1.5 h, 18 h or 3 days of incubation, final PF-68 concentration was measured in the medium, and the proportion of remaining PF-68 was calculated (Final PF-68 concentration x 100 /Initial PF-68 concentration). Mean ± SEM (n=3) are shown. Uptake of labeled and unlabeled PF-68 were found to be not statistically different.

5.4.6 Tracking of internalized labeled PF-68

To examine if cells were able to discard intracellular labeled PF-68, either by lysosomal degradation or by excretion, 1×10^6 cells/mL were incubated 18 h with labeled PF-68, then rinsed with fresh medium not containing PF-68 and cultured 7 days without the polymer. After 7 days the fluorescence due to labeled PF-68 was still intense in chondrocytes (Figure 5-7 A) whereas it was barely visible in CHO cells (Figure 5-7 B). CHO cells exhibited a fast growth and were passaged once during this experiment (after 3 days of culture), meaning that labeled Pluronic might have been partly diluted among daughter cells. Cells could have also degraded or excreted the labeled polymer. In contrast to CHO cells, chondrocytes cultured in suspension and in a serum-free medium exhibited a slow growth, and labeled PF-68 could not have been diluted by cell division. Therefore, there was no clear sign of labeled PF-68 degradation or excretion in chondrocytes.

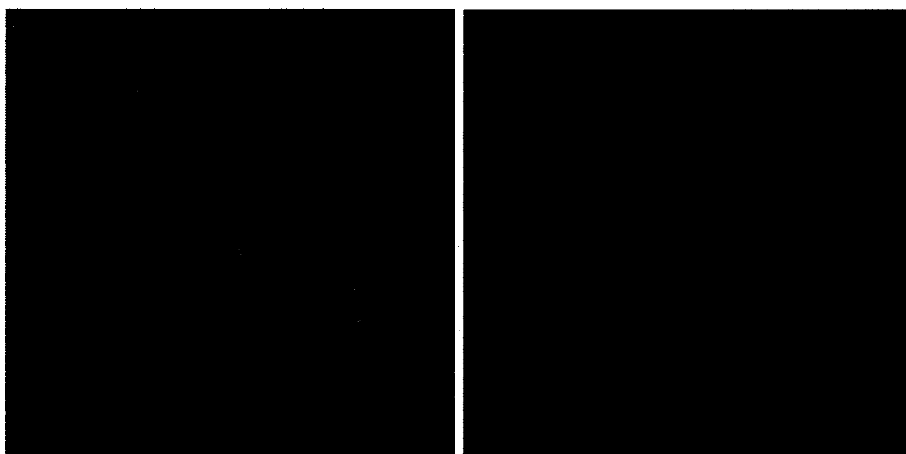

Figure 5-7 : (A) Chondrocytes or (B) CHO cells at 10^6 cells/mL were incubated with 0.1 % (w/v) of labeled PF-68 for 18 h and then cultured 7 days in a medium without PF-68. Green is PF-68, endosomes/lysosomes are red and the nucleus blue (see Figure 2 caption for details). Scale bar: 20μm.

5.4.7 Comparing the cell uptake of labeled or unlabeled PF-68

Finally, the influence of the fluorescein moiety of labeled PF-68 on cell behavior was evaluated. The amount of internalized Pluronic was found to be the same for labeled or unlabeled Pluronic, and for both cell types over a 72 h time period (Figure 5-6 A and B). Growth and cell viability of CHO cells were found to be similar over a 3 day culture in the presence of labeled or unlabeled PF-68 (data not shown). For chondrocytes, there was no cell count because cells form tryspin-resistant aggregates when grown in suspension. Nevertheless, cells behavior and aggregation kinetics were found to be similar using labeled or unlabeled PF-68 (data not shown).

5.5 Discussion

PF-68 is a surfactant commonly used to protect cells from shear stress. Some studies demonstrated that it interacts with cell membranes but it was still unknown where the polymer is able to enter the cells. This work shed light on cell-PF-68 interactions by combining the use of a fluorescent derivative of PF-68 to track it in the cells and evaluation of cellular Pluronic uptake.

We clearly demonstrated that PF-68 is taken up by the mammalian cells studied and that its fluorescent derivative is internalized in cells and transit in the endocytic pathway where it colocalized with Dextran-Alexa 647 (MW, 10 kDa), a molecule commonly used to stain endosomes and lysosomes (Berthiaume et al., 1995; Bright et al., 2005; Lencer et al., 1990; Wang and Goren, 1987). The typical endocytic pathway consists in the internalization of endocytosed molecules in early endosomes at first, which are localized just beneath the plasma membrane. Then, molecules can be recycled back to the plasma membrane or, after 5-15 min are directed to late endosomes, which are close to the Golgi apparatus and the nucleus. Finally, endocytosed materials travel to lysosomes for degradation or accumulation. Localization of Dextran molecules in the cell depends on their MW and also on the time lap between Dextran removal (after a pulse of Dextran) and cell observation (chase period). For example, Bright et al., 2005 observed an accumulation of fluorescent Dextran (10 kDa) in late endosomes after a 5 min chase and in lysosomes after a 20 h chase. In all experiments done in the present study, Dextran-Alexa 647 was always incubated at least 18 h, and was then left in the medium so that the whole

endocytic pathway was probably stained, i.e. lysosomes and endosomes. But as there was a delay between removal of fluorochromes and microscopic observations (about 10 min), it can be estimated that only late endosomes and lysosomes were stained by Dextran-Alexa 647 (i.e. not early endosomes) at the time of observation. Thus, the co-localization of labeled PF-68 with Dextran-Alexa 647 shows that labeled Pluronic accumulates in late endosomes and lysosomes. Labelled PF-68 did not accumulate in the cytoplasm or in the nucleus as previously observed with PP-85 and PL-121 (Batrakova et al., 2003). It may seem surprising that fluorescein-labeled Pluronic can be detected in acidic compartments as lysosomes. Fluorescein is known to be pH-sensitive, and its fluorescence is greatly reduced at low pH (Leonhardt et al., 1971). The labeled PF-68 also exhibits pH sensitivity but the fluorescence intensity decreases by only 37 % from pH 7 to 5 (data not shown). Thus, the fluorescence intensity of labeled PF-68 in lysosomes remained high enough to be detected.

In the case of chondrocytes, fluorescent PF-68 after 1.5 h seemed to be accumulated near to the membrane at defined areas but was not co-localized with Dextran-Alexa 647. It is difficult to determine if the staining was extracellular (accumulation on the outer surface of the membrane), at the membrane (accumulation within the membrane) or intracellular (in early endosomes). The latter is the more probable hypothesis considering the punctuate staining observed. This two-steps process was not observed in CHO cells, where the polymer was already co-localized with Dextran after 1.5 h. Thus, the labeled Pluronic trafficking was faster in CHO cells since the polymer arrived in late endosomes/lysosomes earlier.

We expected to observe a continuous membrane staining, at any incubation time, as the shear protectant effect of PF-68 is supposed to be due to its interaction with the plasma membrane. For instance, it has been reported that PF-68 decreased the plasma membrane fluidity (Ramirez and Mutharasan, 1990), and increase membrane strength (in TB/C3 hybridoma, Zhang et al., 1992). Moreover, Pluronic polymers PL-61 and PP-85 are known to induce drastic changes in the microviscosity of cell membranes, especially in tumor cells (Melik-Nubarov et al., 1999). The authors attributed these changes to the alterations in the structure of the lipid bilayer as a result of adsorption of the block copolymer on the membrane. In some of our samples, the cell periphery was faintly stained with labeled PF-68, but it was difficult to distinguish this staining from auto-fluorescence, whereas concentration in intracellular vesicles was detectable and distinct from autofluorescence. For instance, this accumulation of PF-68 in chondrocytes corresponded to a significant level at about 8 times cellular DNA content (7 pg/cell, Kim et al., 1988) for example. Thus, shear protection conferred by PF-68 may be partly due to alteration and stiffening of cytoplasmic cell mechanical properties by the high PF-68 concentration in intracellular vesicles.

Another interesting finding arising from this study is that PF-68 uptake in cells was found to be cell-type dependant. CHO cells absorbed 11.7 ± 6.7 (SEM) μg PF-68/10^6 cells and chondrocytes 56 ± 10.9 (SEM) μg/10^6 cells. This dependence can rely on differences in cell surface composition, but also from differences in the endocytic pathway. As the shear protective effect of PF-68 has been reported to be proportional to its concentration (Ma et al., 2004; Zhang et al., 1992), it is important to know if this

uptake phenomenon can significantly affect the Pluronic content in the culture medium. For CHO cells, a 0.2 % (w/v) PF-68 concentration is commonly used (Keane et al., 2003; Schroder et al., 2004) and we showed that the presence of cells only slightly affects this concentration. At 0.2 % (w/v) with 4×10^6 cells/mL, only a 2.4 % decrease of the initial concentration was observed, after 18h. At the end of a classical bioreactor culture, cell densities can reach up to 10×10^6 cells. Since PF-68 concentration decrease in the medium is proportional to the cell density, it can result in a decrease of 6 % of the initial Pluronic concentration, strictly from cell uptake. In the case of chondrocytes the changes in PF-68 concentration can be problematic as these cells uptake more Pluronic. When an initial PF-68 concentration of 0.01 % (w/v) was used, up to 60 % of the Pluronic content was taken up by the cells after 18 h, in presence of 1×10^6 cells/mL. This result means that if cell density doubles, there will be no PF-68 remaining in the medium. Finally, PF-68 uptake by CHO cells and chondrocytes on a per cell basis was found to be independent of initial Pluronic or cell concentration, and was similar for agitated or static culture conditions.

Subsequently, it was observed that CHO cells but not chondrocytes were able to diminish the intracellular labeled PF-68 content to a point it can barely be detected after 7 days. This can be explained either by labeled PF-68 dilution by cell division, and/or by exocytosis which is known to occur in CHO cells (Adams et al., 1982; Buckmaster et al., 1987). On the contrary, results seemed to indicate the inability of chondrocytes to eliminate Pluronic, hence the use of these chondrocytes cultured in the presence of PF-68 for cartilage repair therapy needs to be evaluated. PF-68 has already been studied in

clinical trials for its hemorheological properties as a treatment for sickle cell disease (patient where hemoglobin molecules tend to aggregate and block blood vessels) and is known to be well tolerated (Adams-Graves et al., 1997; Orringer et al., 2001). These clinical studies infused of several hundred mg/Kg of PF-68. By comparison, in the case of cartilage repair, 5×10^6 cells are used for 1.6 to 6.5 cm^2 defects (Brittberg et al., 1994), corresponding to about 75 μg of PF-68 carried by these cells. From this point of view, culture of mammalian cells in PF-68 prior to transplantation may not present an obvious risk. However the effect of this polymer on cell phenotype is still not known and need to be characterized.

One limitation of this study was that the stability of labeled PF-68 in the cell is unknown. Lysosomes are the principal sites of intracellular digestion and are filled with hydrolytic enzymes. In these compartments, the bond linking fluorescein to PF-68 could have been hydrolyzed, resulting in two independent molecules. However, a lot of studies have been done with labeled polymer as dextran or other Pluronics to study the endocytic pathway and lysosomes, without noticing a separation of the dye and the polymer. For instance, Dextran-FITC has been shown to be stable *in vivo* and *in vitro* (Schroder et al., 1976). Another uncertainty lies in the unknown effect of the fluorescein moiety on PF-68 trafficking in cells. However it is believed that the effect of fluorescein is weak or absent. First, the uptake of unlabeled PF-68 and labeled PF-68 by CHO cells and chondrocytes were compared (Figure 6) and were not found to be different. Secondly, in a previous study (Batrakova et al., 2003) using a similar approach, the intracellular localization of four different Pluronics (F108, P85, L35 and

L121) labeled with fluorescein were compared. Depending on the Pluronic used, some cells were not stained, or shown a cytoplasmic or an endocytic staining. In can then be concluded that the distribution of Pluronic in cells is unlikely to be driven by the fluorochrome. On the contrary, Berthiaume et al., 1995 studied endocytosis with similar dextran but conjugated to fluorescein or Texas Red and they observed similar dextran uptake and localization in cells, for both dyes. Once again it is an indication that the fluorochrome has no or little influence in the polymer localisation in the cells.

