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(54) Title: CARTILAGE REPAIR

(57) Abstract: The invention relates to a method for isolating a stable articular cartilage cell population wherein the cells of the population are characterised by the retention of their pericellular matrix. Further the invention concerns the use of the cell population in tissue repair.

CARTILAGE REPAIR

The invention relates to a novel method for isolating a progenitor cell population from articular cartilage and also a method for producing a chondro-progenitor cell population therefrom; the use of said chondroprogenitor cell population in tissue repair and particularly cartilage repair; an implant comprising or including said chondroprogenitor cells and the aforementioned progenitor cell population or chondroprogenitor cell population.

INTRODUCTION

Articular cartilage is avascular, aneural and contains no lymphatic vessels with a low level of metabolic activity compared with that of other connective tissues such as bone and muscle. It also has an extensive extracellular matrix, which it relies upon to provide cartilage with its characteristic properties. The two main constituents of articular cartilage are the highly specialised chondrocyte, which is unique to cartilage and the matrix, composed of a complex, interconnecting arrangement of proteoglycans, collagens and non-collagenous proteins (Buckwalter and Hunziker, 1999).

Articular cartilage can be divided into four main zones through its depth. These are the superficial; transitional; upper and lower radial; and calcified cartilage zones running from the outer articular surface to the deep subchondral bone, respectively. Although named zones are present there are no 'actual' boundaries, which can be visualised between the zones. In each zone there are biomechanical and morphological variations (Dowthwaite *et al*, 2003), which include differences in cell morphology (size and shape), cell packing, metabolic activity and the thickness of the layers. Differences in matrix composition also

exist between zones, with variations in the types and quantities of various collagens, proteoglycans, and non-collagenous proteins.

At most, articular cartilage is only a few millimetres thick, but it can reach up to 7mm in a large joint such as the hip. But in spite of being only a few millimetres thick it still manages to provide stiffness to compression and resilience, and displays the ability to be able to distribute loads, thus in turn, reducing high stresses placed upon subchondral bone (Buckwalter and Hunziker, 1999).

Chondrocyte

Normal articular cartilage contains one cell type, the highly specialised chondrocyte surrounded by extracellular matrix (Buckwalter, 1998). In the majority of cases the chondrocyte is "cytoplasmically isolated" (Archer and Francis-West, 2002) from its adjacent cells, never forming cell-cell contacts. Each chondrocyte, therefore, is completely surrounded by matrix with which it freely interacts. Chondrocytes differ in their morphology and metabolic activities between the zones of articular cartilage. Generally the chondrocyte has a rounded or polygonal morphology, except at tissue boundaries where it may appear flattened or discoid, i.e. at the articular surface of joints (Archer and Francis-West, 2002). The principal role of the chondrocyte is in the maintenance of the intricate extracellular matrix of cartilage in particular the soluble, hydrophilic structures such as hyaluronan and aggrecan (Knudson, 2003). Intracellularly, the chondrocyte contains organelles that are typical of that of a metabolically active cell (Archer and Francis-West, 2002) that play a pivotal role in matrix synthesis, continually working to synthesise and turnover a large

matrix to volume ratio, primarily composed of proteoglycans, glycosaminoglycans and collagens (Buckwalter and Hunziker, 1999). Some chondrocytes also contain short processes or microvilli, which can detect mechanical alterations in the matrix. This is achieved as they extend from the cell directly into the matrix. Intracytoplastic filaments, lipid, glycogen and secretory vesicles enable chondrocytes to interact with the matrix. Mature chondrocytes are easily distinguished from other cells as they have a spheroidal morphology. They also have abundant amounts of type II collagen, large aggregating proteoglycans and specific non-collagenous proteins interwoven within a meshwork, which forms a cartilaginous matrix that covers and binds to their cell membranes (Buckwalter and Hunziker, 1999).

Zones

Superficial zone

The superficial zone (Figure 1) is extremely thin and consists of two layers. The most superficial layer is acellular and consists of a thin, clear film of amorphous material known as the lamina splendens which overlies a sheet of fine, densely packed collagen type II microfibrils (Buckwalter and Hunziker, 1999). The deeper cellular layer is composed of flattened, discoid chondrocytes enclosed within a collagen matrix, which lie parallel to the articular surface (Dowthwaite *et al*, 2003). These cells synthesise matrix, which is abundant in collagen, fibronectin and water, and low in proteoglycans content compared to that of the deeper zones. These chondrocytes secrete surface zone proteoglycans, (a surface zone protein) called PG4, which lubricates the surface of cartilage (Dowthwaite *et al*, 2003 and Flannery *et al* 1999).

The dense layer of collagen fibrils have an orientation parallel to that of the surface and provide cartilage with its characteristic mechanical properties which include having high tensile strength and being able to resist shear force put upon it (Buckwalter and Hunziker, 1999). The meshwork of collagen fibrils also permits the movement of molecules into and out of cartilage such as antibodies and large cartilage molecules respectively.

Various studies have shown that the surface zone of articular cartilage is involved in the regulation of tissue development and growth. The surface zone has been found to be a signalling centre due to the expression of various growth factors and their receptors, which play a pivotal role in the morphogenesis of the diarthrodial joint via differential matrix synthesis (Dowthwaite *et al*, 2003). Furthermore *in vivo*, the surface zone has been shown to be responsible for the appositional growth of articular cartilage (Dowthwaite *et al*, 2003) and recent *in vitro* studies have shown that the surface zone of articular cartilage contains a progenitor cell population (Dowthwaite *et al*, 2003).

Matrices

In cartilage, chondrocytes are anchored within lacunae that are in turn encased by a territorial matrix, which is intensely basophilic in comparison with the interterritorial matrix, which is more distinct from the cells. The lacunar area contains a narrow pericellular area of matrix, which surrounds and encases the chondrocytes. The pericellular matrix is 1.3μm thick, which is very thin compared to the territorial matrix that can reach up to 50μm thick. The pericellular and territorial matrices differ considerably in structure from the interterritorial matrix. The pericellular and territorial matrices form the boundary

between the chondrocyte and the rest of the tissue, i.e. the interterritorial matrix, therefore they are thought to control matrix synthesis and turnover (Goldberg and Toole, 2005).

Pericellular matrix

5 Chondrocytes possess an extensive, highly ordered pericellular matrix, which is very closely associated with their cell surface. As a whole the composition of the pericellular matrix is similar to that of the cartilage extracellular matrix, however there is a high concentration of hyaluronan and the chondroitin sulphate/keratin sulphate hybrid proteoglycan (Knudson *et al*, 1996).

10 The chondrocytic pericellular matrix is composed predominantly of aggrecan. But its overall structure depends on the scaffold of hyaluronan and then its interactions with the plasma membrane. A particle exclusion assay first developed by Claris and Fraser (1968) enables visualisation of the chondrocytic pericellular matrix.

15 As in many tissues carefully regulated cell-matrix interactions maintain cartilage homeostatis, with these cell-matrix interactions being mediated via transmembrane receptors. Articular chondrocytes express integrin (e.g. $\alpha 1\beta 1$, $\alpha 1\beta 3$ and $\alpha 5\beta 1$) (Knudson *et al*, 1996) as well as non-integrin receptors. Interactions of these receptors with their principal ligands enable chondrocytes

20 to pick up changes in the extracellular matrix.

The chondrocyte matrix receptor CD44 (expressed as several alternatively spliced variants) plays a pivotal role in these cell-matrix interactions (Knudson, 2003). It is the principal cell surface receptor for the extracellular matrix glycosaminoglycan hyaluronan. It has been suggested that the

interactions of CD44 with the matrix allows two-way communication into and out of the cell. The spatial organisation of CD44 at the cell surface allows it to establish and mediate the pericellular matrix dependent upon the presence of a hyaluronan scaffold (Knudson, 2003).

5 Type VI collagen is also present in the pericellular matrix surrounding chondrocytes. It is involved in cell-matrix interactions, to help enable chondrocytes to bind to the matrix, and it also stabilises the fibrillar collagens (Hagiwara *et al* 1999, Marcelino & McDevitt, 1995).

Extracellular matrix

10 The extracellular matrix of articular cartilage is composed of tissue fluid and structural macromolecules. The interactions between these components provide the tissue with its characteristic mechanical properties such as compressive resistance (Buckwalter & Mow, 1992; Mow & Rosenwasser, 1988). The three main macromolecules of the extracellular matrix are collagens, 15 proteoglycans and non-collagenous protein, which are present in varying amounts within the tissue fluid.

Water contributes 80% to the wet weight of the tissue fluid and its interaction with the various matrix macromolecules directly influences the mechanical properties of the tissue. The tissue fluid also comprises gases, 20 small proteins, metabolites and a high concentration of cations to balance the negatively charged proteoglycans. The volume, concentration and behaviour of the tissue fluid within the tissue depends mainly on the interactions with the structural macromolecules particularly the large aggregated proteoglycans that are continually working to sustain matrix fluid levels and fluid electrolyte

concentrations (Mow & Rosenwasser, 1988; Buckwalter *et al*, 1990).

The other 20-40% of the wet weight of the tissue is made up of collagens, proteoglycans and non-collagenous proteins. Collagens make up 60% of the dry weight, 25-30% is proteoglycans and the remaining 15-20% is composed of glycoproteins and non-collagenous proteins (Buckwalter and Hunziker, 1999). The tensile strength and form of cartilage is provided by the collagen fibrillar meshwork (Buckwalter & Mow, 1992). Proteoglycans and non-collagenous proteins then bind to the collagenous meshwork becoming mechanically encased within it, with water then filling the spaces within this framework.

A few non-collagenous proteins organise and stabilise the matrix macromolecular framework, whilst others work to bind chondrocytes to the macromolecular components of the framework.

Hyaluronan and CD44

Hyaluronan is a ubiquitous constituent of the extracellular matrix of many animal tissues. Hyaluronan is a linear macromolecule that is composed of repeating disaccharide units: β-1,4-glucuronic acid- β-1,3-N-acetyl-D-glucosamine. It is a member of the non-sulphated group of glycosaminoglycans (GAGs) (Knudson, 2003).

