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Cartilage regeneration: Injectable matrix for bone marrow stem cells

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Abstract

Human articular cartilage heals poorly in adults and current surgical procedures do not provide long-term repair. Cell therapy and tissue engineering may provide a solution for this problem. In this study the effectiveness of gels consisting of hyaluronic acid, fibrin and collagen were investigated with respect to cartilage formation from bone marrow stromal cells. Gelling properties, morphology and cell differentiation was determined in gels of different compositions. Histology showed that the cells organized in lacunae and deposited matrix in all gels. Immunohistochemistry confirmed that the matrix consisted of collagen type II, a typical marker for mature chondrocytes. Gels based on hyaluronic acid were inducing chondrogenesis more efficiently than collagen based ones, which could be due to interactions between the hyaluronic acid and the cells during differentiation.

Keywords

Cartilage, fibrin, hyaluronic acid, bone marrow stromal cells, chondrocytes, collagen type II

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Cartilage regeneration:

Injectable matrix for bone marrow stem cells

Sammanfattning

Broskskador är ett stort problem för många människor, t ex förslitningsskador och idrottsskador, då denna vävnad läker dåligt i vuxna individer. Brosk är en av de mest transplanterade vävnaderna och det finns idag ingen effektiv metod för långsiktig läkning.

I denna studie har injicerbara geler bestående av fibrin/hyaluronsyra och fibrin/collagen undersökts som bärare för stamceller i syfte att regenerera broskvävnad.

Användning av en injicerbar gel ger flera fördelar gentemot dagens tekniker: det krävs ingen operation för att få stamcellerna på plats, den kan anta samma form som skadan och den kan innehålla terapeutiska faktorer genom enkel blandning i den flytande gelkomponenten.

Gelningsegenskaper hos matrisen samt tillväxt och mognad av stamcellerna till broskceller har undersökts *in vitro*, d.v.s. i provrör på lab. Stamceller som mognat till en viss celltyp, i detta fall broskceller, sägs vara differentierade. Differentierade broskceller utsöndrar sin egen extracellulärmatris, och histologi visade att geler baserade på hyaluronsyra ger mer utsöndrad matris än collagenbaserade. Immunohistokemi konfirmerade att matrisen bestod av ett protein som fungerar som en markör för mogna broskceller, collagen typ II.

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Februari 2004

1. Introduction

Damage to cartilage is a major problem for millions of people because of the limited self-renewal of this tissue. Articular cartilage defects do not heal adequately and often progress to osteoarthritis.[1] Cartilage is one of the most frequently transplanted tissues, but still there is no surgical procedure in use today that effectively provides long-time repair. The use of cell therapy and tissue engineering may be the answer we are looking for.

Tissue engineering has been defined as the development and manipulation of laboratory-grown cells to replace or support the function of defective or injured body parts.[2] It takes advantage of the combined use of cultured living cells and three dimensional scaffolds or matrices to deliver vital cells to the damaged site of the patient.

The use of stem cells could be the ultimate solution to tissue engineering since they have the potential to become any type of tissue. The conventional definition of a stem cell is an undifferentiated cell with the capacity of self-renewal and the ability to produce at least one type of differentiated progeny.[3]

Bone marrow stromal cells (BMSC) are adult stem cells that can easily be expanded and grown *in vitro*. They have the capacity to differentiate primarily into cartilage, bone and adipocytes, but recently it has been shown that BMSC also can generate cell lineages like neurons and cardiomyocytes.[4]

Creating new materials for implantation that are biocompatible and do not induce inflammation and forming of scar tissue is a challenge for today's scientists. Different approaches for cartilage repair involve the transplantation of chondrogenic cells in preformed biodegradable scaffolds[5], which serve as provisional templates into which cells can penetrate during growth and differentiation.[6]

Another method, currently used in clinic, is based on autologous chondrocyte implantation. It involves two surgical steps, one in which a biopsy of healthy cartilage is removed from the knee joint and a second operation where the chondrocytes expanded *in vitro* are replaced in the lesion which is covered by a sutured perosteal flap to keep the cells in place.[7]

Recently the idea of using injectable, gel-forming matrices have been reported.[8] They are of special interest since they may offer several advantages over the preformed scaffold, such as the ability of assuming any shape of a defect, capacity to contain therapeutic agents by simple mixing and there is no requirement of surgical procedure for placement.

For chondrogenesis, a gel consisting of hyaluronic acid would be more preferable since it is the main component of the extracellular matrix (ECM) in cartilage tissue.[9] A combination of hyaluronic acid and fibrin will provide transplant fixation, a compliant microenvironment and long-term viability of the transplanted cells.

The hypothesis is that successful cartilage regeneration will be achieved using a matrix consisting of fibrin and hyaluronic acid, incorporated with BMSC that can proliferate and differentiate into cartilage tissue.

2. Aim

The objectives of this study were to evaluate the effectiveness of gels consisting of hyaluronic acid-fibrin and collagen-fibrin as injectable matrices for cartilage formation *in vitro*, using human BMSC and also investigate the properties of such matrices.

The biomaterial scaffold in tissue engineering applications functions as a temporary artificial ECM, to guide the cells in their three dimensional growth. To have a successful growth, the matrix has to be biodegradable, biocompatible and provide a proper microenvironment. The latter includes characteristics like pore structure, mechanical properties such as modulus and delivery of growth factors and other morphogenic factors.[10]

Considering the advantageous gel forming properties of fibrin, its adhesive quality and an excellent tissue tolerance as well as the inducing role of hyaluronic acid during chondrogenesis, a gel consisting of these two components would provide a suitable basis for cartilage regeneration. Gels of collagen are frequently used in biomaterial scaffolds and were investigated as a comparison.[11,12]

BMSC can easily be isolated from a patient's marrow and after *ex vivo* expansion they still possess stem features [2]. This makes them an interesting target for use in cell therapy.

3. Theoretical background

3.1 The extracellular matrix

Cells in the connective tissue produce their own environment called the extracellular matrix.[13] Simply put, it consists of entangled macromolecules like collagen, elastin, proteoglycans and plasma proteins. The ECM functions as lubrication, barrier, cell anchor and cell mediator. It also provides mechanical stability and is storage of nutrients, lipids and water.[14]

3.2 Cartilage and connective tissue

There are several types of connective tissue, which are responsible for providing and maintaining form in the body. The major constituent of connective tissue is ECM, unlike other tissues (epithelium, muscle and nerve), which mainly consists of cells.[15]

Cartilage consists of cells called chondrocytes, deriving from mesenchymal cells, or BMSC, and an extensive ECM enriched with glucosaminoglycans and proteoglycans.[16] Chondrocytes synthesize and secrete the ECM and the cells themselves are located in matrix cavities called lacunae, as illustrated in figure 1.