5.6 Conclusion

In this study, it was clearly demonstrated for the first time that PF-68 enters mammalian cells, and strong evidences indicate that it accumulates in the endocytic pathway. PF-68 uptake was also found to be cell-type dependant, but to be cell concentration independent as well as initial PF-68 concentration independent. Finally, it was established that cellular uptake of PF-68 was similar for agitated and static culture conditions. In the light of these results, one should keep in mind that PF-68 concentration can be significantly affected by the cells in culture, especially when high cell density cultures are performed. This study also suggests cell inability to eliminate PF-68, which is a concern in biomedical issues when cells have to be implanted in a patient.

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CHAPITRE 6. CHONDROCYTE AGGREGATION IN SUSPENSION CULTURE IS GFOGER-GPP AND β1 INTEGRIN DEPENDENT

6.1 Abstract

Phenotypically stable isolated chondrocytes form aggregates in suspension culture that maintain chondrocyte phenotype in a physiological pericellular environment. The molecular mechanisms involved in chondrocyte aggregation have not been previously identified. Using this novel system, we performed mRNA and protein expression analysis along with immunohistochemistry for potential cell adhesion molecules (CAM) and extracellular matrix integrin ligands. Inhibition assays were then realised using specific blocking agents. We found that i) direct cell-cell interactions were not implicated in chondrocyte aggregation ii) chondrocytes in aggregates were surrounded by a matrix rich in collagen II and cartilage oligomeric protein (COMP) iii) aggregation depends on a β1-integrin which binds a triple helical GFOGER sequence found in collagens iv) integrin α10 subunit is the most highly expressed α subunit among those tested, including α5, in aggregating chondrocytes. Taken together, this body of evidence suggests that the main molecular interaction involved in aggregation of phenotypically stable chondrocytes is the α10β1-collagen II interaction.

6.2 Introduction

Cell-matrix and cell-cell interactions in cartilage are of crucial importance in mediating cartilage development (1), homeostasis (2), degradation (3), and cell survival (4). These interactions have been extensively studied in the context of limb bud mesenchymal cell condensation, an early stage of cartilage development that is believed to be mediated by cell-fibronectin interactions. In this case, antibodies against fibronectin interrupted cell condensation in limb bud mesenchymal micromass culture (5), and fibronectin depletion, or inhibition of cell binding to fibronectin by RGD peptides, interfered with aggregation of prechondrogenic cells (6). After this initial condensation stage, cell-cell contacts are strengthened by direct cell-cell adhesion implicating the homotypic cell adhesion molecules, Neural Cell Adhesion Molecules (NCAMs) and N-Cadherins (6,7). Perturbation of NCAM and N-Cadherin activity by blocking antibodies or transfection with mutated genes resulted in reduced or altered cell aggregation and chondrogenesis *in vitro* and *in vivo*. However, NCAMs and N-Cadherins then disappear from the forming cartilage and are not expressed by differentiated chondrocytes *in situ* (8,9). In healthy adult cartilage, there are no cell-cell contacts but rather functional cell-matrix contacts, primarily integrin-mediated, between differentiated chondrocytes and components of the ECM.

Immunostaining of tissues as well as FACS and western-blot analyses on freshly isolated chondrocytes enabled localization and detection of several integrins. In adult and fetal articular cartilage, integrin subunits $\alpha5$, αV and $\beta1$ are easily detected while

the expression of other subunits depends on the specific type of cells or tissue, or the technique used. For example, the expression of $\alpha 1$ and $\alpha 2$ varies between published studies, as does $\alpha 3$ (10-12) . The $\alpha 6$ subunit was detected in fetal or new born cartilage (11, 13) but not in adult cartilage (10). A priori, chondrocytes can therefore potentially use integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$ and $\alpha V\beta 3$ to bind ECM ligands.

In terms of ligand specificity, $\alpha 3\beta 1$ integrin has been characterized as a receptor for laminins and failed to show a role in chondrocyte adhesion (11,14) . In contrast, $\alpha 5\beta 1$ is the main chondrocyte receptor for fibronectin, as blocking antibodies almost completely abolished cell adhesion to this molecule (11,15). $\alpha 5\beta 1$ can also bind denatured collagens type VI and II (16,17). $\alpha 6\beta 1$ is one of the chondrocyte receptors for laminin (13) while $\alpha V\beta 3$ is known to bind osteopontin, bone sialoprotein and vitronectin (18,19) found in bone or calcified cartilage (20,21) . Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are collagen receptors (22), as is $\alpha 10\beta 1$ which is thought to be a major collagen-binding integrin during cartilage development and in mature hyaline cartilage (12,23). Integrin $\alpha 11\beta 1$, also binds collagens but is expressed in the region just outside cartilage in cells around ribs, vertebrae and intervertebral disc, and in human embryonic tissues, showing a non-overlapping staining pattern with collagen type II (24). Finally, non-integrin collagen receptors are also expressed by chondrocytes *in situ* and *in vitro*, including Discoidin Domain Receptor 2 (DDR2) and annexin V (25) , which are cell-surface molecules that can bind fibrillar collagens.

The central role of cell-matrix interactions in controlling multiple processes in chondrocytes necessitates further investigation into the specific molecular interactions at play. Unfortunately monolayer culture dedifferentiates chondrocytes (26) while encapsulation in gels such as agarose impedes cell contacts and is cumbersome, rendering molecular analyses more difficult. An alternative to examine cell-cell and cell-matrix interactions for chondrocytes is suspension culture, where phenotypically stable chondrocytes are first dispersed in the culture medium and then proceed to form aggregates (27). This aggregation process likely occurs in a manner that reproduces *in situ* interactions, since phenotype is maintained in aggregates or pellet cultures which also bear high similarity to the native cartilage environment (27-29). Given this similarity to the *in situ* chondrocyte environment, and the relatively fast dynamic process of chondrocyte aggregation seen in suspension cultures, we report here a series of suspension culture experiments that elucidates the specific molecular mechanisms involved in forming chondrocyte aggregates, taking into account the potential for both cell-cell and cell-matrix interactions.

Previous work in suspension culture has provided some insight into the aggregation process. For example anti-fibronectin serum or hyaluronidase failed to prevent aggregation (4,30); hyaluronidase could even promote aggregation (4,31). On the other hand, the presence of a blocking antibody against the β1 integrin subunit reduced cell condensation (4,30), suggesting integrin involvement in aggregation of avian embryonic chondrocytes. These studies also showed that addition of collagen type I to chondrocytes in suspension increased aggregation, whereas collagenase

inhibited it (4,30). Although these previous studies suggest a collagen-integrin interaction is at play in the formation of aggregates of avian embryonic chondrocytes, the specific integrins and collagens involved, as well as the relevance of these findings for phenotypically stable mammalian chondrocytes, are not known. In this study we have therefore hypothesized that the aggregation of phenotypically stable mammalian chondrocytes in suspension culture is mediated by cell-matrix interactions rather than cell-cell interactions and involves a β1 integrin with known binding properties to collagen type II, namely integrins α1, α2, α10 or α11β1. To test this hypothesis, expression analysis of candidate cell adhesion molecules and potential ECM ligands in suspension culture was performed. We then carried out several inhibitions of aggregation assays using blocking antibodies and peptides to further identify classes of molecules involved in chondrocyte aggregation.

6.3 Experimental procedures

6.3.1 Culture media

Serum free medium (SFM). The SFM is a 1/1 (v/v) mix of calcium-free HAM's F12 and calcium-free DME low glucose (both from US Biological) supplemented with 1mM $CaCl_2$, 0.4 mM proline, 1.5 mM glutamine, 22mM sodium bicarbonate, non-essential amino acids 1X, penicillin-streptomycin 1X. *Supplemented SFM* is SFM with ITS+1 (Sigma-Aldrich), EGF, PDGF-BB, FGF-2 all 2ng/mL (R&D Systems), 10^{-8} M dexamethasone, $5x10^{-5}$ M β-mercaptoethanol, and 30 μg/mL ascorbate, added fresh.

6.3.2 Chondrocyte isolation and culture

Chondrocytes were isolated from the femoropatellar groove of a 1-2 month old calf. Briefly, cartilage was sequentially digested, first by protease Type XIV (Sigma-Aldrich Canada) at 56 U/mL and by collagenase CLS2 (Worthington) at 752 U/mL and released cells were filtered, and washed with SFM. After isolation, chondrocytes were either cultured as static cell suspensions for 18h or used to perform aggregation assays, (see "Aggregation assays"). For static suspension culture, cells were seeded at 0.4×10^6 cells/mL in supplemented SFM in Petri dishes previously coated with 2% (w/v) agarose to prevent cell adhesion. Microscopic observation of cell aggregates was performed with an inverted microscope (AXIOVERT S100TV, Carl Zeiss) equipped with a 5X objective (NA 0.12), a QICAM Fast 1394 camera (QImaging) and NORTHERN ECLIPSE image acquisition software (Empix imaging). At different times after seeding (t=0, 4h, 18h) cell samples were taken and lysed in buffers for RNA or protein analyses (see below). At t=18h, cell aggregates were also fixed for immunostaining (see below).

6.3.3 Immunoblotting

Cell proteins were extracted in RIPA buffer (150mM NaCl, 1% w/v Triton X-100, 1% w/v sodium deoxycholate, 0.1% w/v SDS, 10mM Tris pH 7.2) with protease inhibitors (P8340, Sigma-Aldrich) and electrophoresed on 7.5% acrylamide/bisacrylamide gel. Proteins were then transferred onto a polyvinilidene fluoride (PVDF) membrane, and probed first with primary antibody (to NCAM, AB5032, to integrin β1, AB1952 both from Millipore, to N-Cadherin, clone 3B9 from

Zymed) and then secondary antibody, coupled to horseradish peroxidase, which was detected by ECL (GE-Healthcare).

6.3.4 mRNA Isolation and Quantitative Reverse Transcription PCR

Total RNA was isolated with the RPN kit (Sigma-Aldrich). QRT-PCR were performed as previously described (27). Quantitative PCR occurred in the ROTORGENE 6000 (Corbett Research) using SYBRGREEN to quantify cDNAs produced from various gene mRNA, using primers (from BioCorp) listed Table 6-1. Relative mRNA abundance was calculated following the Liu and Saint method (32). The housekeeping gene Elongation Factor 1α (EF1α) was used to calculate relative mRNA expression of target proteins. Elongation Factor 1α was tested and found to be an appropriate housekeeping gene for isolated chondrocytes cultured in suspension.

Tableau 6-1 : Primer pairs used for PCR.