Hyaluronan interacts with cells via specific hyaluronan binding proteins or hyaluronan receptors. Hyaluronan plays an integral role in maintenance of the extracellular matrix and when present at the cell surface it can affect cell behaviour via adhesion, migration and differentiation (Knudson, 2003). Hyaluronan's ability to influence cell behaviour and have close cellular associations, is mediated by two types of binding proteins: The Structural matrix

hyaluronan binding proteins which interact with hyaluronan within the extracellular matrix proper and Cell surface hyaluronan binding proteins that interact with hyaluronan at the plasma membrane of cells. The term hyaladherins was adopted by Toole (1990) for this family of molecules that display a high binding affinity for hyaluronan; CD44 is a cell surface hyaladherins (Knudson, 2003). Matrix hyaladherins, such as link protein and aggrecan, form large proteoglycan aggregates with hyaluronan, which have strong viscoelastic properties that influence osmotic potential of the cartilage matrix, thus establishing the characteristic biomechanical properties of cartilage, being able to dissipate compressive loads.

Cell surface hyaladherins function as hyaluronan receptors (Figure 2). There are vast numbers of CD molecules, which have a variety of different functions within cells. The primary receptor for hyaluronan is CD44 (Aruffo *et al*, 1990), it is a multifunctional, transmembrane glycoprotein rector (Knudson, 2003) that is present in a wide range of cell types. As a multifunctional receptor, CD44 has a crucial role in cell adhesion, tumour cell metastasis, endocytosis and cell signalling and matrix formation. Tissues undergoing embryonic development are abundant in CD44. CD44 serves as the critical link for the retention of hyaluronan-proteoglycan aggregates to the chondrocyte cell surface (Chow *et al*, 1995).

<u>Cartilage repair</u>

Articular cartilage has a limited capacity for self-repair. There are several limitations in the ability of cartilage to repair itself in terms of restoring a long-term function diarthrodial joint. Chondral repair tissue has an intermediate

structure and composition between hyaline cartilage and fibrocartilage, rarely, if ever replicating the actual structure of articular cartilage. There is disruption to the orientation and organisation of the collagen fibrils, failure to make important interactions between macromolecules, in particular the proteoglycans and the
5 collagen fibrillar network, thus resulting in a decrease in stiffness and in the ability to resist compressive loads. A major factor contributing to the low reparative capacities of articular cartilage is that the tissue is avascular and aneural.

Treatments are being developed to try and overcome the problems that
10 are faced when trying to treat articular cartilage defects. Potential treatments need to successfully integrate a tissue into a defect that has the same mechanical and structural properties as articular cartilage. Current cell based transplantation treatments involve the use of expanded autologous chondrocytes for transplantation into the defect to generate a repair tissue hopefully similar to
15 that of the native articular cartilage. This cell based transplantation treatment is known as Autologous Chondrocyte Implantation (ACI) and was described by Brittberg *et al* (1994) for the treatment of full-thickness cartilage defects. The problem with this technique, is that it involves the extraction of healthy articular cartilage from a non-injured, non-weight bearing region of the joint.
20 Contemporary research is looking into the potential use of mesenchymal stem cells (MSCs) as a cell source for use in tissue engineering and their infiltration into biodegradable scaffolds. Bone marrow derived MSCs have been focused on extensively but many other tissue types are now being considered as MSC sources such as cartilage and synovium.

However, we have obtained a new source of cells for use, primarily but not exclusively, in tissue repair. Our cells, advantageously, are highly clonable and so give rise to a large amount of stable, chondrogenic tissue.

STATEMENTS OF INVENTION

5 According to a first aspect of the invention there is provided a method for isolating a cartilage progenitor cell population comprising:

a) obtaining articular cartilage tissue; and

b) digesting this tissue, to release progenitor chondrocytes or chondrocytes, by using enzymes that digest all but the said chondrocytes and their pericellular
10 matrix.

In a preferred method of the invention said articular cartilage is digested with dispase and collagenase and more preferably the articular cartilage is exposed to 2-5U/ml + ideally 3.78U/ml of dispase and 0.1-1.0U/ml + ideally 0.74U/ml collagenase. Typically, enzymatic digestion is undertaken at 37°C
15 and, ideally, the digestion takes place whilst the digestant is being agitated for, ideally, up to a period of 5 hours.

In yet a further preferred method of the invention the digested material is, optionally, filtered in order to isolate the released progenitor chondrocytes and chondrocytes.

20 In yet a further preferred method of the invention the filtration referred to above employs the use of a filter with a pore size no less than 40μm and is, ideally, followed by a process of centrifugation before the released cells are resuspended in tissue culture media. The centrifugation is undertaken at 300g to 700g and more preferably at 620g (or 2000 rpm for 5 minutes).

In yet a further preferred embodiment of the invention the digested, and where applicable the filtered product, is, optionally, resuspended in tissue culture medium. More preferably still, the digested product, and where applicable the filtered and resuspended product, is exposed to hyaluronan and those cells
5 which bind relatively rapidly, in the order of 1 to 15 minutes, with the hyaluronan are selected for further use. The hyaluronan may be prepared as a suspension or as a coating on a substrate. The latter form is preferred because cells binding to plated hyaluronan form a flattened colony which are easier to identify and isolate.

10 Reference herein to rapid binding to hyaluronan includes reference to the binding to hyaluronan within minutes, as opposed to hours.

In yet a further preferred embodiment of the invention the released cells are plated on hyaluronan coated substrates for approximately 1 to 15 minutes and ideally 10 minutes.

15 In yet a further preferred method of the invention the articular cartilage tissue is surface or superficial cartilage tissue. More preferably still the cartilage tissue is taken from an individual to be treated and therefore represents autologous tissue.

Our method for isolating an articular cartilage progenitor cell population
20 involves not only the release of the progenitor chondrocyte population but also each cell's pericellular matrix. We believe that the retention of the pericellular matrix is advantageous because we have, surprisingly, discovered that chondrocytes that retain their pericellular matrix have enhanced colony forming abilities and so are capable of forming large colonies which we have discovered

can be successfully cloned and expanded in culture. This colony forming ability is best appreciated when one realises that, typically, chondrocytes lose their ability to form cartilage after seven populations doublings. Whereas, our pericellular retaining progenitor chondrocytes can undergo 41 population
5 doublings thus producing in the region of 10^{12} cells. As those skilled in the art will appreciate, this number of cells is highly beneficial because it enables a large amount of cartilage to be replaced.

Using conventional techniques, viable chondrocytes are isolated from their surrounding extracellular material so that they are free of all matrix matter
10 including pericellular matrix using a fairly aggressive enzymatic digestion technique. This technique involves a sequential digestion in tissue culture medium containing 2% FCS supplemented with pronase (700 U/ml) for one hour at 37°C and followed by collagenase digestion (300 to 900 U/ml) for 3 hours at 37°C. This conventional method is known to isolate the chondrocytes from all
15 the extracellular matrix components including the pericellular coat.

C. W. Archer, J. McDowell, M. T. Bayliss. M. D. Stephens and G. Bentley. Phenotypic modulation in sub-populations of human articular chondrocytes in vitro. Journal of Cell Science 97, 361-371 (1990)

According to a yet further aspect of the invention there is provided a
20 progenitor cell population isolated from articular cartilage according to the method described herein.

Moreover, the results presented herein show that a progenitor cell population isolated from articular cartilage can be successfully cultured and cloned in order to produce a stable population of chondrocytes.

Reference herein to a stable population of chondrocytes includes reference to a population of cells that exhibit a chondrogenic phenotype that is conserved when grown in an environment permissive for chondrogenesis. The chondrogenic phenotype is described herein with reference to the chondrocyte 5 and the chondrocytic zones.

According to a further aspect of the invention there is provided the use of an articular cartilage progenitor cell population in tissue repair.

According to a further aspect of the invention there is provided the use of a population of cells exhibiting a stable chondrogenic phenotype, manufactured 10 in accordance with the methods described herein, in tissue repair.

According to a further aspect of the invention there is provided an articular cartilage progenitor cell population which is characterised by either or both of the following:

a) the existence of a pericellular matrix; and/or

15 b) the ability to rapidly bind hyaluronan

Notably, the isolation of the progenitor cell population based upon adhesion to hyaluronan is of further importance in relation to the clinical application of these cells in tissue engineering and in tissue repair as this method of isolation does not utilise flow cytometry. Currently, there are no 20 accredited flow cytometers for use in clinical practice and therefore the isolation of the progenitor cells using hyaluronan is a more beneficial procedure.

According to a further aspect of the invention there is therefore provided a method for isolating an articular cartilage progenitor cell population wherein the cells are isolated by their rapid binding to hyaluronan.

According to a yet further aspect of the invention there is provided an implant for use in tissue repair comprising an articular cartilage progenitor cell population wherein the cells retain their pericellular matrix or are characterised by the ability to rapidly bind hyaluronan or cells exhibiting a stable chondrogenic
5 phenotype.

In a preferred implant of the invention the said cells are further characterised by being manufactured according to the methods described herein.

The implant may comprise any conventional scaffolding structure on
10 which the cells are conventionally grown. A scaffolding structure suitable for use in relation to these cells is described in WO 02/10348.