There are three types of cartilage; each with a different composition of the matrix components adapted to different biomechanical needs.[14] The most common type is hyaline cartilage, where type II collagen is the principal collagen type. These proteins interact with proteoglycan aggregates consisting of mainly hyaluronic acid. Cartilage is an avascular tissue, and chondrocytes exhibit low metabolic activity. The tissue is nourished by the diffusion of nutrients from capillaries in the adjacent connective tissues or by synovial fluid from joint cavities.[17]

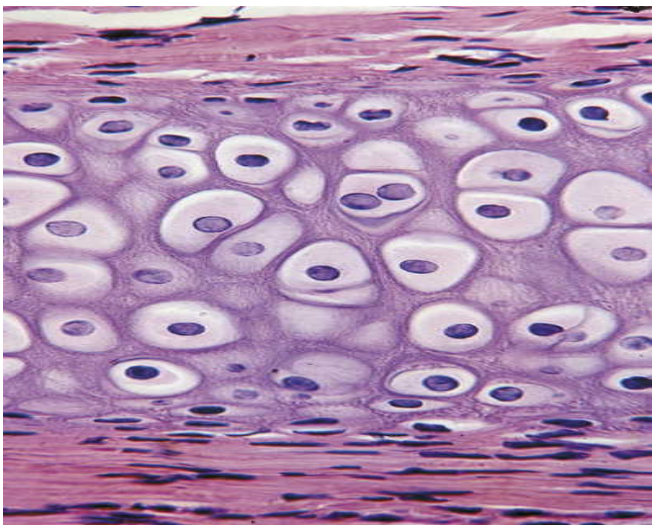


Figure 1. Hyaline cartilage stained with hematoxylin and eosin. The chondrocytes are located in matrix lacunae. Picture shown with permission from the McGraw-Hill Companies.[14]

Damage to cartilage is a major problem for millions of people because of the limited self-renewal of this tissue. Articular cartilage defects do not heal adequately and often progress to osteoarthritis. Another problem is meniscal tear, a common injury in contacts sports. The lack of vascularisation and penetration of lymphatic vessels severely limits healing of cartilage defects that are superficial to the subchondral plate.[18] Deeper lesions penetrating the vascularized subchondral bone are repaired to some extent but with a poorly functional fibrocartilaginous tissue.[17] Cartilage is one of the most frequently transplanted tissues, but still there is no surgical procedure in use today that effectively provides long-time repair.

3.3 Bone Marrow Stromal Cells

The bone marrow stromal system is defined as the connective tissue elements providing structural and functional support for hemopoiesis. BMSC are pluripotent adult stem cells that reside in the postnatal bone marrow cavity, and have been shown to undergo osteogenic, chondrogenic and adipogenic differentiation. Their progeny give rise to skeletal tissue: cartilage, bone, tendon, ligament, fat, muscle and other connective tissues[19] and are responsible for the continuous bone turnover throughout life.[20] It has been shown that they after *ex vivo* expansion still possess stem features[19], and this fact together with the ease of which they can be isolated from a patients marrow and expanded *in vitro* makes them an interesting target for use in cell therapy.

To be used for clinical therapy, the BMSC system needs to be highly standardized and reproducible in bone and cartilage formation efficiency. At the Advanced Biotechnology Centre in Genoa, Italy, professor Cancedda and co-workers have standardized the culture conditions for BMSC, using fibroblast growth factor-2 during expansion.[21, 21]

The *in vitro* and *in vivo* microenvironment has profound effects on the properties of BMSC, and survival and differentiation of the cells after for example transplantation is dependent on environmental cues rather than intrinsic properties of BMSC.[22] Chondrogenesis in vitro is induced by TGF-β1 (transforming growth factor β1), dexamethasone and ascorbic acid.[23] Ascorbic acid is an important cofactor required for the hydroxylation of collagen molecules and a basic medium commonly used to induce matrix organization in three dimensions while TGF-β1 and dexamethasone are strong chondrogenic factors.[17]

3.4 Fibrinogen and fibrin

Fibrinogen is a 340 kDa plasma protein, consisting of two identical molecular halves held together in a trinodular structure. Elaborate studies have been made considering its structure, function and interactions with other molecules. Fibrinogen and fibrin plays an important role in blood clotting, a phenomenon first described in the late 17th century as a process similar to freezing of water. In the middle of the 19th century it was suggested that there was a precursor of fibrin called fibrinogen that spontaneously formed the fibrous fibrin. But not until later it could be shown that fibrinogen did not form clots spontaneously, but at the addition of a "thrombic" enzyme.[24]

Fibrinogen is an inert molecule that circulates the body.[25] It is activated by the enzyme thrombin, which cleaves the molecule and hence exposes the polymerisation sites which results in start of fibrin assembly. The transition of fibrinogen into fibrin consists of three major steps:

(1) binding of thrombin and subsequent cleavage of fibrinogen into fibrin monomer,
(2) construction of half-molecule overlapping double-stranded protofibrils, and
(3) lateral association of protofibrils to form thick fibrin bundles and networks.[26]

Fibrin is an active molecule that is reactive toward several proteins and cell types. The fibrinogen-fibrin system is complex and many factors are involved in its activation. The

polymerisation is activated by thrombin, and the concentration of thrombin affects the polymerisation time and the architecture of the network.[27] Factor XIII is an enzyme mediating the cross linking of fibrinogen to fibrin, and the amount of present factor XIII affects the properties of the forming clot. Higher concentration of factor XIII increases the amount of covalent cross-linking in the fibrin network, resulting in a stronger gel.[28] Another protein often co-administrated with the fibrinogen component is the fibrinolysis inhibitor aprotinin.

The fibrin gel has a sticky character and is used mainly in sealants and adhesives. The main advantages of fibrin are that it is haemostatic, adheres to connective tissue, promotes wound healing, and that it is biodegradable with excellent tissue tolerance. [13]

The polymerisation time of fibrin is mainly dependent on the thrombin concentration. In an injectable gel it is favourable to have instantaneous or almost instantaneous gel formation upon mixing, so that the matrix solidifies in the shape of the defect or lesion keeping the cells in place and do not diffuse or get flushed away.