Gene, PUBMED accession number Sequence	
Bovine type II collagen, X02420	**Bovine integrin sub-unit α11,** XM_602058
5'-GAA CCC AGA ACC AAC ACA ATC C-3' (forward)	5'- AACTGCACCAAGCTCAACCT-3'(forward)
5'-TCT GCC CAG TTC AGG TCT CTT AGA GA-3' (reverse)	5'- CCATGTAGGTCTGGCACCTT-3'(reverse)
Bovine type I collagen, S64596	**Matrilin 1**, XM_589498
5'-TGG CCC AGA AGA ACT GGT-3' (forward)	5'- TGT GGA CAC GCT GGA TGT AT-3'(forward)
5'-AGG AAG GTC AGC TGG ATG-3' (reverse)	5'- GTG AAG ACA ATG CCC ACC TT-3'(reverse)
Bovine Type VI collagen, XM_582924	**Matrilin 2**, XM_027543
5'-AGT CTG GAG GCA GAA GTC CA-3' (forward)	5'- GCA AGC AAG GCT ACA TCC TC-3'(forward)
5'-AGT GCG ACC ACA AGA GTC CT-3' (reverse)	5'- GGT TTT CTG AGG CGC AGT AG-3'(reverse)
Bovine Type IX collagen, XM_582924	**Matrilin 3**, XM_591137
5'-ATG GCT GCG AGT CTG AAG C-3' (forward)	5'- TCC AGA CCC ATT CGG ATA AG-3'(forward)
5'-TTT GGG ACC TCT TCC TGG G-3'(reverse)	5'- ACG GCG TAC AGC TCA ATA CC-3'(reverse)
Bovine Fibronectin , K00800	**Matrilin 4**, NM_001098865
5'-ACT GCC CAC TCC TAC AAC CA-3' (forward)	5'- CCT GCT GGA GAA CCT CAA AG-3'(forward)
5'-CAA AGG CAT GAA GCA CTC AA-3' (reverse)	5'- TGA CAG GGT CCA GAC ATT CA-3'(reverse)
Bovine NCAM , BT020673	**TSP1**, AB005287
5' CCG GAG ATG CCA AAG ATA AA 3 (forward)	5'- ACA CGA CTG CAA CAA GAA CG-3'(forward)
5'-CGA TGT TGG CGT TGT AGA TG 3' (reverse)	5'- GGT TGG GGC AAT TAT CCT TT-3'(reverse)
Bovine integrin sub-unit β1, U10865	**TSP2**, NM_176872
5'-TCA AAT CCA GCC ACA GCA GC-3' (forward)	5'- GCT TCG TCC GCT TTG ACT AC-3'(forward)
5'-CCG TGT CAC ATT CCA CCA AC-3' (reverse)	5'- TAG GTG AGG TCC AGG GTG TC-3'(reverse)
Bovine N-Cadherin, X53615	**TSP3**, XM_001251901
5'- GGA CAT CGG GGA CTT CAT TAA TG -3' (forward)	5'- TGC CTC CTG GTC CTG ATA AC-3'(forward)
5'- TGG TTT GCA GCC TCT GCC AAA G -3'(reverse)	5'- GTT GGG TCA ATC TGA GCA T-3'(reverse)
Bovine integrin sub-unit α1, XM_616068	**TSP4**, BC104630
5'-CAA GCA TGA CTT TCG GGA TT-3'(forward)	5'- ACA ACG TGG ACC AGA GGA AC-3'(forward)
5'-GCC CTT TTC TGT GGT GGA TA-3'(reverse)	5'- GGC AGT TGT CCA GGA TGT TT-3'(reverse)
Bovine integrin sub-unit α2, L25886	**TSP5/COMP**, X74326
5'-TTT CCG AGC CTT AAG CGA AA-3'(forward)	5'- ATG CGG ACA AGG TGG TAG AC-3'(forward)
5'-CAG TGC CAT ATT TCG GCT TCG CAA CTG AT-3'(reverse)	5'- TCT CCA TAC CCT GGT TGA GC-3'(reverse)
Bovine integrin sub-unit α5, XM_872198	**Annexin V**, NM_001040477
5'-TTT GCT GTG AAC CAG AGT CG-3'(forward)	5'-AGA TGA TGT GGT GGG GGA TA-3'(forward)
5'-TAA GAG AGA CCT GGG CCT GA-3'(reverse)	5'-TCC CCA TTT CAG TTC TCC AG-3'(reverse)
Bovine integrin sub-unit α10, XM_582982	**DDR2**, BC134426
5'- AGG CCT CAC CTC AGA CAA GA -3'(forward)	5'-GCA GAG GCT GAC ATT GTG AA-3' (forward)
5'- ACC CTC GTC CTT TCC AAA CT -3'(reverse)	5'-CTT CAC CCA GCT TCT CCT TG-3' (reverse)
EF1α, AJ238405	
5'-AGC TGA AGG AGA AGA TTG ATC-3'(forward)	
5'-GGC AGA CTT GGT GAC CTT G-3'(reverse)	

6.3.5 Aggregation assays

600,000 cells/mL were inoculated into agarose-coated 48 well-plates with supplemented SFM (control), supplemented SFM without $CaCl_2$, or in the presence of the reagents listed in Table 6-2. The aggregation assays took place at 37°C, for 4 h. Cell aggregation was monitored by microscopy. To quantify aggregation after 4h incubation, single cells were counted and the % aggregation was calculated as 100 × (1-'single cell concentration'/'initial cell concentration').

In order to verify the activity of blocking antibodies with bovine cells, adhesion assays were performed as positive controls. 96-well plates were coated with either collagen Type I at 100 μg/mL, following the manufacturer recommendations, or with fibronectin (both from Sigma) at 1 μg/mL in PBS, and then blocked with 10mg/mL heat-denatured BSA. Bovine chondrocytes, expanded in monolayer, were treated with trypsin (Gibco; 0.25% w/v in 1mM EDTA), and incubated at 400,000 cells/mL in supplemented SFM with the different blocking agents (see Table 6-2) for 30 min, at room temperature. Then cell suspensions were transferred to coated wells and allowed to adhere 30 min for fibronectin, or 1h for collagen at 37C. Thereafter wells were washed, adhered cells fixed with 5% (w/v) glutaraldehyde, and stained in 0.1% (w/v) in crystal violet. After additional washes the retained dye was solubilized with 100μL of 10% (v/v) acetic acid, and the absorbance read at 570 nm.

Tableau 6-2 : Reagents used in aggregation assays. * Department of Biochemistry, University of Cambridge, United Kingdom

Reactant	Concentration	Supplier/ Comments
Blocking antibodies		
Integrin β1	20μg/mL	Clone 4B4, Beckmann. Control: IgG1.
Integrin α1	20μg/mL	Clone FB12, Millipore. Control: IgG1.
Integrin α2	20μg/mL	Clone P1E6, Millipore. Control: IgG1.
Integrin α5β1	1/40	Clone JBS5, Millipore. Control: IgG1.
Blocking peptides		
GRGDS	0.1-0.5mg/mL	Negative control is SDGRG. Both from Sigma.
GFOGER-GPP	0.1-1 mg/mL	Provided by Dr. Farndale*. Native triple helical collagen sequence recognized by integrins (Knight et al., 2000) with negative control non-triple helical GFOGER-GAP
Other		
EDTA	5mM	Chelates all divalent ions in the medium

6.3.6 Immunochemistry

Chondrocyte aggregates after 18h in suspension culture were fixed in 0.4% paraformaldehyde in PBS, 10min, and blocked in 10% v/v Serum, in PBS. All subsequent steps were carried out in 1.5% v/v Serum (complemented with 0.1% w/v Saponin for integrin α5), in PBS, unless otherwise mentioned. Samples were stained for the antigens described Table 6-3. All samples were counterstained for Actin with PHALLOIDIN-ALEXA 488 (1/40, Molecular Probes,), in 1.5% v/v Serum, 0.1% w/v Saponin, in PBS and the nucleus stained with HOECHST 33258 (0.5μg/mL, Molecular

Probes) in PBS. Stained samples were mounted in 16.7% (w/v) Mowiol 4-88 (Fluka, Buchs,), 33.3% (v/v) glycerol, 0.75% (w/v) n-propyl gallate (Sigma-Aldrich Canada) in PBS. Confocal imaging was performed using an APOCHROMAT 40X/NA 1.2 water immersion objective mounted on an AXIOPLAN 2 microscope equipped with an LSM 510 META confocal laser scanning module, an AXIOCAM HRM camera and the LSM510 acquisition software (all from Zeiss).

Tableau 6-3 : Antibodies used for immunostaining. DSHB: Developmental Studies Hybridoma Bank. * from Shriners Hospital, Montreal, QC. ** from Institute from Biochemistry II, Medical Faculty, University of Cologne, Germany.

Staining	Antibodies
Type II Collagen	Clone II-IIB3 (1/10), DSHB
Denatured Type II Collagen	Clone 3/4m. Provided by Dr. A.R. Poole*
Fibronectin	Biogenesis 4470-3589
Type I Collagen	Clone Col 1 (1/100), Sigma
COMP	(1/1000) Provided by Dr F. Zaucke **
Integrin $\alpha 5$	AB 1928 (1/1 000) , Millipore
Integrin $\beta 1$	Clone 4B4 (1/200), Beckmann

6.3.7 Scanning Electron microscopy (SEM)

Aggregates after 18h suspension culture were first fixed with 0.4% Paraformaldehyde in PBS for 10min., then blocked 4h in 10% Goat serum in PBS. All subsequent steps were carried out in 1.5% v/v Goat Serum in PBS, unless otherwise mentioned. Samples were probed with the first primary antibodies, for type II Collagen

and COMP (see Table 6-3) and with secondary antibodies conjugated to 20nm or 10 nm gold particles (1/40, British Biocell International and 1/40 Aurion). Aggregates were then post-fixed in 2.5% glutaraldehyde in PBS and 5% glutaraldehyde in water, 30min each. Finally a silver enhancement was performed with Aurion R-GENT SE-EM (Aurion). Samples were observed using an environmental SEM (Quanta 200 FEG, FEI Company) in environmental mode (2.5 Torr and 0°C) or in High Vacuum mode after gold coating with the AGAR SPUTTER COATER (Canemco Inc. & Marivac Inc). The dimensions of gold particles and collagen fibrils were measured with XT DOCU software (FEI Company).

6.4 Results

6.4.1 Chondrocytes aggregate in static suspension over agarose

When cultured in static suspension, chondrocytes aggregate rapidly within the first hour and these aggregates reach sizes of more than 1mm after 18 hours (Figure 6-1). The rapidity of this aggregation process suggests that adhesion molecules mediating aggregation were present at early stages of the culture just after enzymatic isolation that is known to remove most cell surface proteins. Expression of candidate adhesion molecules during culture was assessed by quantitative Real-Time PCR (QRT-PCR) and Western-blot.

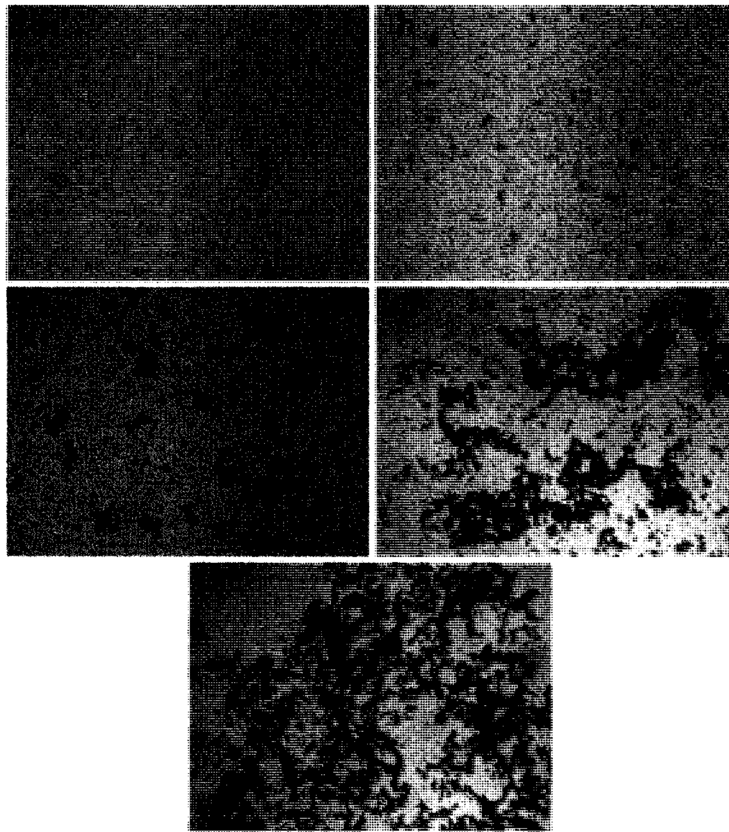

Figure 6-1 : Aggregation of chondrocytes cultured in static suspension. Freshly isolated chondrocytes were cultured at 0.4×10^6cells/mL in suspension for up to 18 hours in growth factor supplemented serum free medium (SFM), with ascorbate. Cell aggregation was rapid, beginning at ~1 hour and increasing thereafter. Scale bar: 500μm.