The invention will now be described with reference to the following where:

Figure 3 are graphs showing the results of the differential adhesion assay for determining the optimum adhesion time, including the initial adhesion data
15 and the CFE for day 3, 4 and 6. Figure (A) shows the initial; adhesion of matrix intact chondrocytes after differential adhesion assay to hyaluronan to determine the optimum adhesion time, colony counts were taken at day 1. There is a significant difference between cells plated down at 1 minute and cells plated down at 10 minutes ($p < 0.05$). Figure (B) shows the CFE of colonies >4 cells
20 (no significance differences were found), Figure (C) shows the CFE of colonies of >12 cells (see appendix for other significant differences) and Figure (D) shows the CFE of colonies of >33 cells (see appendix for other significant differences); at day 3, day 4 and day 6 respectively;

Figure 4 are graphs showing the results of the differential adhesion assay

of the matrix intact (MI), matrix depleted (MD), and CD44 depleted chondrocytes at the optimum point of 10 minutes; including the initial adhesion data, and the CFE data. Figure (A) the initial adhesion cell count for the 3 digests at day 1 after differential adhesion to hyaluronan for 10 minutes. Figure (B) CFE for colonies of >4 cells at day 3 and Figure (C) CFE for colonies of >33 cells at day 6 for the MI, MD and CD44 depleted chondrocytes. No significant differences were calculated;

Figure 5 are erythrocyte exclusion assay (3 representative areas were taken from the wells). Matrix intact chondrocytes with their native pericellular matrices intact (A,B,C). Matrix depleted chondrocytes with disrupted pericellular matrices (D,F,E) and CD 44 depleted chondrocytes with no visible pericellular matrices, as the erythrocytes are completely touching the chondrocytes;

Figure 6 shows representation of the expansion of a 52 cell colony (A) taken from the optimum adhesion time of 10 minutes at day 6 and then when it had reached confluence (C) before pellet cultures were established. Figure (A) shows the 52 cell colony at magnification x10 and (B) at magnification x20. Figure (C) shows the expanded colony at confluency prior to the establishment of pellet cultures. The population doubling (PD) at this stage was 21;

Figure 7 shows comparison of the gross morphology of 7 day pellets after treatment with ITS, TGFβ1, and ITS/ TGFβ1 (magnification x25);

Figure 8 is a graph showing the results of the DMMB assay carried out on the pellets treated with ITS, TGFβ1, and ITS/ TGFβ1. There is a significant difference between GAG concentration of the pellets treated with ITS and ITS/ TGFβ1, and also TGFβ1 and ITS/ TGFβ1 where (*) is $p<-0.05$. No other

significant differences were observed; and

Figure 9 shows analysis of adipogenic and osteogenic differentiation potential after 2 weeks in monolayer culture. (A): Supplementation with medium promoting adipogenesis (B) Control, supplementation with medium containing 10% FCS. (C): supplementation with medium promoting osteogenesis. (D) Control, supplementation with medium containing 10% FCS. Images (E) and (F) are after alkaline phosphatase stain has been applied; (E) is chondrocytes treated with osteogenic medium and (F) is chondrocytes after treatment with media + 10% FCS. Images (G) and (H) are after von kossa stain has been applied; (G) is chondrocytes treated with osteogenic medium and (H) is chondrocytes after treatment with media + 10% FCS.

Chondrocyte isolation

Before dissection, the feet were scrubbed with detergent, the skin removed, the feet were then sprayed with 70% Industrial Methylated Spirits (IMS; Fisher UK). Surface zone bovine articular cartilage was removed from the metacarpalphalangeal (MCP) joints of 7 day-old animals by fine dissection under sterile conditions.

In all cases cartilage shavings were collected in a sterile 50ml tube and chondrocytes were released from surrounding matrix by digestion in 3.78U/ml dispase (Gibco) plus 0.74U/ml collagenase (Sigma) mix in serum free media (DMEM/F12; Gibco) (see appendix for composition) and incubated for 5 hours at 37°C on a roller.

Matrix Intact (Lee et al, 1997)

Cells were passed through a 40μm pore filter (BD Falcon, France),

centrifuged at 2000rpm for 5 minutes and resuspended in 10ml serum free media. Cells were counted using a haemocytometer (Bright-Line) and adjusted to give a final concentration of 4000 cells ml^{-1} in serum free media.

Matrix Depleted Aguiar et al, 1999)

5 Cells were passed through a 40μm pore filter (BD Falcon, France), centrifuged at 2000rpm for 5 minutes and resuspended in 3U/ml Streptomyces hyaluronidase (Sigma) in DMEM/F12 containing 10% Foetal Calf Serum (FCS; Gibco) (see appendix for composition) and incubated for an hour at 37°C on a roller. Cells were counted and adjusted to give a final concentration of 4000
10 cells ml^{-1} as before.

CD44 Depleted (Aguiar D.J., et al 1999)

Cells were passed through a 40μm pore filter (BD Falcon, France), centrifuged at 2000rpm for 5 minutes and resuspended in serum free media containing 0.25% trypsin (Sigma) and incubated for 30 minutes at 37°C on a
15 roller. Cells were counted and adjusted to give a final concentration of 4000 cells ml^{-1}.

Cell Counts

Cell counts were made in this study on a haemocytometer (Bright-Line). Each chamber of the haemocytometer was filled with approximately 10μm by
20 capillary action. Cells within the 1mm centre square and four corner squares (see appendix) were counted in the chamber. Each square of the haemocytometer, with the cover slip securely in place, represents a total volume of 10^{-4}cm. It can be taken that $1cm^3$ is equivalent to approximately 1ml, so the cell concentrations per ml and total number of cells were found using the

following calculations:

Cells per ml = Average cell count (from 5 squares) x dilution factor x 10^4

Total cells = cells ml^{-1} x original fluid volume

Hyaluronan Coated Plates

5 Under sterile conditions, 1.5ml/well of 100μg ml^{-1} of hyaluronan solution (see appendix) was added to 6 well plates and left overnight on a shaker at room temperature. Plates were then washed 3 times with sterile Phosphate Buffered Saline (PBS; Sigma) (see appendix) then air dried under UV light in sterile conditions for 30 minutes.

10 Differential Adhesion Assay to Hyaluronan

Determine adhesion time for optimum Colony Forming Efficiency (CFE) for matrix intact

Exactly 1ml 4000 cells ml^{-1} of cell suspension from the digest was plated into a well of the 6 well hyaluronan coated plates and left for the following 15 intervals of time; 1, 5, 10, 15, 20 and 30 minutes (NB. 2 wells/time point) at 37°C, 5% CO_2.

After the adhesion assay, media was removed and 2ml of DMEM/F12 (Gibco) containing 10% FCS (Gibco) was added to each well, incubated at 37°C, 5% co_2. At day 1 an initial cell count of the total number of cells that adhered to 20 each well was recorded using an inverted phase contrast microscope (Zeiss). A colony count was taken at day 6 of colonies which contained > 33 cells, so the Colony Forming Efficiency (CFE) could be determined to assess the colony forming ability of the adhered chondrocytes. Additional colony counts were made at; day 3 for colonies of four cells, at day 4 for colonies of 12 cells and at

day 5 for colonies of more than 33 cells for comparative purposes.

CFE Equation

The Colony Forming Efficiency was worked out using the following equation:

5 Colony Forming Efficiency (CFE) =

$$\frac{\text{Number of Colonies (at defined time point)}}{\text{Initial Cell Count (d0)}}$$

Compare CFE for 3 digest conditions (n of 2)

The adhesion assay was then repeated at the optimum adherence time
10 for all 3 digests, with the matrix depleted and CD44 depleted (Aguiar *et al*, 1999) wells as controls. The isolated cells from all 3 digests (Matrix intact (Lee *et al*, 1997), Matrix depleted and CD44 depleted (Aguiar *et al*, 1999)) were resuspended in serum free media at a concentration of 4000 cells/ml. Exactly 1ml of 4000 cells/ml of the cell suspension was added to the correctly labelled
15 wells of the 6 well plates and left for the optimum adhesion time at 37°C, 5% CO_2.

After the adhesion assay, media was removed and 2ml of DMEM/F12 (Gibco) containing 10% FCS (Gibco) was added to each well. Cells were then incubated at 37°V, 5% CO_2. At day 1 an initial cell count of the number of cells
20 that adhered to each well was recorded using an inverted phase contrast microscope (Zeiss).

Erythrocyte Exclusion Assay

To visualise the pericellular matrix of the chondrocyte an exclusion of particles using fixed erythrocytes can be carried out. The erythrocyte exclusion

assay was carried out on the matrix intact, the matrix depleted and the CD44 depleted digests (the latter two acted as controls).

Freshly isolated cells were counted and adjusted to give a concentration of 1 x 10^6 cells ml^{-1} and plated straight onto uncoated dishes. They were
5 incubated at 37°C, 5% CO_2 for an hour to allow adherence of the cells to the culture plastic. Non-adherent cells and media were removed and discarded, and 750 μl of fixed erythrocytes (Sigma) at 1 x 10^8 cells ml^{-1} in PBS containing 0.1% bovine serum albumin (BSA; preservative) were added to the culture dishes. The erythrocytes were allowed to settle onto the 6 well plates for 15 minutes at
10 37°C, and were subsequently observed and photographed using an inverted microscope. For obtainment of an accurate result, each assay for each digest was completed in 2 wells of the culture dish and photographs were taken from 3 representative areas of the culture dishes.

Cloning

15 At day 6, colonies of more than 33 cells were cloned. Colonies of greater than 33 cells were identified and marked on the underside of the relevant wells (only colonies that were tightly packed and isolated from any other colonies were selected). Under sterile conditions the media was removed from the marked well and the well was washed with sterile HBSS (Ca and Mg free) (Gibco, UK).
20 An autoclaved, sterile polystyrene-cloning ring (Sigma; ID 4.7mm x H 8mm) was placed over the marked colony using petroleum jelly (Vaseline) to provide a waterproof sealant. Exactly 200 μl of 0.05% trypsin/EDTA (Gibco) was pipetted into the cloning ring, and the 6 well plate was put into the incubator for 5-10 minutes at 37°C, 5% CO_2 to release the adhered colony. The cells were

removed from the incubator and gently tapped on the base of the well to remove any remaining adhered cells. The trypsin cell suspension from each well was removed and placed into separate sterile Eppendorffs. DMEM/F12 (Gibco) containing 10% FCS (Gibco) was added to each well to remove any remaining cells and added to the cell suspension to also deactivate the trypsin. The cell suspension was centrifuged (Microcentaur; MSE) at 1500rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in media containing 10% FCS. Each colony was plated down onto a sterile 6 well plate (1 colony/well) containing a further 1ml of DMEM/F12 (Gibco) plus 10% FCS (Gibco) and incubated at 37°C, 5% CO_2.

Maintenance and Expansion of Clones

Feeding of clones

After 1 week in culture the colonies had expanded and cell proliferation was rapidly occurring. Fresh DMEM/F12 (Gibco) containing 10% FCS (Gibco) was required to provide the proliferating cells with the relevant nutritional requirements for growth. Under sterile conditions the existing media was removed from the well and fresh DMEM/F12 media containing 10% FCS was added to each culture dish and incubated at 37°C, 5% CO_2.

Passage of clones

When the cells reached confluence they were transferred into larger flasks to allow further expansion. Under sterile conditions the media was removed from each dish and discarded. Each dish was washed with sterile HBSS (Ca and Mg free) (Gibco, UK) and enough 0.05% trypsin/EDTA solution was added to completely cover the monolayer of cells. The culture dishes were

then incubated at 37°C, 5% CO_2 to allow successful trypsinisation to occur. Equal quantities of DMEM/F12 (Gibco) containing 10% FCS (Gibco) was added to deactivate the trypsin to inhibit further enzymatic digestion. The trypsin/media cell suspension from each culture dish was placed into separate 50ml centrifuge
5 tubes. The culture dish was washed with media containing 10% FCS and added to the relevant centrifuge tubes. The cell suspension was centrifuged at 2000rpm for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in desired amount of fresh media containing 10% FCS. Cell counts were recorded and cells were either plated into fresh sterile culture
10 vessels or were used for differentiation studies. Cells were fed using media containing 10% FCS every 48-96 hours.