3.5 Hyaluronic Acid

Hyaluronic acid is a high molecular weight polysaccharide found in the connective tissue of all vertebrates.[29] It consists of repeating disaccharide units of D-glucoronic acid and N-acetyl-D-glucosamine linked together by β-1,4 and β-1,3-linkages shown in figure 2.[30]

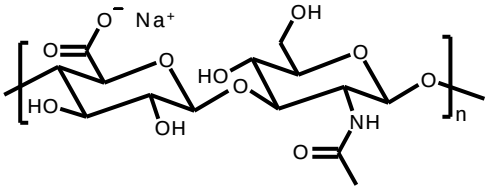


Fig 2. Repeating unit of hyaluronic acid

Hyaluronic acid plays an important role in cell motility and proliferation as well as in supporting and maintaining tissue or organ morphological features.[31] The ECM of cartilage contains mainly hyaluronic acid, and interaction between hyaluronic acid and the CD44 receptor in chondrocytes is important for the organization of the ECM during chondrogenesis.[32]

In solution hyaluronic acid adapts a random coil formation producing a network of entangled molecules with viscoelastic properties.[33] High molecular mass hyaluronic acid forms a tertiary structure when the polymer chains interact and form β-sheets based on 2-fold helices.[34] Due to a short turnover rate in the body hyaluronic acid needs to be chemically modified to obtain a stable material. In this work this is achieved by mixing it with another gel forming component.

3.6 Collagen

Collagens are a family of proteins that provides rigidity, elasticity and strength in multicellular organisms. It is the most abundant protein in the body and is present in skin, bone, cartilage, smooth muscle and basal lamina. There are at least twenty different types of collagens with different structures and functions, each being dominant in a specific tissue.[35]

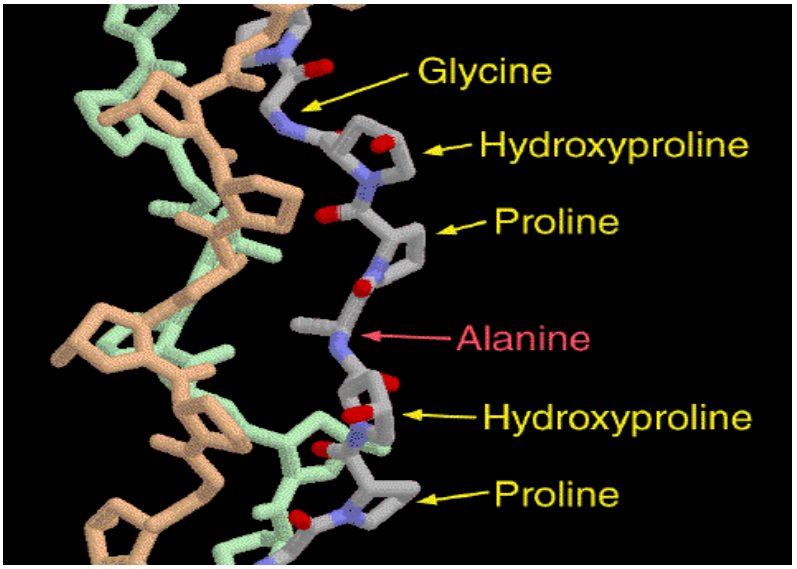


Figure 3. Collagen is composed of three chains, wound together in a tight triple helix. Every third amino acid of each chain is glycine, a small amino acid that fits perfectly inside the helix. The picture is used with permission from PDB data bank.[36]

The basic structure of all types of collagen is the characteristic triple helix shown in figure 3, and variations among them are restricted to the length of the nonhelical fraction, the length of the helix itself and the network forming properties.

Collagen type I is a reticular collagen, which forms a porous gel when it polymerises. It can be kept fluid at conditions such as low pH or low temperature and the polymerisation can be induced by increase in temperature or pH.[37] This makes it favourable for using in an injectable gel.

Collagen is one of the most frequently used natural polymers when fashioning biomaterials today. For example it is used for tissue augmentation, in wound healing or in implants.

4. Materials and Methods

The project was divided into two phases; one in which the matrix was investigated and characterized, and another where cell viability and differentiation in the matrix was investigated *in vitro*.

4.1 Materials

4.1.1 Matrix components

Fibrinogen solution of concentration 6.6 mg/ml or 66.3 mg/ml suspended in 1000 IE/ml aprotinin was provided by Baxter AG, Austria.

Hyaluronic acid derived from streptococci zooepidemicus with a molecular weight of 10^6 Da was used as received from Q-med, Uppsala, Sweden. Concentrations used were 7.5 mg/ml, 12 mg/ml or 20 mg/ml mixed with 83 U/ml thrombin.

Bovine collagen type I from Symantese, France was used in concentrations of 7.25 mg/ml or 12 mg/ml mixed with 83 U/ml thrombin. It was dialyzed in PBS (phosphate buffered saline, pH 7.2) overnight at 4°C in order to increase the pH and ionic strength without gel formation.

4.1.2 Cells

Human BMSC came from iliac crest marrow from healthy donors and human chondrocytes were from patients that had gone through total knee replacements.

4.1.3 Chemicals

Three different media were used: Coon's modified Ham's F12 medium, complete medium consisting of Coon's modified Ham's F12 medium supplemented with 10 % Fetal Calf Serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin and finally pellet medium composed of Coon's modified Ham's F12 supplemented with 5.35 mg/ml bovine serum albumin, 1 mM sodium puryvate, 1.25 µg/ml Linoleic acid, 6.25 µg/ml bovine insulin, 6.25 Hapo Transferrin.

Antibody anti-collagen II (CIICI) came from the Developmental Studies hybridoma Bank, University of Iowa, Iowa City, IA, USA. Chondrogenic factors used for induction were TGF-β1 from Pepro Tech RC Ltd, England, ascorbic acid from Sigma and dexamethasone also from Sigma. During expansion FGF-2, fibroblast growth factor-2, from Austral Biologicals, San Ramon, CA, USA was used.

Stain used for viability test was Tryphan blue from Sigma and for histology and immunohistochemistry Toluidin blue from Merck and Carbazol from Sigma were used.

Enzymes for digestion and detaching were hyaluronidase and 0.05% trypsin-0.01% EDTA from Sigma and Collagenase I and II provided by Worthington Chemical Corporation

4.2 Matrix characterisation

The polymerisation time of fibrinogen with respect to thrombin concentration was investigated by measuring the time it takes from mixing until the gel turns opaque and hardens, which it does upon polymerisation. This was also measured by means of rheology (Advanced Rheometer, TA Instruments, New Castle, USA), where modulus of viscosity (G'') and elasticity (G') is plotted as a function of time.