6.4.2 Integrin subunit β1 is more highly expressed than NCAM and N-cadherin

QRT-PCR and Western-Blot analysis of freshly isolated chondrocytes (FIC) or after 4h or 18h in suspension culture, revealed that NCAM and N-cadherin adhesion molecules were expressed at low levels relative to integrin subunit β1 (Figure 6-2). mRNA levels for NCAM and N-cadherin remained low even after 18h of culture (Figure 6-2 A), and none of the bands corresponding to the 131kDa positive control for N-cadherin or the 3 bands at 131, 180 and 197 kDa of the NCAM positive control were detected by Western Blot (Figure 6-2 B). NCAM are known to present 3 isoforms (120, 140 and 180kDa, named 120-, 140-, or 180-NCAM) that can be differentially glycosylated (33).

Integrin subunit β1 was strongly expressed and the protein level was found to increase with time, similar to the kinetics of aggregation (compare Figure 6-2 B to Figure 6-1). The Western blot revealed bands at 135 and 120 kDa (Figure 6-2 B); the 120kDa band was the intracellular precursor of the integrin sub-unit β1 and the 135kDa its mature, transmembrane counterpart (34). In freshly isolated chondrocytes, the upper band, corresponding to the transmembrane protein was weakly detected, as expected since the enzymatic digestion used to isolate cells digested most cell surface proteins. After 4h, the 135 kDa band became much more intense, indicating chondrocytes had synthesized new functional β1 integrins.

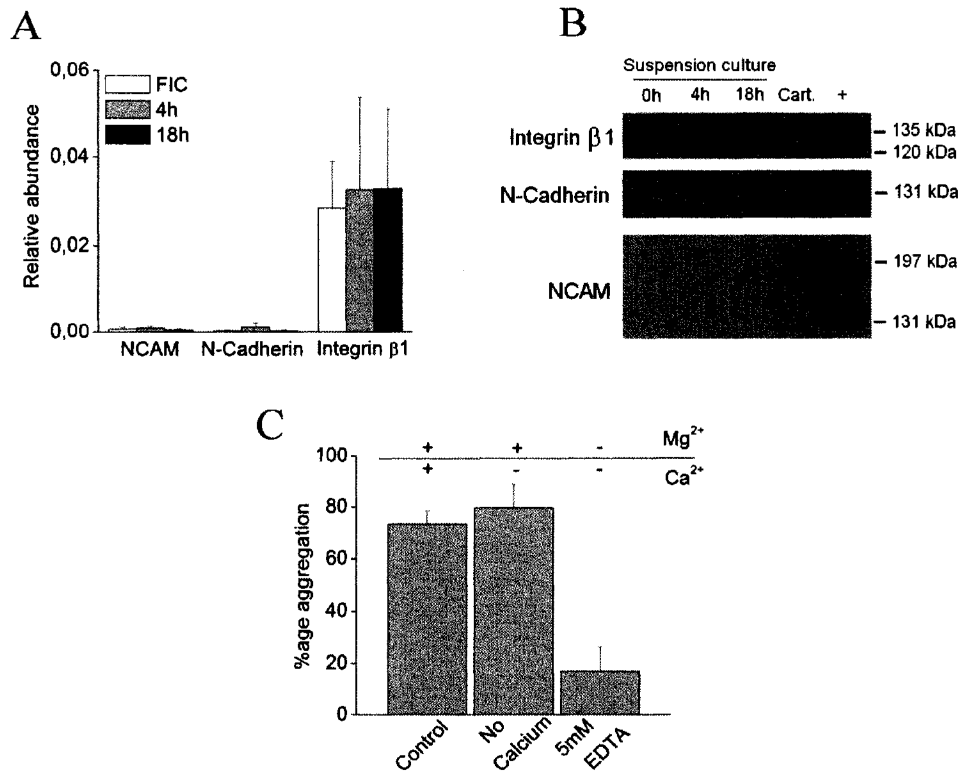

Figure 6-2 : Chondrocyte aggregation is driven by integrins. FIC were cultured in suspension at 0.4 × 10^6 cells/mL. Expression of integrin β1, N-cadherin and NCAM mRNA were analysed by QRT-PCR at different culture times (**A**) and corresponding protein expression was analysed by Western Blot (**B**). Integrin β1 was found to be highly expressed by chondrocytes while NCAM and N-cadherin were not. Aggregation persisted in the absence of calcium (**C**) demonstrating that N-cadherin was not required for aggregation. Addition of EDTA, on the other hand, to chelate all divalent ions, blocked aggregation, indicating that NCAMs were also not involved in chondrocyte aggregation. Positive controls for NCAM and N-Cadherin were bovine brain acetone powder, while for integrin β1, extracts of passaged chondrocytes were used. Error bars are SD with N=3.

6.4.3 Chondrocyte aggregation requires divalent ions but does not require Ca^{2+}

Previous work demonstrated the insensitivity of chondrocyte aggregation to calcium depletion (27) in long term suspension culture (10 days). This type of experiment was repeated here but as a short aggregation assay where aggregation in complete medium (containing 1mM Ca^{2+}, 0.7 mM Mg^{2+} and 1.5μM Zn^{2+}), in a calcium-depleted medium or in a divalent ion-depleted medium (with EDTA) were compared (Figure 6-2 C). Here again the absence of calcium did not inhibit chondrocyte aggregation, affirming that calcium-dependent cadherins are not implicated in chondrocyte aggregation. On the contrary, chelation of divalent ions by EDTA inhibited cell-cell adhesion. NCAM and numerous other CAMs from the Immunoglobulin-like CAM family, are not divalent-ion dependent and thus are insensitive to EDTA suggesting that CAMs are also not responsible for chondrocyte the aggregation. However, integrins are Mg^{2+}/Mn^{2+}dependent, and could also be inhibited by calcium in the mM range, or in the complete absence of calcium, depending on the ligand and the integrin type (35-37). Importantly, we previously measured ~14μM calcium in the "calcium-free" serum-free medium (SFM), likely from the added growth factors in this serum-free formulation (27). This residual concentration is sufficient to avoid integrin inhibition observed by others (35) in complete absence of calcium. Therefore a primary role of integrins in chondrocyte aggregation is entirely compatible with these divalent ion-dependent results (Figure 6-2 C) and is further reinforced by the mRNA and protein expression patterns of integrin β1 (Figures 6-2 A and B). Taken together, these results

suggest that chondrocyte aggregation in suspension culture is primarily mediated by one or multiple β1 containing integrins.

6.4.4 Strong expression of collagen II indicates phenotype maintenance and a collagen-mediated aggregation

In an effort to identify possible ligands for β1 integrin, and to examine to what extent aggregating chondrocytes synthesise a cartilage like pericellular matrix, several ECM molecules were analysed. Among possible ligands, collagens and fibronectin could serve to link integrins on adjacent chondrocyctes. The expression and presence of fibronectin and collagen types II, VI and IX in aggregates was assessed by QRT-PCR and immunostaining. In addition, collagen type I which could be synthesized by dedifferentiating chondrocytes, and denatured collagen type II which may arise from enzymatic treatment during cell isolation were examined. Collagen type II and fibronectin were positively immunostained (Figure 6-3 A) while collagen type I, and denatured collagen type II were not detected (data not shown). Fibronectin was found only in small amounts and exclusively at the aggregate periphery (Figure 6-3 B), whereas collagen type II was strongly detected throughout the whole aggregate, appearing to fill the intercellular space. QRT-PCR confirmed these results since collagen type II was highly expressed by aggregating chondrocytes, while collagen types VI, and IX and fibronectin were expressed at much lower levels, and collagen type I was not detected (Figure 6-3 C).

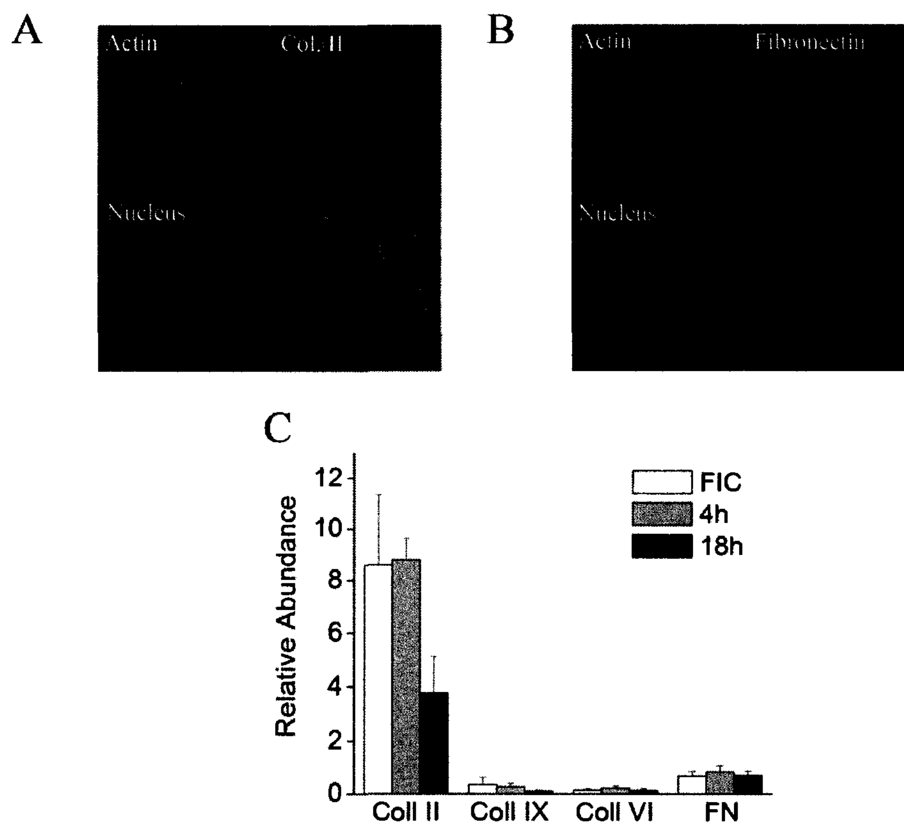

Figure 6-3 : Collagen and fibronectin expression by aggregating chondrocytes. Immunostaining (**A-B**) and QRT-PCR (**C**) of chondrocyte aggregates cultured 18h in suspension revealed that collagen type II is highly expressed and accumulates between cells. On the contrary fibronectin was weakly expressed by QRT-PCR and could only be immunodetected at the periphery of the aggregate. Error bars are SD with N=3. Scale bar: 10μm.

6.4.5 Expression of thrombospondins and matrilins in chondrocyte aggregates highlight TSP5/COMP as potential mediator of aggregate formation

Thrombospondins (TSPs) and matrilins (MATs) could be implicated in aggregation since they have the ability to bind ECM components and cells membrane receptors (38-40). Their mRNA levels were quantified by QRT-PCR. Among TSPs, TSP5/COMP was the most highly expressed followed by TSP1 with a 4-fold lower expression (Figure 6-4 A). In comparison to TSPs, matrilins were weakly expressed with matrilin 2 showing the highest relative abundance (Figure 6-4 B). These low levels of matrilins appear to exclude them from playing a central role in chondrocyte aggregation, whereas TSP1 and in particular TSP5 could certainly contribute to aggregate formation and organisation. The significant presence of TSP5/COMP in aggregates was confirmed by immunostaining (Figure 6-4 C) where COMP was found between cells, throughout the aggregate, in regions where collagen type II was also detected.