Freezing cells

Under sterile conditions media was removed from the culture dish and washed with HBSS (Ca and Mg free) (Gibco, UK) to remove all traces of serum.
15 Enough 0.05% trypsin/EDTA (Gibco) solution was added to completely cover the monolayer and was incubated at 37°C, 5% CO_2 for 3-5 minutes. Following trypsinisation at least an equal volume of fresh growth medium containing serum was added to prevent further trypsinisation. The resultant cell suspension was transferred into a centrifuge tube and centrifuged at 2000rpm for 5 minutes at
20 room temperature. The supernatant was then discarded and the cell pellet was resuspended in FCS and 10% dimentylsulphoxide (DMSO). The FCS/cell suspension was then transferred into clearly labelled sterile cryotubes and placed in a polystyrene box and stored at –80°C. In all cases cell counts were performed prior to freezing.

Re-plating frozen down cells

The cells stored in cryotubes at –80°C were rapidly thawed by transferring the cryotubes containing the frozen cells directly into a 37°C water bath. The contents of the cryotube were then transferred into an appropriately sized sterile culture dish containing DMEM/F12 (Gibco) containing 10-20% FCS (Gibco). The cell suspension was pipetted drop by drop into the culture flask (to avoid the chance of cell shock) whilst swirling the media and incubated at 37°C, 5% CO_2. Fresh media was added after 24 hours to remove any traces of the DMSO.

Population doublings

The age of the cultures were expressed as a Population Doubling level instead of a passage number. The population doubling was calculated using the following equation: $n = (\log Y - \log X) / 0.301$

Where: n = Population Doubling (PD)

Y = Final Cell Count

X = Inoculation Cell Count

Pellet Cultures

Pellet Cultures were established using a suspension of cells at a concentration of 1×10^6 cells ml^{-1}. Exactly 1 ml of the cell suspension was pipetted into sterile 1.5 ml Eppendorff and spun at 1000 rpm for 3 minutes. The resultant cell pellets were incubated at 37°C, 5% CO_2 for a week and fed every 48-72 hours. Cell pellets were cultured in the following three media types:

- Insulin Transferrin Selenium (ITS) – Base medium (see appendix for composition) plus 1% ITS.

- Transforming Growth Factor β 1 – Base medium plus 5ng/ml TGFβ 1 (see appendix for composition).
- ITS/ TGFβ 1 – Base medium plus 5ng/ml TGFβ 1 plus 1% ITS.

During the culture period, when the pellets were fed, media was removed
5 from the 3 pellets at 48, 96, and 144 hours and stored in clearly labelled Eppendorff. A DMMB assay was then carried out on the 3 pellets and their 3 media samples were taken at 48, 96, and 144 hours to show the glycosaminoglycan content of the pellet and the amount of glycosaminoglycans released into the media at the specified time points giving a total
10 glycosaminoglycan content for each pellet.

Monolayer Culture

Induction of adipogenesis or osteogenesis

Cells were plated down onto 12 well plates and were adjusted to give a final concentration of 10,500 cells ml^{-1} (3000 cells/cm^2) recommended by a
15 variety of protocols.

Cells were grown in DMEM/F12 containing 10% serum until 80% confluence was reached. After this time the plates were incubated at 37°C, 5% CO_2 and supplemented with differentiation medium supporting either osteogenesis or adipogenesis (see appendix for composition). Media was
20 changed every 48 hours.

Analysis of chondriogenic differentiation

Dimenthylmethylene Blue (DMMB) Assay

Pellet cultures were digested in Papain (4.46 μl m^{-1}) (Sigma) in a buffer containing cysteine (0.78 μg ml^{-1}) and Sodium-EDTA (1.86 μg ml^{-1}) overnight at
5 65°C.

Pellets digested in papain, and media samples collected at 48, 96, and 144 hours, were subjected to a DMNB assay to show the glycosaminoglycan content of the pellet and the amount of glycosaminoglycans released into the media giving a total glycoasminoglycan content for each pellet.

10 Ninety-six well plates were used for this assay. Chondroitin sulphate standards were set up at the following concentrations 0, 10, 20, 30, 40 μg ml^{-1}. Exactly 40 μl of standard was added to duplicate wells, 30 μl of sample plus 10μl of chondroitin sulphate (10 μg ml^{-1}) were added to duplicate wells, followed by 200 μl of DMMB using a multi-channel pipette. The plates were
15 then read using a multiscan ms plate reader (Labysystems, UK) at 540nm. The concentration of glycosaminoglycan (μg ml^{-1}) was determined; 10μg ml^{-1} was subtracted from the final glycosaminoglycan concentration for each sample. From this data, averages of duplicate sample were calculated and the total glycoasminoglycan concentration for each sample. From this data, averages of
20 each duplicate sample were calculated and the total glycoasminoglycan concentration from the media and pellet samples were calculated.

Osteogenic Differentiation Analysis

Alkaline phosphatase staining

An alkaline phosphatase kit was used following the manufacturers instructions containing Sodium Nitrite solution, Naphthol AS-BI Alkaline solution and FBB-Alakaline solution (Sigma) (see appendix for composition).

Media was removed from each well washed in PBS (Sigma) and then cells were fixed in 10% Neutral Buffered Formalin Solution (NBFS; Sigma) for 30 minutes at room temperature. Cells were then washed with distilled water prior to the addition of the alkaline phosphatase solution. The solution was left on for 30 minutes, after which time the wells were then washed with PBS and mounted under 16mm circular coverslips (RA Lamb) using aquamount (RA Lamb). Staining was observed under microscopic analysis.

Von Kossa staining

Media was removed from each well, wells were washed with PBS (Sigma) and then cells were fixed using 10% NBFS for 30 minutes. Cells were then washed 3 times with distilled water and stained with fresh 5% silver nitrate (Sigma) for 30 minutes under UV light. Cells were washed 3 times with distilled water and developed with fresh 5% sodium carbonate (BDH), made up in 10% NBFS for 5 minutes. Cells were again washed 3 times with distilled water and fixed with 5% Sodium thiosulphate (Sigma) for 2 minutes. A final 3 washed with distilled water was carried out and then the cells were mounted under 16mm circular coverslips (RA Lamb) using aquamount (RA Lamb). Staining was observed under microscopic analysis.

Note: Extra care had to be taken when washing out silver nitrate so a false positive result did not occur.

Statistical analysis

Results of the differential adhesion assay for determining the optimum
5 adhesion time and the comparison of the CFE for all digests at the optimum adhesion time, and the results of the DMMB assay were statistically analysed using a 1 way analysis of variants (ANOVA) and a multiple comparison test (lsd) to a 95% confidence level ($p<0.05$).

RESULTS

10 Optimum adhesion time for matrix intact chondrocytes

To determine the optimum adhesion time for matrix intact surface zone chondrocytes to adhere to the hyaluronan coated dishes, a differential adhesion assay was carried out at the following intervals of time: 1, 5, 10, 15, 20 and 30 minutes (at an n of 3). The optimum initial adhesion time for the surface zone
15 matrix intact chondrocytes to adhere to the hyaluronan coated dishes is 10 minutes (Figure 3A). Significant differences in the initial adhesion time were evident between surface zone chondrocytes plated on hyaluronan for 1 minute and those plated on hyaluronan for 10 minutes (Figure 3A: *$p<0.05$).

If these cells with a high affinity for hyaluronan are a population of
20 chondroprogenitor cells they should have the ability to be able to form large numbers of colonies from an initially low seeding density, such as that with other tissues which have a clearly defined stem cell population (Jones and Watt, 1993). To assess the clonality of the adhesive chondrocytes, the number and

size of colonies of chondrocytes that were subjected to differential adhesion to hyaluronan were counted and recorded.

The Colony Forming Efficiency (CFE) of surface zone chondrocytes initially cultured on hyaluronan for 10 minutes was greater than that of all other time frames, when the definition of a colony is being more than 33 cells was applied (Figure 3D). Using 4 cells, and 12 cells as being indicative of a colony for comparative purposes, the same trend is apparent with matrix intact surface zone cells subjected to differential adhesion to hyaluronan for 10 minutes having a higher CFE at day 3 and day 4 respectively, relative to all other time frames (Figure 3: B&C).

From the initial adhesion data taken at day 1 ad the CFE data at day 3, day 4, and day 6, the optimum time for isolated matrix intact chondrocytes to adhere to the hyaluronan coated dishes is 10 minutes.

Comparison of the CFE of all digests at the optimum adhesion time

The differential adhesion assay was then repeated at the optimum adhesion adherence time of 10 minutes, for the matrix intact chondrocytes, and the matrix depleted and CD44 depleted chondrocytes as controls.

At the initial adhesion colony count taken at day 1, more matrix depleted chondrocytes adhered to the hyaluronan coated dishes compared to a low number of CD44 depleted chondrocytes initially adhering (Figure 4A). The number of adhered matrix intact chondrocytes at the day 1 cell count was consistent with the matrix intact initial cell counts at 10 minutes, taken when the optimum adhesion time was being determined (Figure 4A).

To assess the CFE of the chondrocytes for the 3 digests, the number and size of colonies of chondrocytes subjected to differential adhesion to hyaluronan for 10 minutes were counted and recorded. The CFE potential of the chondrocytes also demonstrates if the adhesion to hyaluronan is CD44 dependent. No significant differences were found between the 3 digests. Although the CFE of matrix intact surface zone chondrocytes initially cultured on hyaluronan for 10 minutes appeared greater than that of the matrix depleted and the CD44 depleted chondrocytes, when the definition of a colony is defined as being greater than 33 cells was applied (Figure 4C).

Using 4 cells as being indicative of a potential colony for comparative purposes, the CFE for the matrix depleted chondrocytes is almost double that of the CFE for the CD44 depleted chondrocytes (Figure 4B), with the curve of the graph resembling that of the day 1 colony count for the initial adhesion of the chondrocytes, from the 3 digests, adhering to the hyaluronan coated dishes (Figure 4: A&B).