Gels were made containing fibrin and hyaluronic acid or fibrin and collagen type I, and their morphology and gel forming properties were studied by scanning electron microscopy (SEM) and rheology. Three different concentrations of fibrinogen were studied, 6.6 mg/ml, 26.5 mg/ml and 66.3 mg/ml, and each of these were combined with either collagen or hyaluronic acid mixed with thrombin in the concentrations shown in table 1.

4.2.1 Scanning electron microscopy

Gels of the different combinations described above were made by means of a two-component syringe with a mixing tip, provided by Baxter Biosciences, Germany, where fibrinogen was held in one compartment and collagen/thrombin or hyaluronic acid/thrombin in the other. The gel was formed upon mixing of the two components. The gels were fixated in a solution of 5 % glutaraldehyde for 1 hour before dehydrated in a series of ethanol solutions (50% for 10 min, 75% for 30 min, 90% for 1.5 hours, 2 x 100% for 1 hour) and dried supercritical. They were then sputtered with gold/palladium (90:10) before imaged.

Table 1. Concentrations of the components of the gel

Fibrinogen	6.6 mg/ml	26.5 mg/ml	66.3 mg/ml
Collagen type I	3.5 mg/ml 7.5 mg/ml	3.5 mg/ml 7.5 mg/ml	3.5 mg/ml 7.5 mg/ml
Hyaluronic Acid	7.5 mg/ml 12 mg/ml 20 mg/ml	7.5 mg/ml 12 mg/ml 20 mg/ml	7.5 mg/ml 12 mg/ml 20 mg/ml
Thrombin	25 U/ml 83 U/ml 125 U/ml	25 U/ml 83 U/ml 125 U/ml	25 U/ml 83 U/ml 125 U/ml

4.2.2 Rheology

The gel forming properties of fibrin were investigated with rheometry. Modulus of viscosity (G'') and elasticity (G') is plotted as a function of time. The polymerisation time of fibrin at different concentrations of added thrombin was studied by a time sweep at constant frequency. The polymerisation is seen when the viscosity contribution, G', increases in the plot. The thrombin concentrations investigated were 0.01 U/ml, 0.25 U/ml and 1 U/ml. The gel forming properties of collagen 3.5 mg/ml was investigated by a temperature sweep from 4°C to 50°C at constant frequency.

4.3 Cell Culture

4.3.1 Bone marrow stromal cells

Human bone marrow cells were obtained from iliac crest marrow aspirates from healthy donors and all procedures were approved by an institutional ethical committee. Bone marrow samples were washed twice with PBS and cells were counted through staining with 0.1 % Methyl Violet in 0.1 M citric acid. They were then plated in 100 mm dishes containing complete medium and 1 ng/ml human recombinant FGF-2 and incubated at 37°C in humidified atmosphere containing 5% CO_2. Medium was changed first after 4 days and then twice a week. When the cells reached confluence, they were detached with 0.05% trypsin-0.01% EDTA counted and replated in 100 mm dishes at 3 x 10^5 cells/plate or frozen for future use. At second passage they were detached by trypsin/EDTA, counted and split to get 800 000 cells/gel. They were then resuspended in fibrinogen solution.

4.3.2 Chondrocytes

Human chondrocytes were isolated from articular cartilage from knee joints of patients that had undergone total knee replacement. The samples were cleaned from adherent muscular, connective or subchondral bone tissues and diced into 1-3 mm pieces. Single chondrocytes were released by repeated 1 hour enzymatic digestions with 400 U/ml collagenase I, 1000 U/ml collagenase II, 1 mg/ml hyaluronidase and 0.25% trypsin. The cells were then plated in 6-well plates precoated with 10% Fetal calf serum. The following day, they were trypsinized, counted and replated at a density of 250 000 cells/well. They were then grown with medium changed twice a week until they reached confluence; thereafter they were trypsinized, counted and resuspended in fibrinogen solution.

Parallel experiments were made with chondrocytes and BMSC. Chondrocytes are easier to induce to chondrogenesis since they are programmed to become cartilage, but BMSC are an easy source of stem cells and also easier to handle during expansion.

4.3.3 Viability Test

The viability of the cells in the gel was investigated after three hours' or one day's culture. The matrix was digested with an enzyme mix of collagenase I and hyaluronidase before the cells were stained with the viability stain Tryphan blue and the living cells counted. After 3 days growth the culture was fixated in 4 % formalin and analysed histological to see the viability and the distribution throughout the matrix.

4.3.4 Gel formation and pellet culture (micro mass culture)

Gels incorporated with cells were formed by means of the two-component syringe. One syringe contained the cells suspended in fibrinogen and the other contained either collagen or hyaluronic acid mixed with thrombin. The concentration of the matrix components and the number of cells of each gel made can be seen in table 2. 100 µl of each component were mixed in the needle of the syringe when injected into a 96-well plate, and after polymerisation they were transferred into 15 ml falcon tubes containing 1 ml complete medium. To find the optimal number of cells for usage in culture, three different cell numbers were tested in the first batch.

Table 2: Table showing number of gels and concentrations of the different components. Each x corresponds to a gel of a specific composition that was tested for chondrogenesis.

BMSC	200 000 cells 2 weeks stimulation	400 000 cells 2 weeks stimulation	800 000 cells 2 weeks stimulation	800 000 cells 3 weeks stimulation
Fibrin 6.6 mg/ml				
Hya 20 mg/ml			x	x
Hya 12 mg/ml		xx	xxx	x
Hya 7.5 mg/ml		xxx	xxx	
Collagen 7.25 mg/ml		xx	xxx	x
Collagen 3.5 mg/ml		xx	xx	
Fibrin 26.5 mg/ml				
Hya 12 mg/ml	x			
Hya 7.5 mg/ml	x	x		
Collagen 7.25 mg/ml	x			
Collagen 3.5 mg/ml	x			
Fibrin 66.3 mg/ml				
Hya 12 mg/ml			x	
Chondrocytes				
Fibrin 6.6 mg/ml				
Hya 12 mg/ml			x	
Hya 7.5 mg/ml			x	

4.3.5 Chondrogenic Induction

The cells were stimulated in pellet medium with addition of the chonrdogenic factors ascorbic acid (500 µg/ml), Dexamethasone (10^{-7} M) and TGF-β1 (10 ng/ml) every other day. After two or three weeks, cells were fixed with 4% formaline in PBS for 20 minutes before being processed for histological analysis. As a control a pellet culture, 2.5 $x10^5$ cells centrifuged for 10 min (2000 rpm), treated in the same way was used.

4.3.6 Histology and immunohistochemistry

Samples were dehydrated, embedded in paraffin, sectioned (4 µm thickness) and stained with toluidine blue before analysed for cartilage formation and matrix deposition using a Zeiss Axiophot microscope (Oberkochen, Germany).