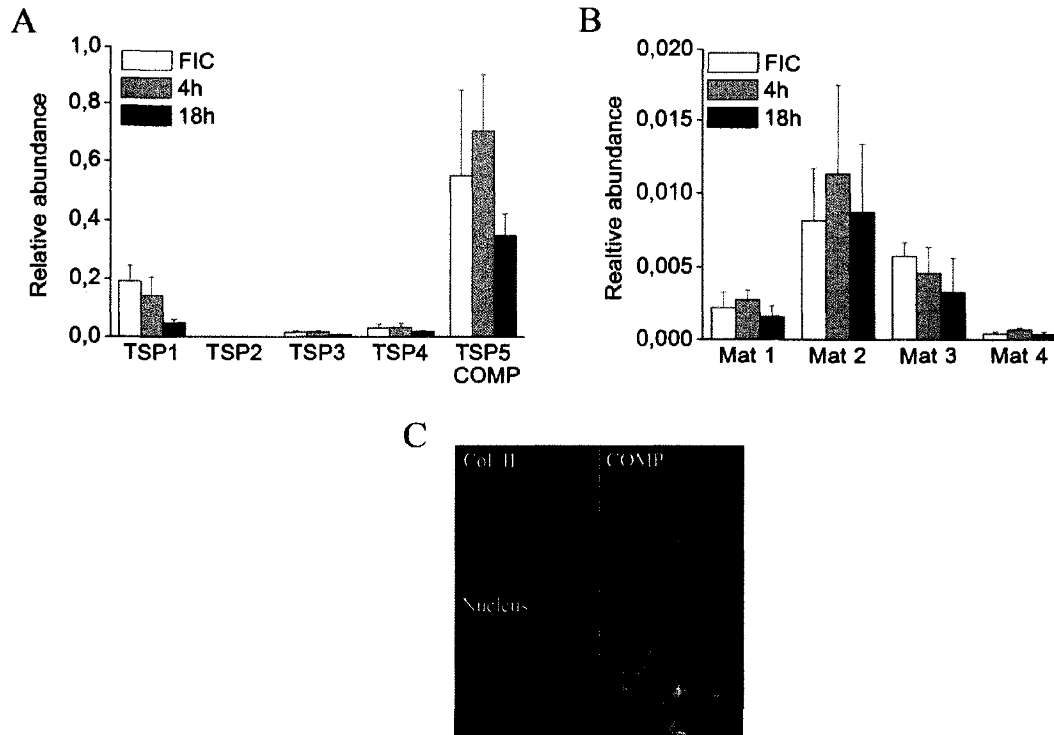

Figure 6-4 : Thrombospondin and matrilin expression by aggregating chondrocytes. FIC cultured up to 18h in suspension were analysed for thrombospondin (TSP) (A) and matrilin (Mat) (B) mRNA expression by QRT-PCR. Among TSPs, TSP5 (or COMP) was the most highly expressed, followed by TSP1. The high level of COMP in aggregates cultured for 18 hours was confirmed by immunostaining (C). Matrilins were found to be expressed at much lower levels than TSPs. Error bars are SD with N=3. Scale bar: 10μm.

6.4.6 Ultrastructural analysis shows cell-cell contacts are absent and the intercellular ECM is fibrillar containing both collagen II and COMP

Scanning electron microscopy (SEM) was used to examine the ultrastructure of chondrocyte aggregates formed in suspension culture (Figure 6-5). These analyses confirmed that chondrocytes in aggregates taken after 18 hours of suspension culture were not in direct contact but were linked by a randomly oriented dense fibril network (Figure 6-5 A), with extensive crosslinking and fibril bonding (Figure 6-5 B). Fibrils appeared to sprout out of the cells; some small fibrils could merge to produce larger structures growing in diameter (Figure 6-5 B). In some locations fibrils were wider, creating a loose fibre network (Figure 6-5 B, D), while other locations displayed a denser network with smaller diameter structures (Figure 6-5 C). Fibril diameter varied from 20 to 180 nm, as measured in environmental mode, since gold coating for high vacuum observations significantly increased fibril width. Immunogold staining revealed that the intercellular fibre network was positive for collagen type II and COMP confirming the presence of these molecules spanning the intercellular space in chondrocytes aggregates formed after 18h (Figure 6-5 C,D), and affirming their possible involvement in aggregate formation and organisation.

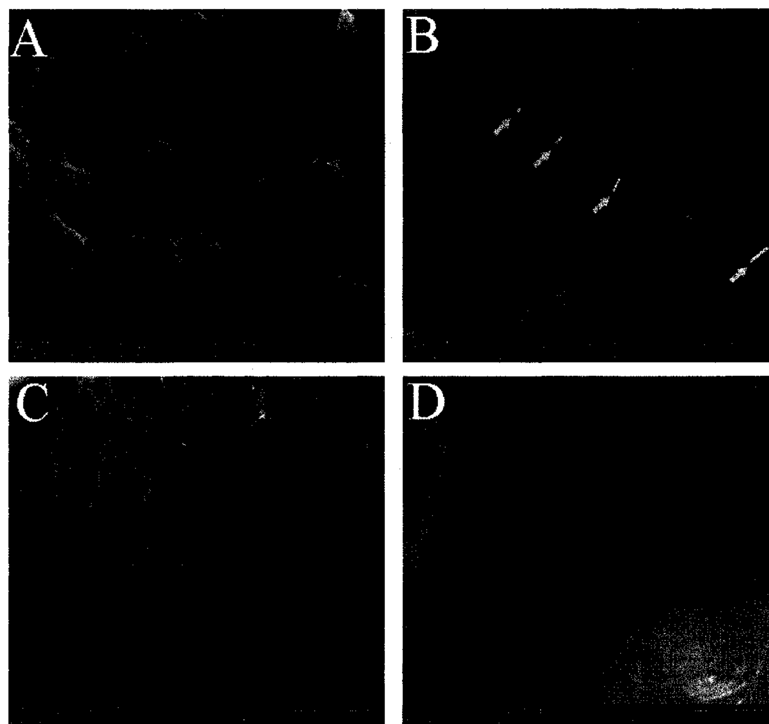

Figure 6-5 : Chondrocyte aggregates after 18h of culture in suspension were observed in scanning electron microscopy in high vacuum mode (A-B) and in environmental mode (C-D). Fibrils arose from chondrocytes (**A**) and could present a loose network of relatively large fibrils containing fibril bonding sites (arrows in B) or a dense network of small diameter structures (**C**). Immunogold-staining for COMP and collagen type II, followed by silver enhancement, to increase particle size, revealed collagen II and COMP to be present across the intercellular space (**C, D**) Larger silver enhanced gold particles (initially 20nm, for collagen type II) are represented in blue and smaller silver enhanced gold particles (initially 10nm, for COMP) are colored pink (C-D).

6.4.7 Integrin sub-unit α10 and annexin V are the most highly expressed collagen receptors

Triple helical collagens such as collagen II can bind integrin receptors α1β1, α2β1, α10β1, and α11β1 or non-integrin receptors as DDR2 and annexin V. On the other hand, TSP5/COMP is recognized by integrin α5β1. mRNA levels for these ECM binding cell surface molecules were analyzed by QRT-PCR, just after cell isolation as well as after 4h and 18h of suspension culture (Figure 6-6 A). Among integrin subunits, α10 was found to have the highest relative abundance at all time points. Annexin V showed even higher mRNA expression, with relative abundances of about twice those obtained for α10. Integrin subunits α1, α2 displayed weaker expression while α11 was lower yet (not distinguishable in Figure 6-6 A but detected with a relative expression of $3.9 \pm 2.7 \times 10^{-4}$). Finally DDR2 showed a level of expression similar to α5, with a relative abundance of 0.024 ± 0.004 for DDR2 and 0.027 ± 0.015 for α5. Thus significant expression of integrins α5β1 and α10β1 were found along with DDR2 and Annexin V. Note that the latter two receptors (DDR2 and Annexin V) are not likely to be involved implicated, considering the divalent ion-dependence of aggregation (Figure 6-2 C). Immunohistochemical staining confirmed high levels of α5 (Figure 6-6 B) and β1 (Figure 6-6 C) subunits.

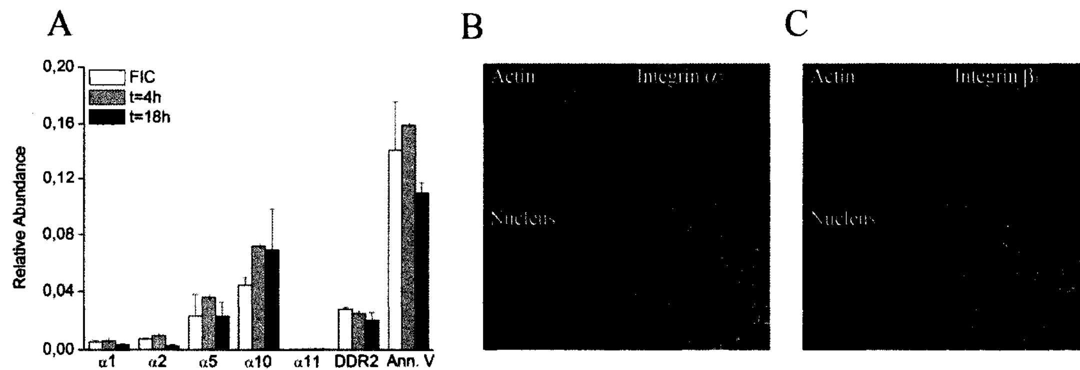

Figure 6-6 : Integrin sub-units expressed by aggregating chondrocytes. Analyses of integrin subunits α1, 2, 5, 10, 11, as well as DDR2 and annexin V mRNA expression by QRT-PCR (**A**) showed that integrin subunit α5 and 10, as well as DDR2 and annexin V were the most highly expressed, suggesting a role in chondrocyte aggregation. Immunostaining (**B-C**) confirmed the strong expression of α5 and β1 integrin subunits in aggregating chondrocytes. Error bars are SD with N=3. Scale bar: 10μm.

6.4.8 Blocking integrin $\beta 1$ inhibits aggregation while blocking $\alpha 1$, $\alpha 2$ and $\alpha 5$ do not

Aggregation assays with blocking antibodies against integrin $\beta 1$, $\alpha 1$, $\alpha 2$ and $\alpha 5\beta 1$ confirmed that a $\beta 1$ integrin is implicated in aggregation since only the integrin $\beta 1$ blocking antibody significantly reduced chondrocyte aggregation from 94±3 % in the control to 36±5% with the integrin $\beta 1$ blocking antibody (Figure 6-7 A). On the contrary, antibodies against $\alpha 1$, $\alpha 2$ and $\alpha 5\beta 1$ had no effect on chondrocyte aggregation in suspension. As positive controls to ensure blocking functionality, all antibodies were tested for their capacity to inhibit bovine chondrocytes adhesion to collagen type I or to fibronectin in monolayer. Antibodies to $\beta 1$, $\alpha 1$, $\alpha 2$ were found to efficiently block cell adhesion to collagen type I in monolayer with 89±2, 90±3 and 40±14% inhibition, respectively (Figure 6-7 B). Similarly, antibodies to $\beta 1$ and $\alpha 5\beta 1$ inhibited adhesion to fibronectin in monolayer with 65±12% and 72±5% inhibition, respectively (Figure 6-7 B), in accordance with previous findings (11,41). Thus, amongst the integrins, only integrin $\alpha 10\beta 1$ remains as a likely candidate receptor for collagen II in the formation of chondrocyte aggregates in suspension. Unfortunately we could not obtain any effective antibody towards the bovine $\alpha 10$ subunit either for immunostaining or for blocking experiments.