The data taken at day 1 of the initial adherence, of the matrix intact, matrix depleted and CD44 depleted chondrocytes, to the hyaluronan coated dishes (Figure 4A), showed that there are very low numbers of CD44 depleted chondrocytes (Mean adherence is 36.7 cells) that adhere to hyaluronan after 10 minutes, whereas, in comparison there are very high numbers of adhered matrix depleted chondrocytes (Mean adherence is 224.83 cells) and a slightly lower number of adhered matrix intact chondrocytes (Mean adherence is 124.67 cells) compared to the matrix depleted chondrocytes.

From the data it is evident that the adhesion of the chondrocytes to the hyaluronan coated plates is dependent on cell surface receptors, i.e. adhesion is CD44 dependent.

Visualisation of the pericellular matrix

5 Immature bovine surface zone chondrocytes with their pericellular matrices intact (MI) produced large pericellular matrices that can be visualised by exclusion of fixed erythrocytes (Figure 5A, B&C). In all photographs, taken from representative areas of the culture dishes, pericellular matrices were visible on the matrix intact chondrocytes, as the erythrocytes are not touching the 10 chondrocytes. There is no visible pericellular matrix for the CD44 depleted chondrocytes after treatment with trypsin (Figure 5D, E&F) as the erythrocytes are completely touching the CD44 depleted chondrocytes. There is a disruption in the pericellular matrices of the matrix depleted chondrocytes treated with *Streptomyces hyaluronidase*, as some erythrocytes are touching the 15 chondrocytes, whereas some are not (Figure 5G, H&I).

Population doublings

Population doubling was calculated prior to the establishment of the clones used for pellet culture. A 52 cell colony was taken from the optimum adhesion time of 10 minutes at day 6 was cloned and expanded in culture until 20 pellet cultures were established. It was calculated there were 21 population doublings from the initial 52 cell colony to when pellet cultures were established (Figure 6).

Pellet cultures

Morphology of the pellets

The pellet cultured with ITS containing media has a flattened, discoid morphology. (Figure 7: ITS). The pellet cultured with TGFβ1 containing media
5 has a rounded morphology typical of that of a high density pellet culture (Figure 7: TGFβ1). The ITS/TGFβ1 media culture also has a rounded and compact morphology, typical of that of a high density pellet culture, except that this pellet appears larger than the pellet cultured with TGFβ1 alone (Figure 7: ITS/TGFβ1).

10 DMMB Assay

During the culture period, when the pellets were fed, media was removed from the pellets at 48, 96, and 144 hours and stored in clearly labelled Eppendorffs. A DMMB assay was then carried out on the pellets and the media to test for glycosaminoglycan content. DNA content could have also been
15 analysed.

Results of the DMMB assay shows that there is a significance difference in the total concentration of glycosaminoglycans between the pellets treated with ITS/TGFβ1 and ITS alone and also between the [pellets treated with ITS/TGFβ1 and TGFβ1 (*p<0.05). The concentration of glycosaminoglycan of
20 the ITS/TGFβ1 treated pellet is double that of the ITS treated pellet (Figure 8).

Analysis of differentiation capacity

Adipogenic analysis

Cells from the monolayer culture treated with adipogenic media died after 2 weeks in culture. Figure 9A shows that the cells have lifted off the culture dish
5 and with nothing to adhere to the cells died, whilst the cells treated with media containing 10% FCS were healthy and confluent within the culture dishes (Figure 9B).

Osteogenic analysis

Cells from the monolayer culture treated with osteogenic media have a
10 different morphology than that of the control cells supplemented with media containing 10% FCS. The cells treated with media promoting osteogenic differentiation appear to have had a plump, spindle shaped morphology in comparison to the chondrocytes treated with FCS (Figure 9: C&D).

Alkaline Phosphatase:

15 Cells from the 2-week-old monolayer culture supplemented with osteogenic media were tested for alkaline phosphatase activity. There was a negative result for the presence of alkaline phosphatase for the cells cultured with osteogenic media. A negative result was also apparent for the control cells cultured with media containing 10% FCS (Figure 9: E&F).

20 *Von Kossa:*

Cells from the 2-week-old monolayer culture supplemented with osteogenic media were analysed with Von Kossa staining and tested for the presence of calcium nodules. There was a negative result for the presence of

calcium nodules for the cells cultured with osteogenic media and the control cells cultured with media containing 10% FCS (Figure 9: G&H).

CONCLUSIONS

We have isolated a progenitor cell population from articular cartilage and,
5 in particular, the surface zone thereof in order to provide a population of progenitor cells that is characterised by either its high affinity for hyaluronan or its ability to form a large number of colonies from an initial low seeding density. This population of cells has a high expression of CD44 which, at least in part, explains its ability to bind hyaluronan. This high affinity for hyaluronan is
10 unusual and is an indicator for the existence of the pericellular matrix. Moreover, we found that our pericellular matrix retaining chondrocytes not only form colonies but can successfully be cloned and expanded in culture. Further, we found that these cultured cells retain their chondrogenic phenotype when grown in an environment permissive for chondrogenesis. We have therefore
15 been able to produce a stable population of chondrocytes for use in tissue repair. Additionally, we have also been able to produce a progenitor cell population from articular cartilage which can either be used directly in tissue repair or, indirectly, after the progenitor cell population has been cultured to produce chondrocytes.

20

CLAIMS

1. A method for isolating a cartilage progenitor cell population comprising:

(a) obtaining articular cartilage tissue; and

(b) digesting this tissue, to release progenitor chondrocytes or chondrocytes, by using enzymes that digest all but the said chondrocytes and their pericellular matrix.

2. A method according to Claim 1 wherein said articular cartilage is digested with dispase and collagenase.

3. A method according to Claim 2 wherein 2-5U/ml of dispase is used.

4. A method according to Claim 3 wherein 3.78U/ml of dispase is used.

5. A method according to Claims 2-5 wherein 0.1-1.2U/ml of collagenase is used.

6. A method according to Claim 5 wherein 0.74U/ml of collagenase is used.

7. A method according to any proceeding claim wherein said digestion is undertaken at 37°C.

8. A method according to any proceeding claim wherein the digestion involved agitation for a period up to 5 hours.

9. A method according to any proceeding claim wherein the digested material is filtered to isolate the released progenitor chondrocytes and chondrocytes.

5

10. A method according to Claim 9 wherein a filter with a pore size no less than 40 μm is used.

11. A method according to Claim 10 wherein filtration is followed by
10 centrifugation between 300g to 700g.

12. A method according to Claim 11 wherein centrifugation is performed at 620g.

15 13. A method according to any proceeding claim wherein the progenitor chondrocytes or chondrocytes are resuspended in tissue culture medium.

14. A method according to any proceeding claim wherein said progenitor chondrocytes or chondrocytes are exposed to hyaluronan.

20

15. A method according to Claim 14 wherein the hyaluronan is coated on a substrate.

16. A method according to any proceeding claim wherein the articular cartilage tissue is obtained from surface or superficial cartilage tissue.

17. A method according to any proceeding claim wherein the cartilage tissue is taken from an individual to be treated and therefore presents autologus tissue.

18. An isolated articular cartilage progenitor cell population which is characterised in that a substantial number of the cells of said population have retained their pericellular matrix.

19. An isolated articular cartilage progenitor cell population when isolated according to the methods of Claims 1 – 17.

20. A population according to Claims 18 or 19 wherein said population is stable.

21. Use of an articular cartilage progenitor cell population according to Claims 18 – 20 for use in tissue repair.

22. An articular cartilage progenitor cell population which is characterised by either, or both, of the following:

(a) the existence of a pericellular matrix; and/or

(b) the ability to rapidly bind hyaluronan.

23. A cell population according to Claim 22 wherein the ability to bind hyaluronan occurs within 15 minutes.

24. An implant for use in tissue repair comprising a cell population according
5 to Claims 18-20 and 22-23.

25. An implant according to Claim 24 wherein the implant comprises conventional scaffolding on which the said cells are grown.

10 26. A method for isolating an articular cartilage progenitor cell population comprising the binding of said cell population to hyaluronan.