To determine whether the deposited matrix was collagen type II, a specific marker for cartilage tissue, peroxidase based immunohistochemistry with monoclonal antibody CIICI was performed. The sections were deparaffinized in xylene for 2 x 10 minutes, hydrated in a series of ethanol solutions (2 x 100% for 2 minutes, methanol and 30% H_2O_2 for 30 minutes, 2 x 95% for 2 minutes, 80% for 2 minutes, 70% for 2 minutes) before washed in PBS for 10 minutes. Antigenic sites were uncovered with enzyme digestion, followed by incubation with

hyaluronidase for 15 minutes at 37°C and were then incubated with serum to eliminate unspecific interactions. After that followed incubation with the first antibody for 1 hour in room temperature and, after washing with PBS, incubation with a labelled second antibody, specific to the first, for 30 minutes in room temperature. Thereafter they were incubated 30 minutes with peroxidase and streptavidine that recognizes the second antibody. Additional washing was followed by staining with carbazol, washing and finally the sections were mounted with cover glasses.

4.3.7 Scanning electron microscopy

After two weeks of stimulation the gels were fixated for 1 hour in a 5% glutaraldehyde-PBS solution. They were then dehydrated in a series of graded ethanol solutions as described earlier and dried supercritically before imaged.

5. Results

5.1 Matrix characterisation

5.1.1 Polymerisation time

The polymerisation time measurements of the fibrin gel showed that thrombin concentrations above 60 U/ml gave instantaneous gel formation, while lower concentrations made the polymerisation time slower, as can be seen in table 3. A thrombin concentration of 83 U/ml was chosen for future use.

Table 3. Polymerisation time of fibrin at different thrombin concentrations

[Fibrin]/ [Thrombin]	6.6 mg/ml	26.5 mg/ml	66.5 mg/ml
500 U/ml	Instant	Instant	Instant
250 U/ml	Instant	Instant	Instant
125 U/ml	Instant	Instant	Instant
83 U/ml	Instant	Instant	Instant
50 U/ml	10 s	10 s	10 s
25 U/ml	19 s	-	-
5 U/ml	47 s	-	-
1 U/ml	75 s	-	-
0.25 U/ml	10 min	-	-
0.01 U/ml	14 min	-	-

5.1.2 Rheology

The gel forming time of fibrin and collagen was also investigated by means of rheology. Fibrinogen with a concentration of 6.6 mg/ml and a thrombin concentration of 0.01 U/ml, 0.25 U/ml and 1 U/ml was used to investigate the gel formation time of fibrin. Fibrin concentrations higher than 50 U/ml gave instantaneous gel formation, and the gel forming procedure could therefore not be followed accurately with the rheometer. As shown in figure 5 a – c, the gel point was reached after 14 min at a thrombin concentration of 0.01 U/ml, after 10 min for 0.25 U/ml and after 1.5 min for 1 U/ml. The modulus is increasing with decreasing thrombin concentration.

Also collagen was examined with regard to its gel forming properties. A collagen solution of 3.5 mg/ml at pH 7 was studied by means of a temperature sweep from 4°C to 70°C. The

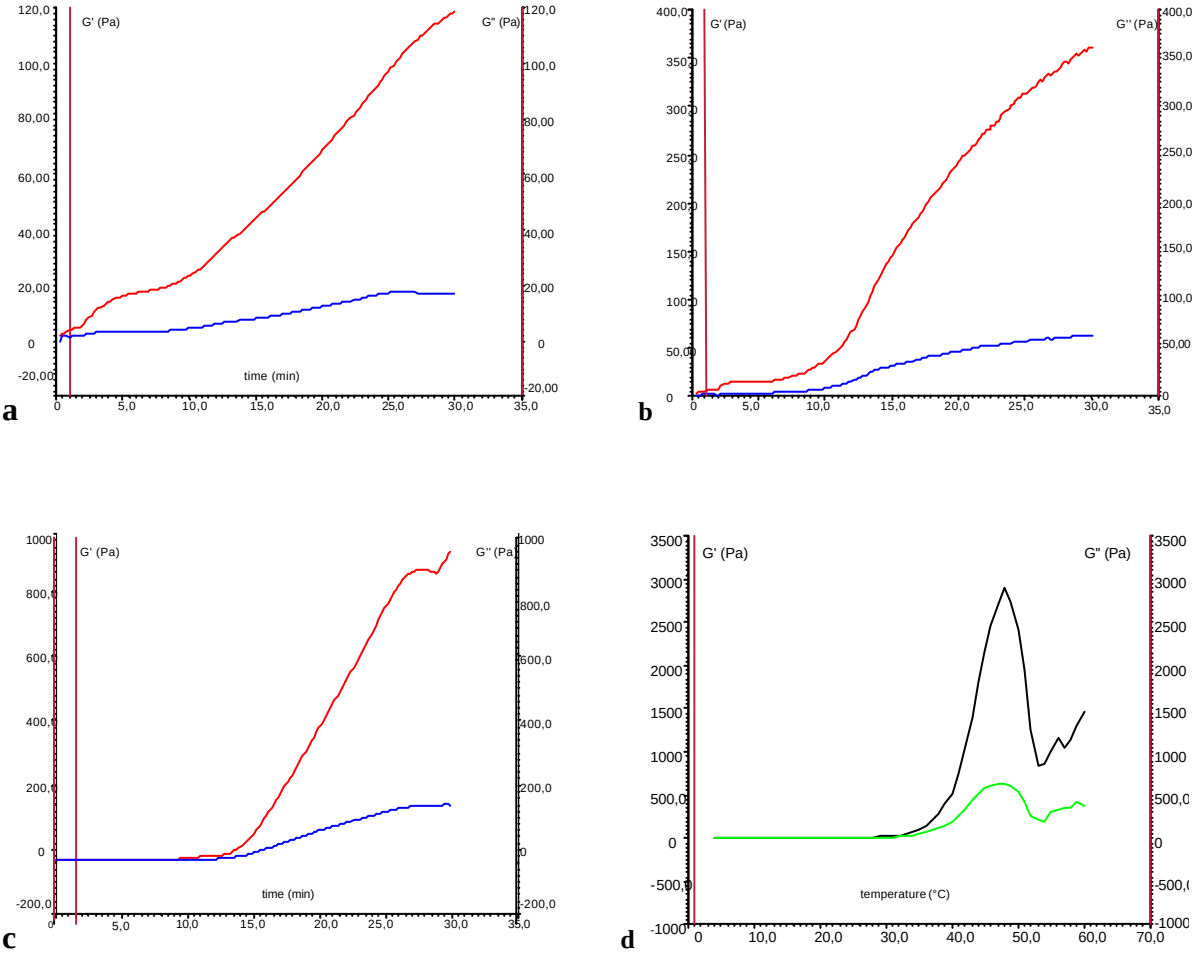


Figure 5. Diagram a-c show time sweep of fibrinogen 6.6 mg/ml, with thrombin concentration a) 1 U/ml b) 0.25 U/ml and c) 0.01 U/ml. Red curve corresponds to viscosity modulus, G' and blue line to elasticity modulus (G''). The polymerisation time of fibrin increases with increasing thrombin concentration, and the modulus increases with decreasing thrombin concentration. The d figure shows a temperature sweep of collagen 3.5