6.4.9 The triple helical GFOGER-GPP peptide significantly inhibits chondrocyte aggregation while RGD peptides do not

To further study the role of collagen II and TSP5/COMP, aggregation assays with peptides mimicking sequences present in these potential matrix molecule ligands, were performed (Figure 6-7). RGD is a cell adhesion motif found in TSP5/COMP (and fibronectin and collagen I) and is recognized by several integrins (42). On the other hand, GFOGER is a triple-helical motif present in collagens I, II and XI and is recognized by collagen specific integrins including $\alpha 1$-, $\alpha 2$- and $\alpha 11\beta 1$ (43). Our data found no inhibitory effect of RGD peptides on chondrocyte aggregation, while we observed triple-helical GFOGER to have a significant dose-dependent inhibition of aggregate formation (Figure 6-7 C). This latter result indicates that collagens are implicated in aggregation, most likely in the form of collagen type II which contains a GFOGER sequence, and was found to be present in the greatest amount in expression profiling (Figure 6-3). Cell adhesion on fibronectin or collagen type I coated surfaces was also assayed in presence of RGD or GFOGER peptides respectively, as a positive control. GFOGER at 500μg/ml was found to inhibit chondrocyte adhesion on collagen type I by 57 ±9% and RGD by 71±6% on fibronectin coated plates.

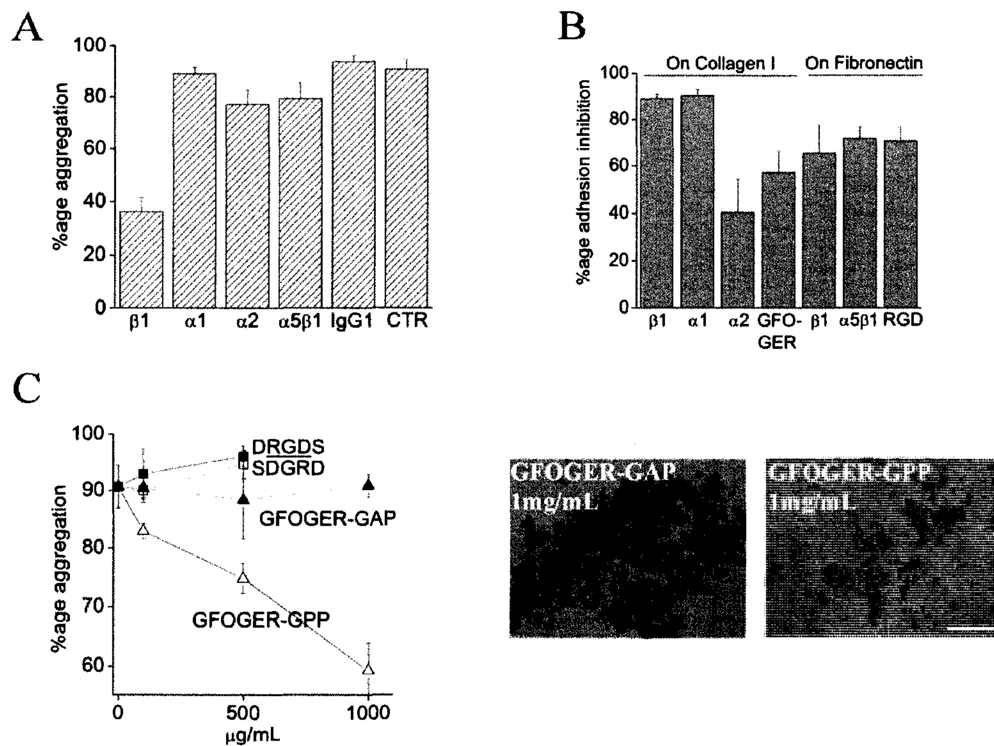

Figure 6-7 : An integrin containing the β1 subunit, most likely α10β1, and collagen II interact to form chondrocyte aggregates. A blocking antibody against the integrin β1 subunit effectively inhibited chondrocyte aggregation while blocking antibodies against α1, α2 and α5β1 did not (**A**). Adhesion assays in monolayer on collagen I or fibronectin confirmed that all blocking agents were biologically active under the conditions used (**B**). Chondrocyte aggregation was not inhibited by DRGDS peptides (negative control SDGRD), while strong inhibition by GFOGER-GPP (negative control GFOGER-GAP), which is known to block certain integrin-collagen interactions, was dose-dependent (**C**). Although no effective blocking antibody against integrin α10 could be obtained, a primary role of α10β1 binding to collagen II is implicated by the expression profiling (Figure 6-6) and the ability of GFOGER-GPP

and β1 blocking antibody to inhibit chondrocyte aggregation. Error bars are SD with N=3. Scale bar: 500μm.

6.5 Discussion

6.5.1 Chondrocyte aggregation is driven by integrin-mediated cell-matrix interactions rather than cell-cell interactions

N-CAM and N-cadherin are known to be expressed and involved in mesenchymal pre-chondrogenic cell condensation, but are no longer found in mature cartilage (8,9). Although these molecules could be re-expressed after enzymatic isolation and contribute to chondrocyte aggregation, QRT-PCR and Western Blot analysis showed that this was not the case (Figure 6-2 A). On the contrary, integrin β1 was strongly synthesized post-isolation and its protein level correlated with the kinetics of aggregation (Figure 6-2 B). Moreover, the divalent-ion dependence of aggregation where calcium was not required but other divalent ions were needed (Figure 6-2), also pointed to an integrin-mediated process, and excluded all calcium-dependent cadherins, and other divalent-ion independent adhesion molecules such as N-CAMs, annexin V and DDR2.

6.5.2 Expression profiling suggests that chondrocytes aggregate through collagen II binding to a cellular collagen receptor

β1 integrins, in association with different α subunits can bind collagens, fibrobectin (41) and other non-collagenous proteins such as matrilins and TSPs (38,40). As expected in this culture, collagen type I was not found while collagen types II, VI

and IX were expressed with collagen type II at a level more than 20 times higher than the others (Figure 6-3). COMP was also found to be highly expressed and to be co-localized with collagen type II both at the light and electron microscopic level (Figure 6-4 C and -5 D), in accordance with its role in promoting collagen fibrillogenesis (44). Although COMP can bind integrins α5β1 or αVβ3 (45) present at the chondrocyte surface, its proximity to collagen type II and lack of any blocking effect of anti-α5β1 and RGD peptides (Figure 6-7 A) suggest its role to be related to collagen fibrillogenesis rather than direct cell binding.

6.5.3 The most highly expressed collagen receptor compatible with the divalent ion dependence of chondrocyte aggregation is integrin α10β1

In principle several matrix molecules analysed in our study are compatible with the divalent ion dependence of chondrocyte aggregation, however only collagen II and COMP were highly expressed and detected in intercellular regions. Four different integrin-based collagen receptors are known, α1β1, α2β1, α10β1 and α11β1 (22), all of which can be expressed by chondrocytes or mesenchymal cells (10-12,24) and therefore could be active in our aggregating freshly isolated chondrocytes. On the other hand, integrin α5β1 has the ability to bind COMP or denatured collagen type VI and type II in an RGD-dependent manner (16,17,45) and is the principal receptor for fibronectin, another abundant ECM molecule. Analysis of these five α subunits by QRT- PCR revealed that α5 and α10 were indeed the most highly expressed (Figure 6-6) where higher expression of α5 in comparison to α1 or α2 is corroborated by the

literature (11,25). The very weak expression of $\alpha 11$ was also expected as $\alpha 11$ was previously detected in mesenchymal cells but not in cartilage (24). Our data appears to be the first to quantitatively compare $\alpha 10$ expression to other α subunits in chondrocytes. We found $\alpha 10$ to be the most highly expressed of all α subunits, suggesting that $\alpha 10\beta 1$ is a main candidate integrin to bind collagen II in aggregating chondrocytes. We also analysed other non-integrin collagen receptors, known to be expressed in cartilage. In particular DDR-2 and annexin V were found to be expressed by aggregating chondrocytes, with annexin V exhibiting the highest mRNA expression among all candidate collagen receptors tested. However, annexin V is primarily located intracellularly and adhesion of annexin V to collagen is not divalent ion dependent (25,46). Similarly no ion binding site has been described for DDR-2 (47). Thus the likelihood of these non-integrin collagen receptors driving chondrocyte aggregation appears to be minimal.

6.5.4 Function blocking antibodies and peptides further identify collagen II binding to integrin $\alpha 10\beta 1$ as a prime mediator of chondrocyte aggregation

Although the above expression profiling and divalent ion-dependence of chondrocyte aggregation has pointed to particular collagens and their receptors as main molecular candidates in the chondrocyte aggregation process, function blocking experiments were required to further specify these interactions and their importance in chondrocyte aggregation. Antibodies to integrin $\beta 1$ subunits strongly inhibited chondrocyte condensation, but not antibodies to $\alpha 1$, $\alpha 2$ and $\alpha 5\beta 1$ (Figure 6-7). Unfortunately a blocking antibody against $\alpha 11$ was not available but, as $\alpha 11$ mRNA

expression was found to be very low, this molecule is unlikely involved in aggregation. We tested a blocking antibody against human integrin $\alpha 10$ (mAb 365 provided by Cartela, Lund, Sweden) but found this agent to be inactive with our bovine cells, even in immunocytochemistry, and thus was not useful in our study.

The absence of a role of $\alpha 5\beta 1$ in the aggregation process was further confirmed by the lack of inhibition of aggregation by linear RGD peptides, the cell adhesion motif recognized by $\alpha 5\beta 1$ (42) that is present in fibronectin, COMP, and denatured collagens type VI and II. On the contrary GFOGER-GPP peptides, a triple-helical motif present in collagen types I, II, and XI (but not in collagen types III, V, VI and IX) efficiently inhibited aggregation in a dose-dependent manner (Figure 6-7). Since collagen type I was not detected in aggregating chondrocytes, while collagen type XI is buried inside collagen fibrils and therefore not accessible, collagen type II appears to be the only collagen implicated in aggregation that is blocked by GFOGER. The GFOGER motif is known to be recognized by integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 11\beta 1$ (43), as well as by integrin $\alpha 10\beta 1$ (Lundgren-Akerlund and Farndale, personal communication).

All of the above results, when taken together in the context of existing literature, lead to the following conclusions concerning the aggregation process of phenotypically stable freshly isolated mammalian articular chondrocytes : 1) $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 11\beta 1$-collagen interactions are not involved due to their weak expression, and the lack of effect of blocking antibodies to $\alpha 1$ and $\alpha 2$. 2) Ineffectiveness of RGD peptides or blocking antibodies to $\alpha 5\beta 1$, also exclude any $\alpha 5\beta 1$ interaction with fibronectin,

denatured collagen type VI or II (16,17) and with COMP/TSP5 (45) in the aggregation process. 3) The inhibition of aggregation obtained with the β1 blocking antibody and with the GFOGER-GPP peptide, indicates that aggregation is mediated by a collagen binding to a β1 integrin, most likely collagen type II binding to α10β1. Note that a lack of importance of DDR2 and annexin V in aggregation is further supported here by the known non-GFOGER binding sites of annexin V which rather bind to the N-telopeptide (48) and DDR-2 which binds most strongly to a very specific motif, GPRGQOGVMGFO in the D2 period of collagen type II, but not to GFOGER (49,50). Other ECM components such as chondroadherin (CHAD), or RGD-CAP, that were not studied here are known to bind collagen and chondrocytes by integrins α2β1 or α1β1 respectively (51-53). Since these two integrins were excluded from being active in the aggregation process it appears that chondroadherin (CHAD) and RGD-CAP are also unlikely to be involved. Hence all of our results converge towards an α10β1-collagen type II interaction that drives the aggregation of freshly isolated phenotypically stable chondrocytes.

6.5.5 Chondrocyte aggregates simulate the in situ chondrocyte environment

The integrin expression profile we found in nascent aggregates is similar to native cartilage where α1β1 and α2β1 were not always detected, or were detected in smaller amounts than α5β1. Our study was the first to identify high expression levels of α10β1 that are compatible with its strong immunostaining in human cartilage (12) while α11β1 expression was not significant also in agreement with its expression in

mesenchymal cells *in situ* but not cartilage (24). We found that ECM composition and structure of aggregates and resemble that of the pericellular matrix of chondrocytes. Aggregates and chondrons (the chondrocyte in its pericellular matrix) both contain collagen types II, VI and IX, in addition to fibronectin (54), and matrilins 1, 3 and 4 (55). COMP has also been detected directly adjacent to chondrocytes *in situ* (56). Moreover, the dense fibrillar network we observed in SEM resembles the chondrocyte pericellular and territorial matrix observed in cartilage (57,58). More specifically, the range of fibril diameters we observed (20-180nm) and the branching filamentous morphology of the intercellular ECM is similar to that seen *in situ* (58-60).