27. A method according to claim 26 wherein said binding is undertaken rapidly and so within 15 minutes.

15

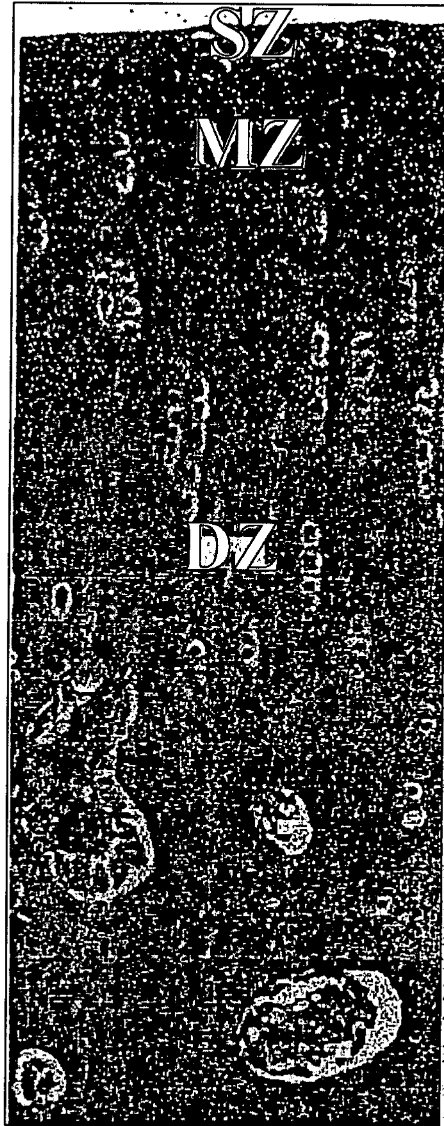

Fig. 1

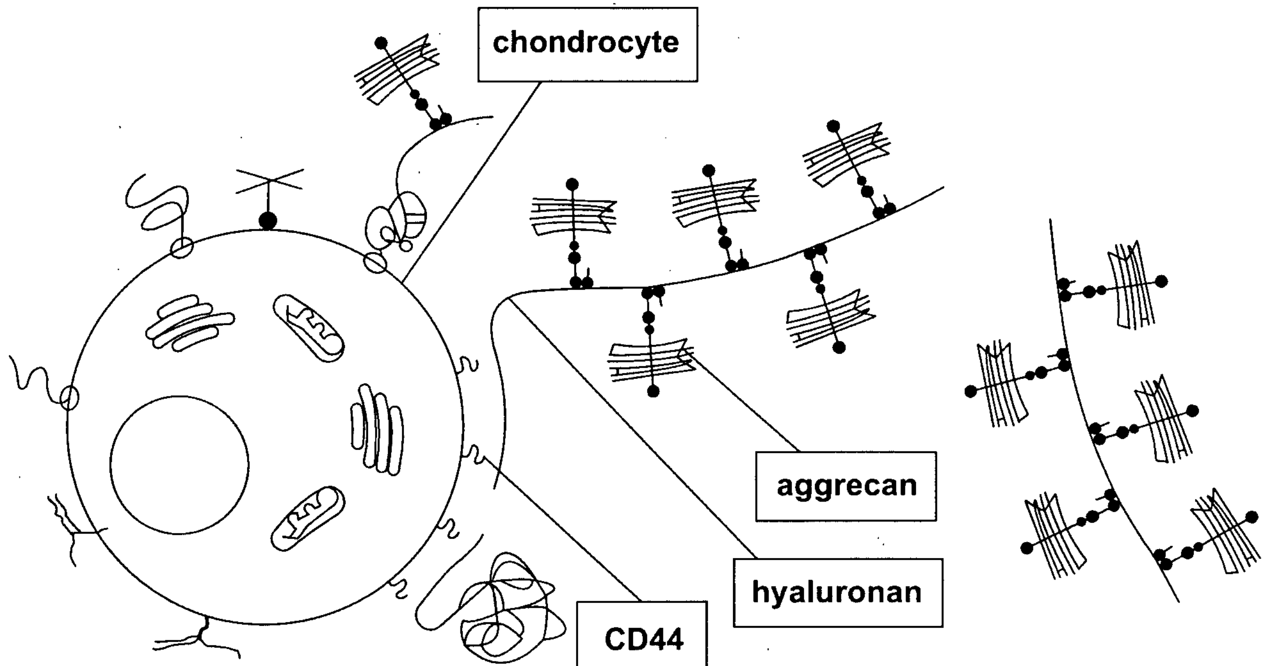

Fig. 2

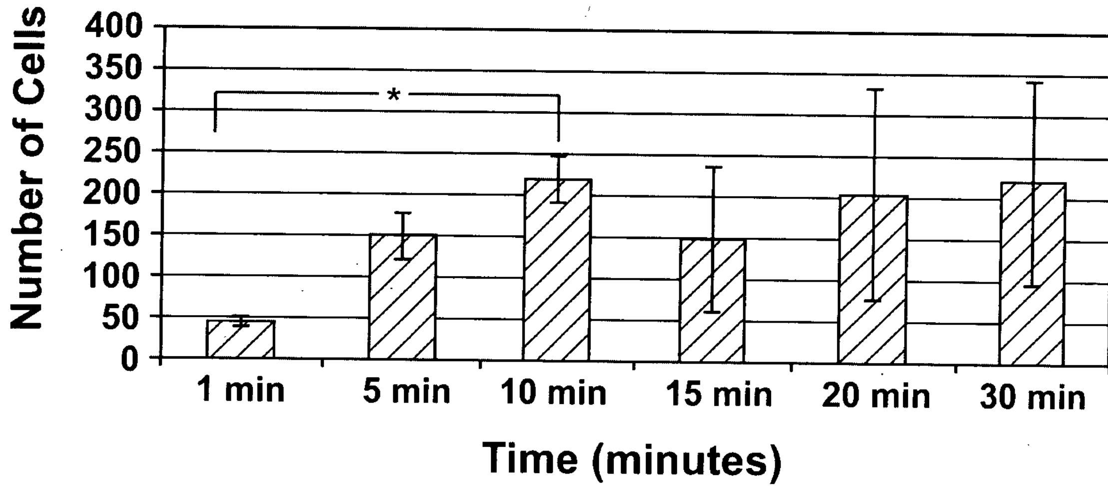

Fig. 3A

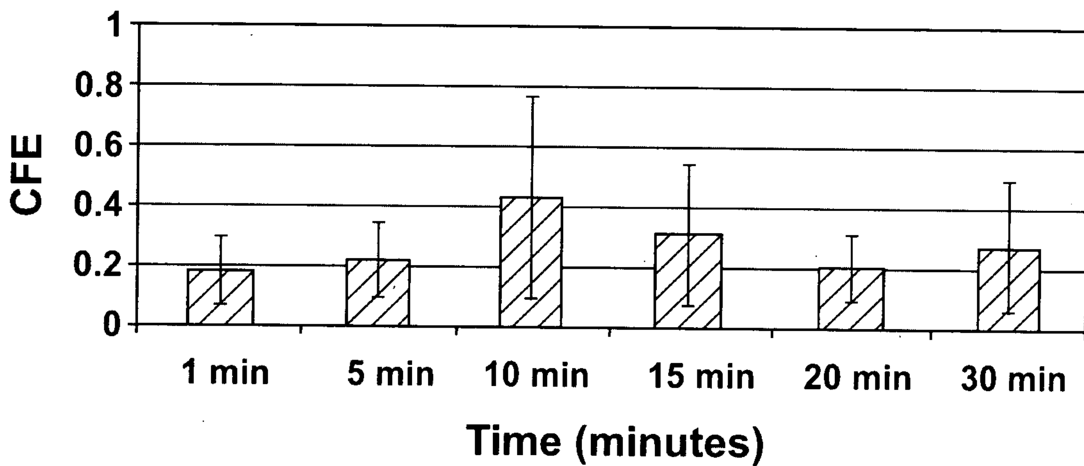