mg/ml pH 7.4. Collagen polymerise at a temperature of 35°C, and further raising of temperature results in denaturation of the protein and destruction of the gel, as can be seen as a drop in G'.

diagram shows the modulus as a function of time. Collagen starts to polymerise at around 35°C, which can be seen as a sharp increase in storage modulus (G'). As seen in figure 5 the viscosity drops at around 47°C, which could be due to denaturation of the proteins and the breaking of bonds holding the gel together.

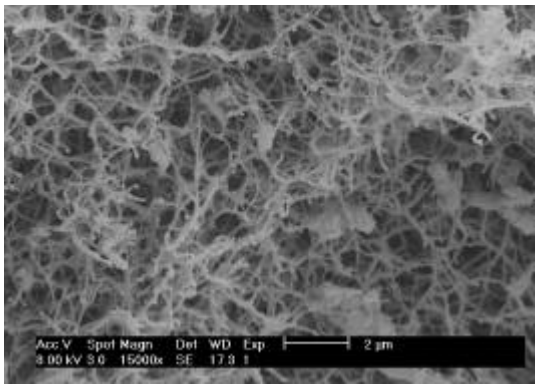
5.1.3 Scanning electron microscopy

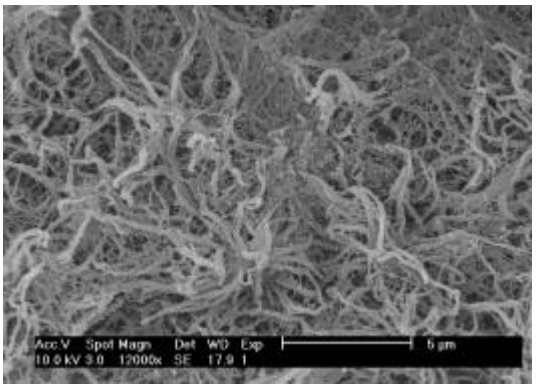
The morphology of the gels was analysed with respect to pore size and network structure. The gel compositions of fibrin, collagen and hyaluronic acid investigated can be seen in table 4.

Table 4. Fibrinogen of three different concentrations, 6.6, 26.5 and 66.3 mg/ml, was combined with each of the concentrations of hyaluronic acid-thrombin or collagen-thrombin shown below. Totally 18 compositions were analysed with respect to pore size and network structure.

Fibrinogen (mg/ml)	Hyaluronic acid (mg/ml)	Collagen (mg/ml)
6.6	7.5	3.5
	12	7.25
	20	12
26.5	7.5	3.5
	12	7.25
	20	12
66.3	7.5	3.5
	12	7.25
	20	12

Gels with a fibrin concentration of 6.6 mg/ml, showed a network with pore sizes in the range of 2-5 µm as can be seen in figure 6. Fibrin concentration of 66.3 mg/ml showed a more compact gel. When comparing fibrin gels, collagen-fibrin gels and hyaluronic acid-fibrin gels, they showed relatively similar morphology according to the SEM micrographs. They had comparable structures probably strongly influenced of the fibrin network structure.





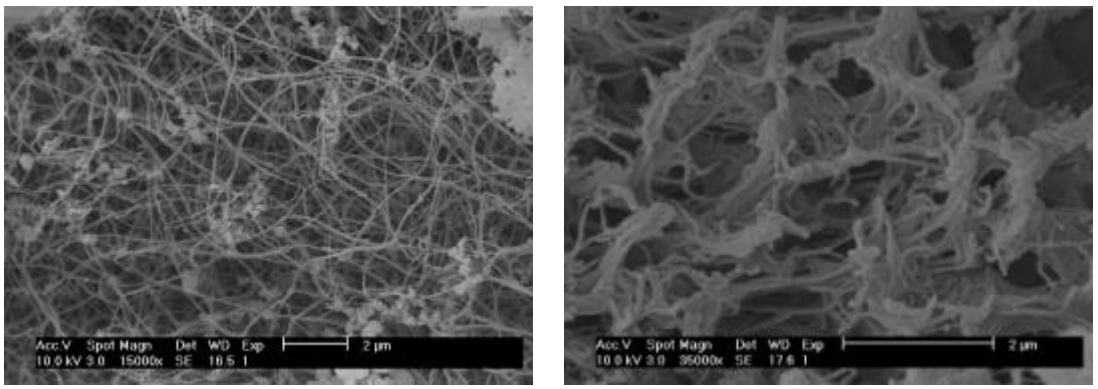


Fig 6. SEM pictures showing the network structure of the gel. Top left is fibrin 6.6 mg/ml and top right 66.3 mg/ml. Both have a thrombin concentration of 250 mg/ml. The lower pictures are showing 6.6 mg/ml (left) and 66.3 mg/ml (right). These have a thrombin concentration of 25 U/ml.

The hypothesis is that larger pores favours cell growth because the easier diffusion of nutrients and factors, while higher concentrations, and hence more compact gels with smaller pores are better for cells to attach and also decreases the degradation rate. Based on these results, gels with thrombin concentration of 83 U/ml were chosen to be used for the cell culture.

5.2 Cell culture

5.2.1 Viability test

The viability of cells in the matrix was tested by staining with Tryphan Blue after growth for three hours or one day. There was no sign of dead cells, neither for BMSC or chondrocytes; hence the cells survived the environment in the fibrinogen and the transfer through the syringe. A histological analysis made of the gel after 3 days, showed that the cells were still alive and evenly distributed in the matrix.

5.2.2 Histology and immunohistochemistry

After 8 or 14 days induction, the gels were fixated and processed for histological analysis. The samples were embedded in paraffin and stained with toluidin blue. After 8 days, the formation of lacunae with cells within could be seen, but no significant signs of deposited matrix was observed neither in the control nor any of the gels (data not shown).