Taken together these results indicate that chondrocyte aggregates cultured in suspension provide a useful *in vitro* model that preserves the phenotype in terms of molecular expression and creates a pericellular ECM that closely simulates the *in situ* situation. This dynamic system could be used to further study and understand cell-matrix interactions in cartilage, and elucidate inside-out and outside-in signalling pathways of chondrocytes in a physiologically representative environment. It can be therefore reasonably assumed that the $\alpha10\beta1$ – collagen type II interaction mediating aggregation *in vitro*, is also an active cell-matrix interaction in cartilage *in situ*.

Chondocytes condense rapidly in suspension culture, forming aggregates rich in extracellular collagen type II and COMP that are organised in a dense fibrillar network, similar to the *in situ* environment. Aggregate formation was not mediated by direct cell-cell contact but by cell-matrix interactions. Cell aggregation was strongly inhibited

by both an antibody against integrin β1 and by the peptide GFOGER-GPP, that mimics the triple-helical integrin-binding motif of collagens, but not by RGD peptides or antibodies to α1, α2 and α5β1 integrins. Therefore it appears that aggregate formation is primarily mediated by a collagen interacting with a β1-containing integrin. Since collagen type II was detected in significant amounts in aggregates while collagen type I, VI and IX were not expressed or minimally expressed, it appears that collagen II is the primary integrin binding ligand active in the aggregation of chondocytes in suspension. Amongst integrin collagen receptors, α10 exhibited the highest mRNA expression, which combined with our blocking studies, strongly suggests that α10β1 integrin mediates aggregation of chondrocytes in suspension. Therefore the most probable receptor-ligand pair responsible for aggregation of differentiated chondrocytes in suspension culture is α10β1-collagen type II.

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CHAPITRE 7. DISCUSSION GÉNÉRALE

Comme il a été précisé antérieurement, un des défis relatif à la culture de chondrocytes est l'obtention d'une croissance cellulaire associée à la préservation du phénotype afin d'être en mesure à la fois d'utiliser ces cellules pour des études physiologiques ou à des fins thérapeutiques.

Parmi les différents types de culture possibles, nous avons privilégié la culture en suspension qui nous apparaissait comme la plus prometteuse. Effectivement, elle permet de conserver l'état différencié des chondrocytes et semble aussi favoriser la synthèse d'un tissu similaire au cartilage. Cependant, ce mode de culture est peu exploité et mal caractérisé, et certains problèmes persistent : malgré un environnement 3D, les cellules finissent par se dédifférencier et la taille atteinte par les agrégats en suspension entraîne une accessibilité limitée des nutriments au centre de ceux-ci.

Afin de pallier ces désavantages, l'utilisation d'un milieu sans sérum a tout d'abord été proposée, pour à la fois limiter la dédifférenciation et l'agrégation. L'impact d'un tel milieu sur des chondrocytes cultivés à haute densité (ce qui assure un meilleur maintien du phénotype) ou à basse densité (entraînant une dédifférenciation plus rapide), en monocouche ou en suspension, a été comparé à celui d'un milieu contenant du sérum. Il a ainsi été observé qu'en suspension, l'absence de sérum permettait de réduire considérablement la taille des agrégats (de 462 ± 287 à 44 ± 20.9 cellules/agrégats), ainsi que d'obtenir un meilleur maintien du phénotype.

Effectivement, en absence de sérum, une baisse importante de l'expression de collagène type I a été observée, alors que l'expression de collagène type II est restée, quant à elle, élevée. Il a également été confirmé que la culture en suspension est favorable pour le maintien des caractéristiques chondrocytiques par rapport à la culture en monocouche. Ces résultats corroborent ceux de la littérature (Mandl et al., 2002; Mandl et al., 2004b; Reginato et al., 1994; van Osch et al., 2001). Parallèlement, l'effet de la teneur en calcium dans le milieu de culture (15 μM, 50μM, et 1mM) a été évalué. L'agrégation s'est avérée être indépendante de la concentration en calcium, contrairement au phénotype mieux maintenu à de faibles concentrations, notamment en monocouche. Ce phénomène est accompagné par un changement de morphologie cellulaire, puisque à la fois en absence de sérum et à concentration en calcium réduite, les cellules arrondies sont plus nombreuses et la formation d'adhésions focales, caractérisées par l'apparition de fibres de stress, est beaucoup moins fréquente. Ces résultats sont également en accord avec certaines études qui montraient que l'utilisation de la dihydrocytochalasin B (qui inhibe la polymérisation d'actine et inhibe la formation de fibres de stress) ou la culture à haute densité qui privilégie une morphologie arrondie, permettent un meilleur maintien du phénotype (Kuettner et al., 1982; Mallein-Gerin et al., 1991; Tew et Hardingham, 2006; Watt et Dudhia, 1988). Par contre, c'est la première fois qu'il a été clairement démontré que de faibles concentrations en calcium (15-50μM) entraînaient un ralentissement de la dédifférenciation.

Cette étude, présentée dans le chapitre 3 de cette thèse, a permis de déterminer que le milieu de culture optimal pour la culture de chondrocytes en suspension est un milieu sans sérum, contenant 1 mM de calcium. Bien que la croissance observée y est plus faible que celle obtenue avec le milieu précédemment utilisé (contenant du sérum et environ 2 mM de calcium), cette nouvelle formulation offre de multiples avantages. Par exemple, le phénotype est maintenu sur une période de 10 jours, la taille réduite des agrégats assure un meilleur transfert de matière au centre de ceux-ci et les cellules présentent une morphologie arrondie. Ce milieu a été utilisé pour tous les travaux ultérieurs.

Par la suite, l'utilisation d'un système agité pour la culture de chondrocytes en suspension, ainsi que d'un surfactant, le PF-68, ont été conjointement investigués. Le PF-68 est un surfactant reconnu pour son action protectrice sur les cellules envers les forces de cisaillement générées par l'agitation. Cet additif communément utilisé à 0.1% (p/v) trouve notamment son application pour la culture à grande échelle de cellules mammifères produisant des protéines d'intérêt thérapeutique et son utilisation n'a que rarement été rapportée dans le cadre du génie tissulaire. En ce qui concerne la culture de chondrocytes, Srivastava et al., (1974) avaient utilisé du PF-68 pour cultiver des chondrocytes en spinner, mais sans insister sur ce point, ni caractériser l'effet de cette molécule sur les cellules. Au demeurant, un certain nombre d'études soulèvent le fait que le PF-68 pourrait s'intercaler dans la membrane plasmique, voir même pénétrer dans les cellules (Ramirez et Mutharasan, 1990; Zhang et al., 1992). De ce fait, si les chondrocytes sont cultivés dans un but thérapeutique, avec pour objectif de les

réimplanter dans une lésion, il est crucial de clarifier si effectivement le PF-68 entre dans les cellules et d'en connaître la destinée. Nous avons démontré dans les travaux exposés dans le chapitre 5 qu'un dérivé fluorescent du PF-68, synthétisé au laboratoire, entre effectivement dans les chondrocytes et s'accumule dans la voie endocytique, sans être dégradé ni ré-excrété. Il a également été évalué que les chondrocytes absorbaient 56 ± 10.9 μg de PF-68/10^6 cellules, indépendamment de la concentration initiale en pluronic ou en cellules. Cette quantité est compatible avec une possible utilisation des cellules ainsi cultivées pour la transplantation autologue de chondrocytes (ACI), puisque l'utilisation du PF-68 a déjà été accréditée pour son usage chez l'humain, dans des quantités supérieures à celles qui seraient injectées dans la lésion via des chondrocytes. D'autre part, le PF-68 est une molécule fréquemment utilisée avec les cellules CHO, la lignée cellulaire la plus utilisée dans les bioprocédés pharmaceutiques. Par conséquent, l'internalisation du pluronic dans ces cellules a également été étudiée. Les cellules CHO absorbent de plus petites quantités de surfactant que les chondrocytes (11.7 ± 6.7 μg/10^6cells) et semblent être capables de se défaire du PF-68 fluorescent, puisqu'il n'est plus détecté dans ces cellules après 7 jours. Un des problèmes lié à l'absorption cellulaire du PF-68 est la baisse de la concentration en surfactant dans le milieu de culture qui en résulte et qui peut se traduire par un effet protecteur amoindri. Il faut donc être conscient de ce phénomène et ajuster, si besoin est, la concentration initiale en pluronic. Par exemple, la concentration de PF-68 initialement choisie pour cultiver les chondrocytes en bioréacteur était 0.01% (p/v). Cependant, à cette concentration, l'absorption cellulaire s'est avérée être significative (60.7% est absorbé

en 18h, par 4.10^6 cellules/mL), et une concentration de 0.05% (p/v) a finalement été adoptée. D'autre part, aucun effet néfaste du PF-68 n'a été détecté, autant chez les chondrocytes que chez les cellules CHO et le comportement cellulaire est identique en sa présence ou en son absence.

Il est donc tout à fait raisonnable d'utiliser cette molécule pour la culture de chondrocytes en spinner, puisqu'elle assurerait une protection vis-à-vis du cisaillement sans *a priori* avoir d'effet néfaste sur la culture. Les spinners sont des systèmes agités de quelques dizaines de millilitres (100 mL dans cette étude) qui permettent d'obtenir un environnement contrôlé (température, composition de la phase gazeuse, agitation). L'agitation permet notamment d'améliorer le transfert de matière, en plus de contrôler la taille des agrégats (Moreira et al., 1995; Sen et al., 2001; Sen et al., 2002). Plusieurs cultures en spinner ont été réalisées avec le milieu optimisé sans sérum additionné de PF-68 à 0.05% (p/v) et i) les chondrocytes y sont viables et forment des agrégats de plus petite taille par rapport au système statique (170-370 μm versus >1mm), ii) la concentration cellulaire double en 7 jours, iii) le phénotype est maintenu puisque le collagène type II est très fortement exprimé par rapport au type I, alors que ce dernier n'est pas détecté dans les agrégats par immunostaining, iv) les agrégats contiennent une matrice riche en GAG et en collagène type II, v) les cellules interagissent avec la matrice, d'une manière probablement similaire aux chondrocytes *in situ*.

En parallèle, deux autres milieux ont été testés : un premier sans PF-68 et l'autre contenant une concentration en facteurs de croissance dix fois supérieure. Dans le premier cas, aucune croissance n'a été observée. Cependant, de nombreuses cellules en

division étaient identifiables dans les agrégats et une croissance aurait probablement été mesurée après quelques jours de culture supplémentaires. Aucune différence majeure, en termes de viabilité, n'a pu être notée. En présence d'une concentration accrue en facteurs de croissance, un résultat particulièrement intéressant a été observé : les cellules présentent une morphologie plus arrondie par rapport aux autres conditions et la synthèse de collagène type I n'est pas du tout détectée, indiquant un excellent maintien du phénotype. Il serait donc intéressant d'investiguer plus en avant un tel milieu de culture. Au terme de cette série d'expériences nous avons donc obtenu un système de culture offrant un environnement relativement contrôlable et homogène, supportant le maintien du phénotype conjugué avec la croissance cellulaire, permettant d'obtenir des agrégats d'une taille compatible avec un transfert de matière efficace (Malda et al., 2004d; Sutherland et al., 1986) et qui sont composés d'une matrice de type cartilagineuse, avec laquelle les cellules peuvent interagir.