Fig. 3B

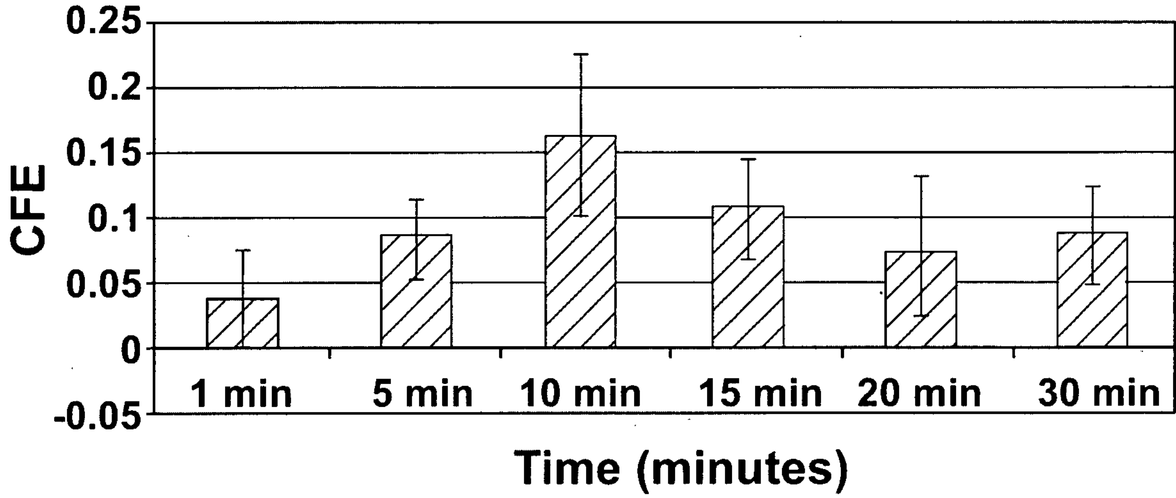

Fig. 3C

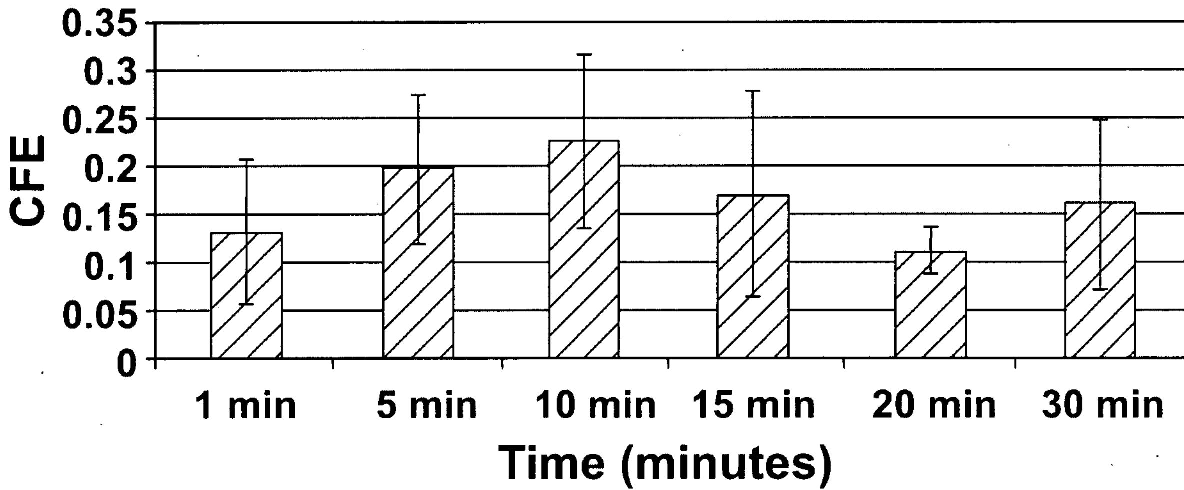

Fig. 3D

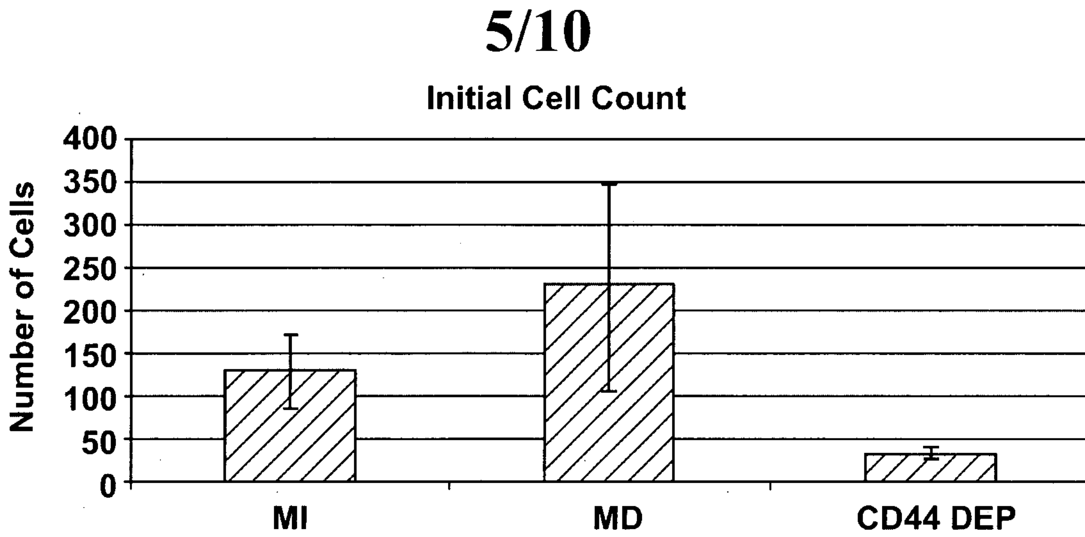

Fig. 4A

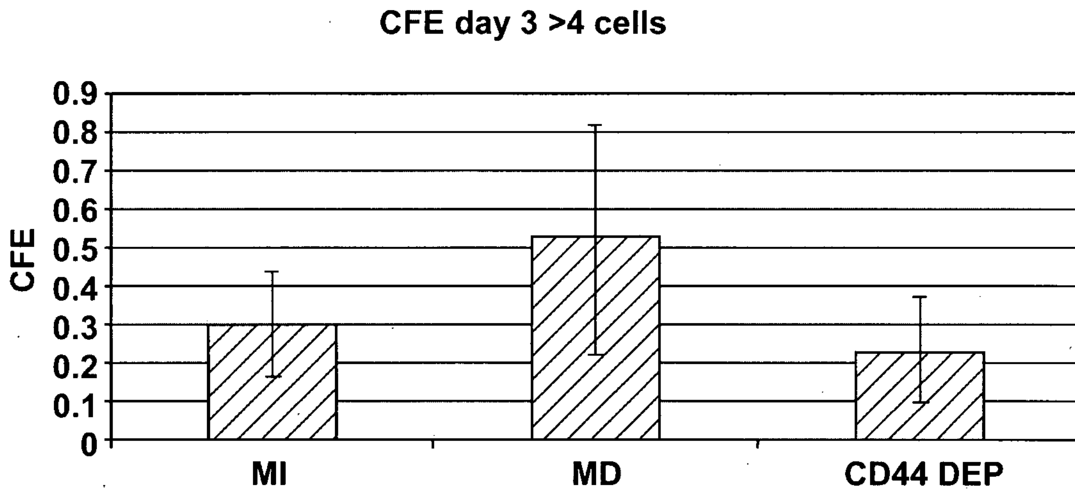

Fig. 4B

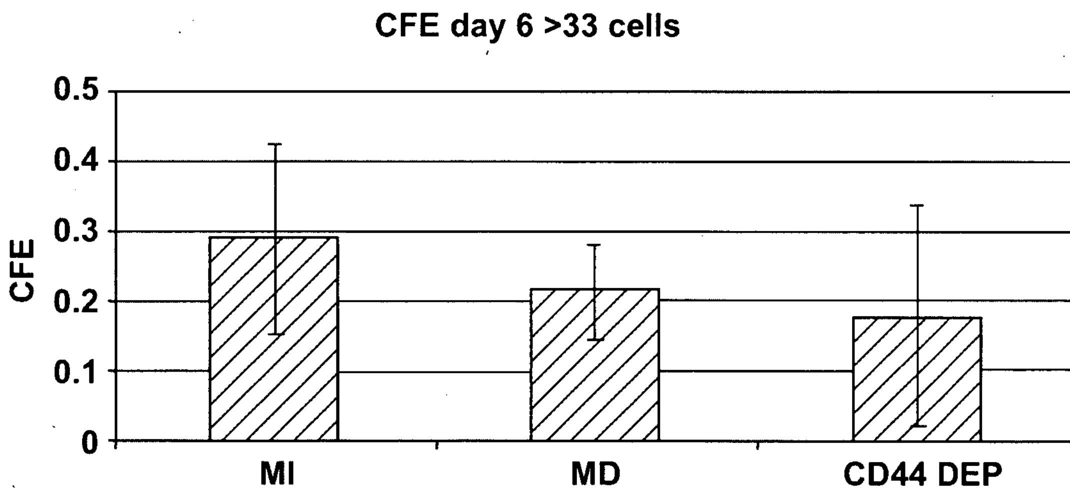

Fig. 4C