Due to problems with contamination during expansion, lack of cells made further testing with chondrocytes impossible.

After induction for 14 days, cells had organized in lacunae and a cartilage-like ECM that showed a deep purple metachromatic staining could be seen. The colour of the matrix was clearly distinguishable from the original fibrin matrix, which stained more blue as seen in figure 7.

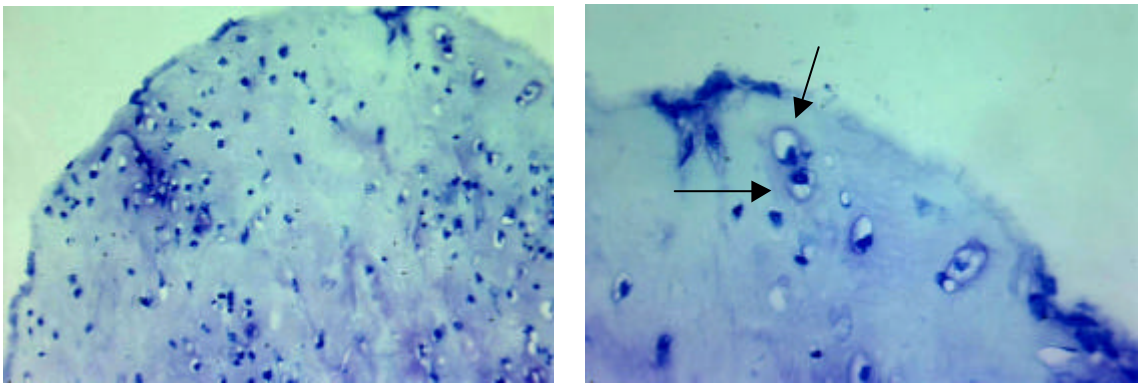


Figure 7. Right: Matrix deposited from matured cells stain more purple than the original matrix containing fibrin and collagen. Left: Chondrocytes are located in matrix cavities called lacunae and when differentiating they start to produce their own matrix, which can be seen as the purple border of the cavity.

The gels had shrunk to approximately 20% of their original size, and were significantly more compact. The shrinkage of the samples was not correlated with the fibrin concentration used, since the gels of 6.6 mg/ml shrunk as much as the ones consisting of 26.5 mg/ml and 66.3 mg/ml, but the cells seemed to grow better in the gels with fibrin concentration of 6.6 mg/ml.

The matrices containing hyaluronic acid, showed a more intense deposition of matrix, compared to the samples with only fibrin or fibrin and collagen. Higher concentration of hyaluronic acid seemed to give more matrix deposition as can be seen in figure 8 (a – f).

a **g**

Gel composed of fibrin 6,6 mg/ml and collagen 3,5 mg/ml.

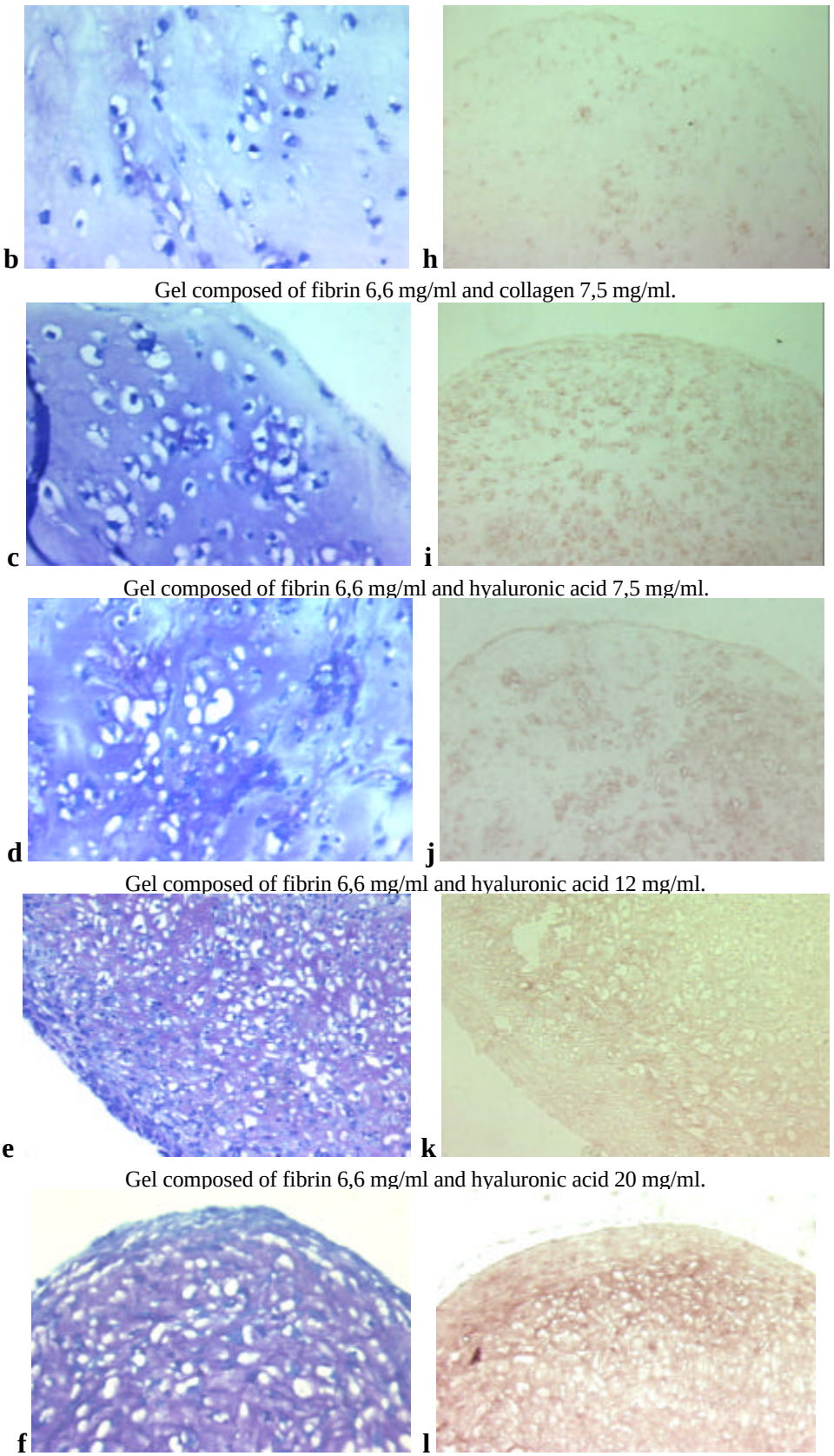


Figure 8. Histology showing cell organization and matrix deposition in gels of different compositions. All gels were seeded with 800 000 BMSC and induced *in vitro* for 14 days. The positive control was treated in the same way as the other samples. Gels containing hyaluronic acid (**c – e)** show more metachromatic staining than gels containing collagen (**a – b**). Higher concentration of hyaluronic acid in the matrix seems to increase the deposition of matrix from the BMSC. The right column (**g-l**) shows immunohistochemistry on sections from the

same samples stained with antibody specific towards collagen type II. It can be seen that all gel compositions are stained positive for collagen type II, meaning they are mature chondrocytes.