Nous avons ensuite voulu mieux caractériser la composition de cette matrice, mais aussi élucider les interactions s'établissant entre les chondrocytes et leur MEC. Ces dernières sont d'une importance majeure puisqu'elles jouent un rôle dans de multiples phénomènes biologiques comme le développement du cartilage, son homéostasie, sa dégradation, la survie et le phénotype cellulaire (Attur et al., 2000; Cao et al., 1999; DeLise et al., 2000; Knudson et Loeser, 2002; Pulai et al., 2002; Shakibaei, 1998; Takahashi et al., 2007; Woods et al., 2007). Une meilleure compréhension de ces interactions dévoilerait potentiellement une des clés de la maintenance phénotypique. En suspension, ces interactions se concrétisent par la formation d'agrégats en

suspension, ce type de culture s'avère donc être l'outil idéal pour les étudier. Une série d'expériences impliquant l'analyse de l'expression de certaines molécules d'adhésion et de la MEC pendant l'agrégation a été réalisée. De plus, des anticorps et des peptides bloquants ont été utilisés pour inhiber spécifiquement certaines interactions. Cette étude, détaillée dans le chapitre 6, a permis de montrer que l'interaction prédominante provoquant l'agrégation implique une intégrine $\beta 1$ et une molécule de collagène contenant une séquence GFOGER en triple hélice. Il s'agit très probablement de l'intégrine $\alpha 10\beta 1$ et du collagène type II. Enfin, un ensemble d'analyses par QRT-PCR (ARNm), Western-Blot (protéine), immunostaining (localisation des protéines dans l'agrégat) et par microscopie électronique à balayage (structure des agrégats et visualisation des fibrilles de collagène), a démontré que le micro-tissu synthétisé par les chondrocytes en suspension était très similaire à leur environnement dans le cartilage. La culture en agrégat est donc un outil puissant pour l'étude de la biologie des chondrocytes. Elle serait également potentiellement applicable pour l'ACI, puisque ce système conjugue à la fois croissance et maintien du phénotype.

Lors de cette thèse, un nouveau mode de culture des chondrocytes a été développé. Ce système est supérieur à ceux déjà existant puisqu'il permet d'obtenir conjointement croissance et maintien du phénotype. De plus, les cellules sont dans un environnement proche de l'environnement *in situ* et établissent très probablement des interactions avec cet environnement qui sont similaires à celles s'établissant dans le cartilage. Ce type de culture est nettement supérieur à la culture en monocouche qui offre des conditions très éloignées des conditions physiologiques, avec pour

conséquence une rapide perte de phénotype. La culture en 3D sur support, même si elle promeut le maintien du phénotype, présente plusieurs désavantages : l'interaction des cellules avec un matériau non physiologique (le support) peut influencer le comportement cellulaire, des problèmes diffusionnels sont susceptibles de survenir dus à la dimension des constructions cellules-support, une toxicité éventuelle des produits de dégradation du support est également à prendre en considération. La culture en suspension en spinner ne présente pas de tels inconvénients.

La culture sous forme d'agrégats ou sphéroides en bioréacteur gagne en popularité depuis quelques années. C'est un moyen de culture qui est moins coûteux et moins laborieux que la culture en monocouche ou sur support, et qui est donc plus économique. Ainsi, des méthodes de culture en bioréacteur ont récemment été développées afin d'amplifier une population avec le phénotype voulu et ensuite utiliser ces cellules pour la transplantation. C'est le cas, entre autres, avec les cellules pancréatiques utilisées pour soigner le diabète (Chawla et al., 2006), les cellules souches mammaires épithéliales pour la régénération de glandes mammaires (Youn et al., 2005) ou les cellules précurseures neurales pour traiter les maladies de Parkinson ou de Huntington (Baghbaderani et al., 2008).

Notre approche s'inscrit dans la même volonté de développer des modes de cultures plus performants, hautement contrôlables et plus économiques pour la culture de chondrocytes.

CHAPITRE 8. CONCLUSION ET RECOMMANDATIONS

La culture des cellules composant le cartilage articulaire, les chondrocytes, présente un double intérêt. Premièrement, elles sont responsables de la synthèse, de l'homéostasie et de la dégradation de ce tissu. Une connaissance approfondie de ces cellules est donc un pré-requis pour une meilleure compréhension de la biologie du cartilage et des mécanismes associés à l'initiation et à la progression des maladies articulaires (comme l'arthrite) ; ce qui permettrait de découvrir de nouvelles cibles thérapeutiques. Ensuite, les chondrocytes peuvent être utilisés *in vitro* pour concevoir des implants cartilagineux destinés à être transplantés dans un patient afin de régénérer une lésion articulaire.

Cependant, jusqu'à présent, aucun mode de culture n'était entièrement satisfaisant. La culture en monocouche, qui permet une croissance cellulaire rapide, entraîne un changement de phénotype. Il s'en suit une perte de capacité de la part des chondrocytes à synthétiser une matrice cartilagineuse. La culture en 3D sur support permet, quant à elle, la maintenance du phénotype, mais la croissance est souvent faible, les conditions de cultures s'avèrent non-homogènes et l'utilisation de biomatériaux (le support) peut influencer le comportement cellulaire. La culture en 3D sans support en suspension, optimisée et caractérisée lors des travaux présentés ici, permet majoritairement de pallier ces problèmes.

Un système de culture en spinner avec un milieu sans sérum optimisé, a été mis au point. Ce milieu est un mélange 1/1 (v/v) de HAM'sF12 et DMEM contenant 1mM de calcium et additionné, entre autres, de proline, de glutamine, d'ITS (insuline, transferrine, sélénium, albumine et acide linoleique), de EGF, de FGF-2 et de PDGF-BB (tous à 2 ng/mL), de dexamethasone et de β-mercaptoethanol. Il s'agit d'une modification du milieu précédemment mis au point par Cancedda et Dozin, 2000. Les chondrocytes cultivés en suspension dans un tel milieu forment des agrégats composés de cellules entourées d'une matrice riche en collagène II et en GAG. Les collagènes VI et IX, la fibronectine, les matrilines 1, 3 et 4 ainsi que COMP y ont été également détectés. Or, toutes ces protéines sont également connues pour être présentes dans les chondrons (qui sont définis comme un chondrocyte entouré de son environnement pericellulaire dans le cartilage). La microscopie électronique a également révélé que la structure de la matrice dans les agrégats est similaire à celle observée dans les chondrons. Enfin, le profil d'expression des molécules d'adhésion (NCAM, N-cadherin, intégrines...) est également équivalent aux observations *in situ*. Dans un tel système, les cellules sont donc dans un environnement physiologique qui permet le maintien du phénotype. Cela permet d'obtenir une culture représentative du tissu duquel les cellules sont issues, le cartilage. Lors de cette étude, les molécules responsables de l'agrégation ont également été identifiées comme étant une intégrine β1 qui se lie à une séquence GFOGER en triple hélice. Plus précisément, le couple intégrine α10β1-collagène II serait le principal acteur du phénomène d'agrégation cellulaire.

Ces agrégats peuvent être cultivés en spinner. Ce dernier permet de fournir un environnement contrôlé, d'améliorer le transfert des nutriments, de stimuler mécaniquement les cellules et, enfin de limiter la taille des agrégats. Dans un tel système, contenant du PF-68, les chondrocytes sont viables, métaboliquement actifs, se divisent et maintiennent leur phénotype puisque la synthèse de collagène I reste très faible. De manière exploratoire, il a également été observé qu'une concentration en facteurs de croissance accrue (20 ng/mL au lieu de 2 ng/mL) pouvait encore améliorer le maintien du phénotype, puisque dans ce cas, les cellules présentaient une morphologie arrondie et une absence complète de synthèse de collagène type I. Le PF-68 est un surfactant utilisé pour protéger les cellules des forces de cisaillement générées par l'agitation en bioréacteur. Bien que nous ayons fait la preuve de son accumulation intracellulaire, il s'est avéré être compatible avec l'utilisation des chondrocytes cultivés en sa présence à des fins éventuelles de transplantation (ACI).

Nous avons donc développé un système de culture qui promeut à la fois la croissance cellulaire et le maintien du phénotype, tout en recréant autour des cellules un environnement similaire à celui qui les entoure dans le cartilage. Il est donc envisageable d'utiliser ce système pour des études physiologiques, en supposant que les chondrocytes dans les agrégats répondent à divers stimuli de la même manière qu'ils y répondraient *in situ*. L'utilisation des agrégats à des fins thérapeutiques comme transplant est également envisageable. Cependant, pour ce faire, une prolifération cellulaire plus rapide serait nécessaire. Communément, il est accepté qu'une multiplication de 20 à 50 fois est nécessaire pour les techniques de transplantation de

chondrocytes. Cependant, dans le cas de l'ACI une quantité importante de cellules est nécessaire puisque la lésion doit être complètement remplie par la suspension cellulaire. Si une culture en 3D sur support est réalisée après l'amplification en monocouche, là encore une grande quantité de chondrocytes est requise car la redifférenciation et la synthèse de matrice nécessite une importante densité cellulaire. En ce qui concerne l'utilisation des agrégats comme implants, la quantité de cellules requise pour la transplantation ne devrait pas être aussi élevée car i) ces agrégats seraient encapsulés dans un gel avant implantation ce qui réduit le nombre de cellules nécessaires et ii) les chondrocyte dans les agrégats sont différenciés et synthétisent une matrice cartilagineuse sans qu'une importante concentration cellulaire ne soit nécessaire dans le spinner. En définitive, une amplification d'environ 5 fois pourrait être suffisante dans le cas des agrégats.

Pour de futures études, il serait tout d'abord judicieux d'utiliser le milieu de culture avec une concentration en facteurs de croissance de 20 ng/mL, avec ou sans PF-68, afin de mieux évaluer l'impact de ce dernier sur la culture de chondrocytes en milieu agité. Un renouvellement régulier ou une supplémentation du milieu en cours de culture pourrait également stimuler la croissance et la synthèse de MEC. Enfin, plusieurs paramètres qui sont aisément contrôlables en bioréacteurs, tels que la concentration en oxygène et la vitesse d'agitation pourraient également avoir un impact sur le comportement cellulaire et seraient intéressants à explorer.

Par ailleurs, une meilleure caractérisation de la composition des agrégats, notamment au niveau protéique, permettrait de mieux les comparer aux chondrons. Il

serait également extrêmement pertinent, à l'aide soit de siRNA, soit d'un anticorps bloquant, d'étudier l'implication du couple intégrine $\alpha 10\beta 1$-collagène II dans l'agrégation. Les interactions cellules-matrices sont les médiateurs de nombreux signaux qui jouent un rôle prédominant dans des phénomènes comme la dédifférenciation ou la survie cellulaire. Identifier ces interactions serait un premier pas vers l'identification et la compréhension de ces signaux.

Suite à ces travaux, il est recommandé, pour des études physiologiques sur les chondrocytes, d'utiliser la culture en suspension dans un milieu sans sérum. Ces cultures peuvent être réalisées en conditions statiques, en plaques multi-puits, ce qui permet de tester de nombreuses conditions. La culture en milieu agité, comme en spinner ou en bioréacteur est cependant préférable, car les agrégats y sont plus petits et le transfert de matière plus efficace. Le bioréacteur, système permettant de contrôler de nombreux paramètres est particulièrement recommandé pour des études portant sur la composition de la phase gazeuse ou l'application de stimulations mécaniques. Plus particulièrement, le contrôle de la concentration en oxygène directement dans la phase liquide de la culture pourrait permettre de moduler la synthèse de MEC, le maintien du phénotype et la croissance chez les chondrocytes.

Dans un but thérapeutique, il est également conseillé d'utiliser les spinners pour amplifier une population de chondrocytes. Leur coût d'utilisation est moins élevé et les passages plus aisés par rapport à la culture en monocouche. Cependant une prolifération plus rapide est souhaitable et, par conséquent, ce système nécessite une optimisation supplémentaire.

Enfin, concernant l'utilisation de PF-68 afin de protéger les cellules du cisaillement, son absorption par les cellules doit être prise en considération et la concentration en Pluronic ajustée en conséquence.

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