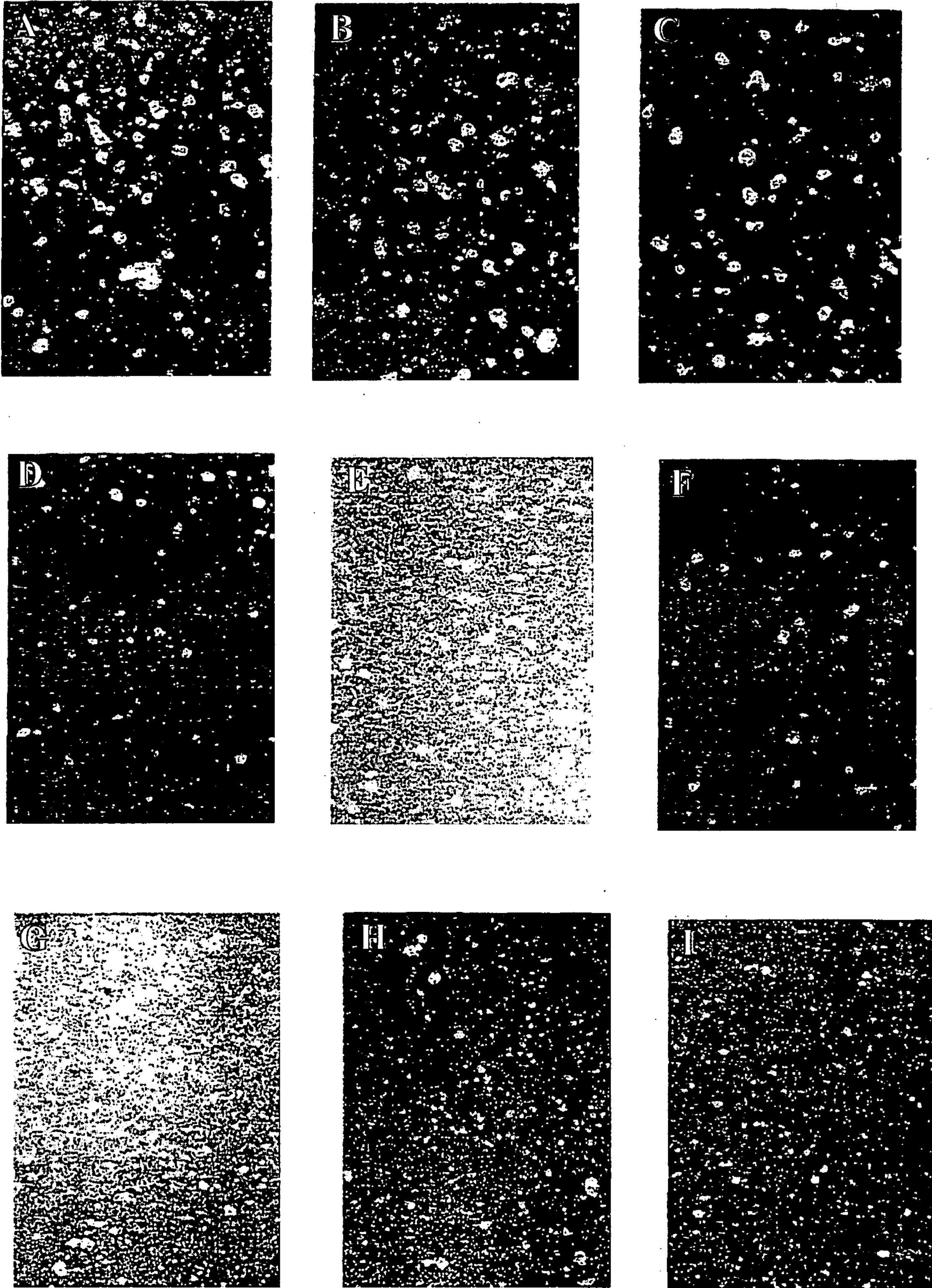

Fig. 5

Fig. 6A

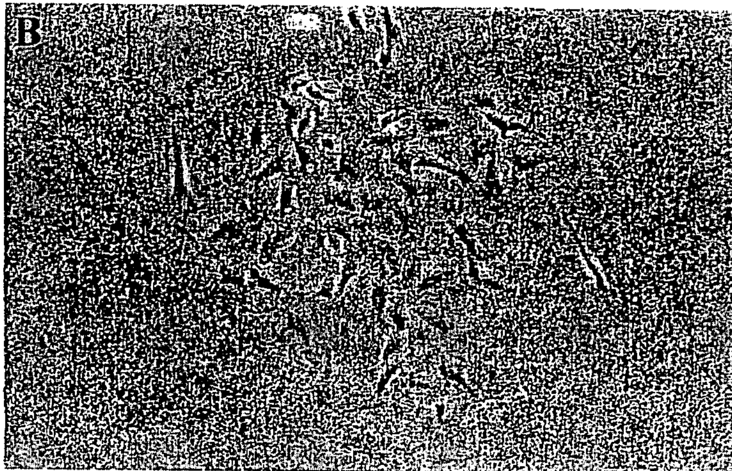

Fig. 6B

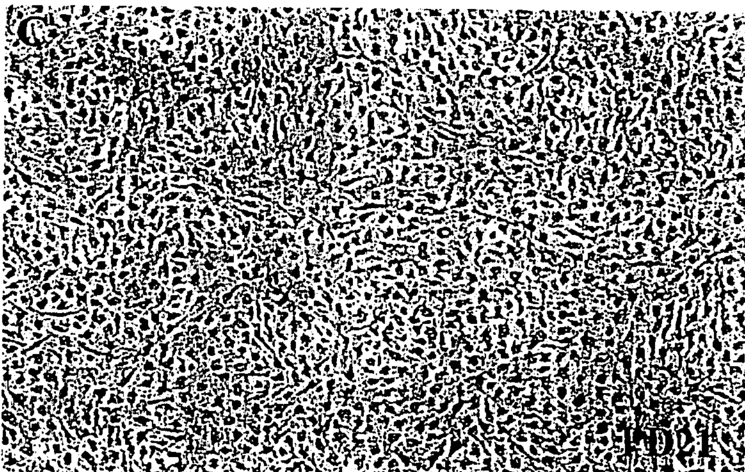

Fig. 6C

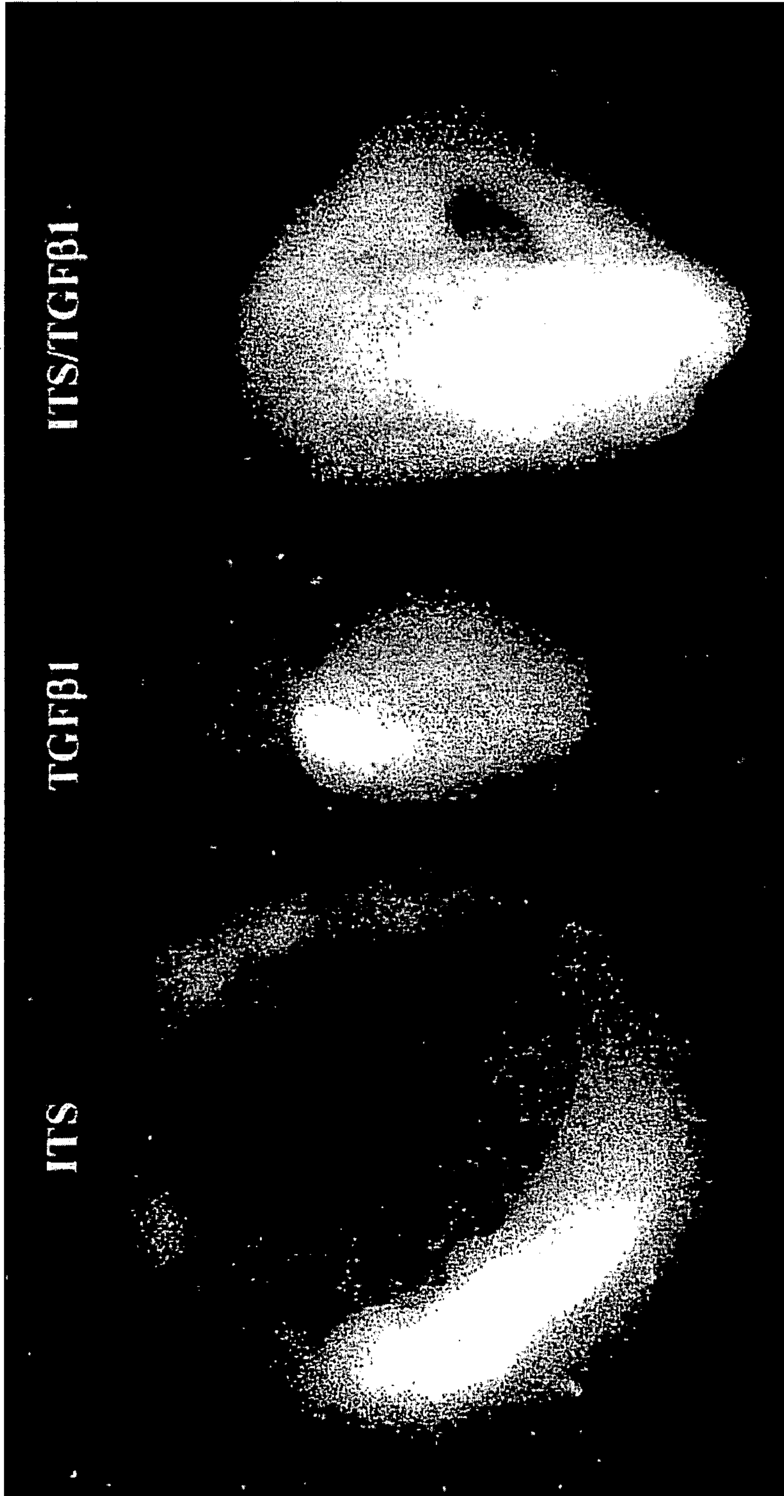

Fig. 7

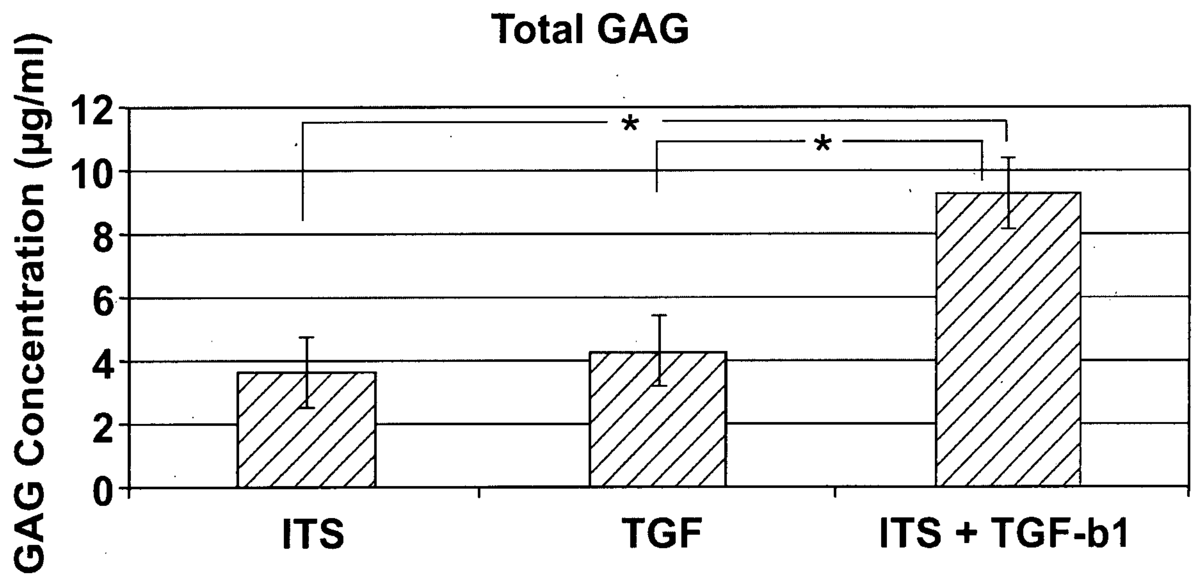

Fig. 8

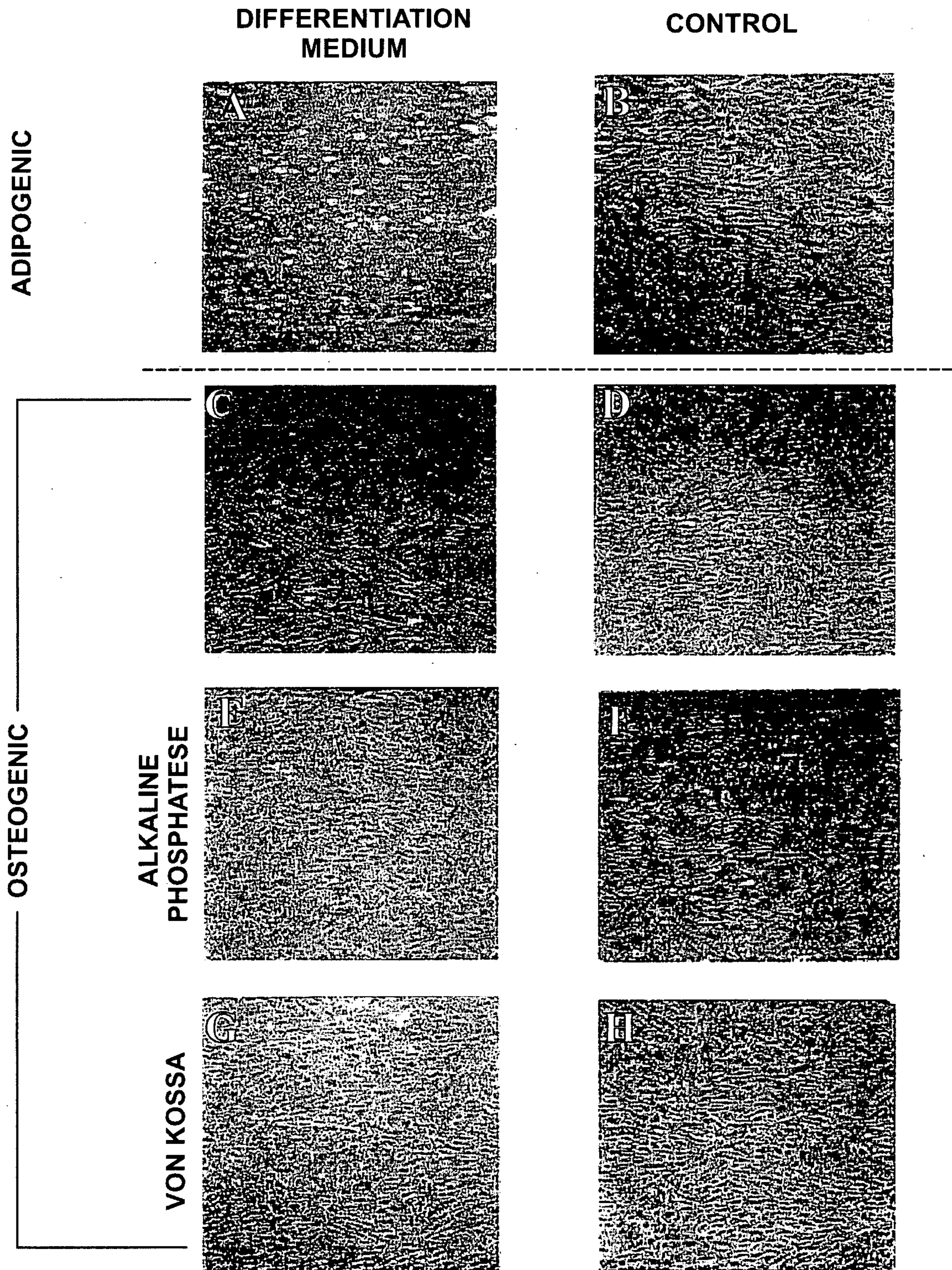

Fig. 9