In order to confirm that the deposited matrix was collagen type II, immunohistochemistry with specific antibody towards collagen type II was performed. The red staining from the peroxidase seen in figure 8 (g – l) verifies that the cells were differentiated.

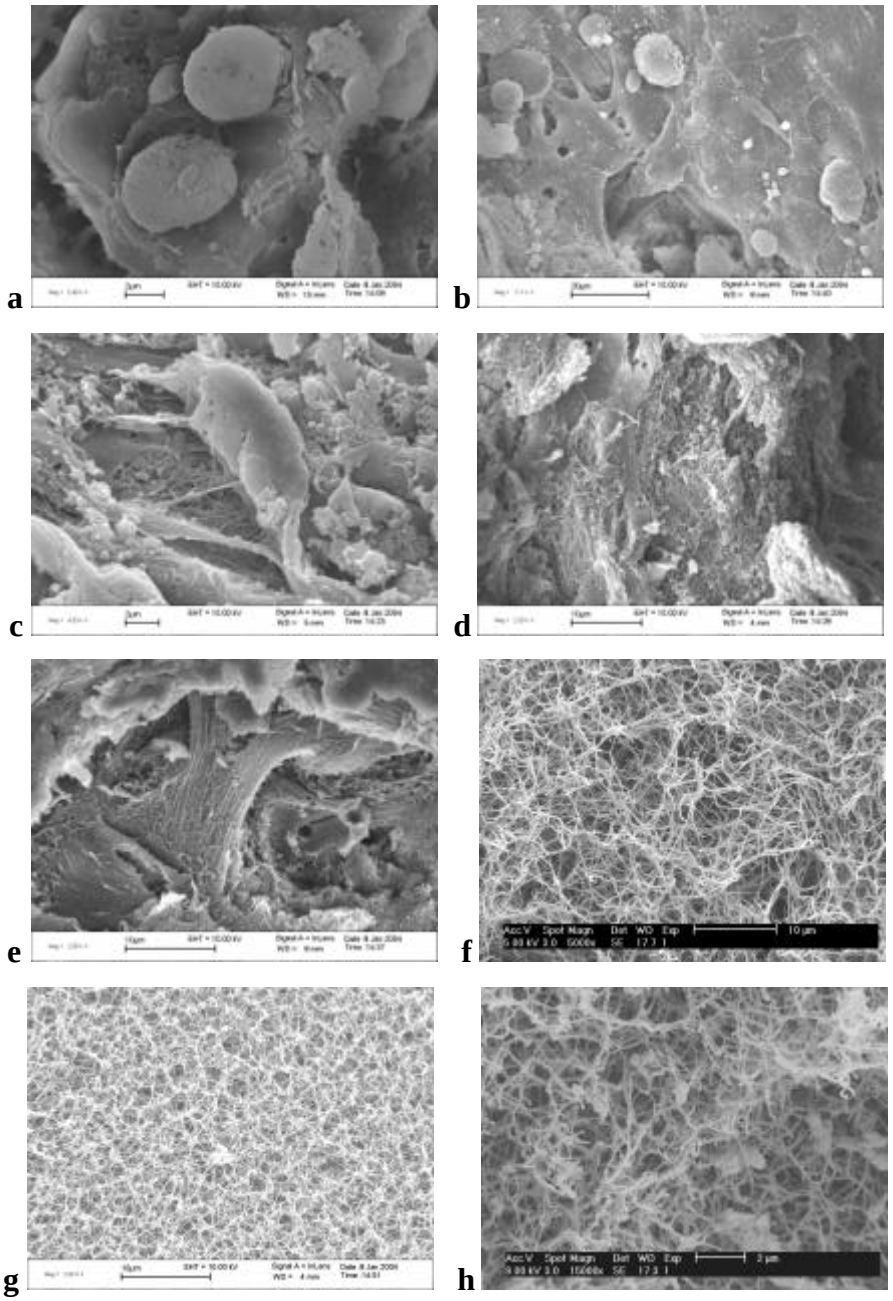


Figure 9. SEM pictures showing differentiated cells in different matrix compositions. The hyaluronic acid gels showed more deposited matrix than fibrin and collagen-fibrin gels. **a)** shows cells of spherical shape in a fibrin 6.6 mg/ml matrix, **b)** shows two cells spanning over a gap in a gel of fibrin 66.3 mg/ml, **c)** is a cell in a matrix of fibrin 6.6 mg/ml and collagen 7.25 mg/ml, **d)** and **e)** is matrices of hyaluronic acid 12 and 20 mg/ml respectively, showing deposited matrix around the cells. Pictures **f) – h)** show hyaluronic acid based matrices not containing any cells. The structure is completely altered when cells are present.

5.3.3 Scanning electron microscopy

SEM of gels induced for two weeks showed that the gels had turned very compact and the matrix was contracted to a pore size of around 0.1 µm after two weeks of stimulation. Compared to gels without cells present, the network structure of the gel had altered completely as seen in figure 9.

6. Discussion

Cell-based regeneration of cartilage requires an initial *in vitro* expansion in order to get a sufficient number of cells to reimplant in the lesion. The use of chondrocytes has shown a functional and efficient way of recreating this type of tissue. So far, some thousands of patients with cartilage lesions have been treated with cultured autologous chondrocytes.[18] In this study the stem cells of the bone marrow compartment, which can differentiate towards chondrocytes, have been investigated on their potential for chondrogenesis in a three dimensional matrix. The cells are easily accessible and expanded *in vitro*, and the use of an injectable gel-forming matrix for placement presents a competitive method for tissue regeneration.

The cell concentrations used were high, 800 000 cells per 200 µl gel, so the ease with which BMSC are expanded is a great advantage compared to chondrocytes, which have a lower metabolic rate and hence are growing slower. BMSC degrade and contract the matrix more than the chondrocytes, which requires a gel with lower degradation rate. The gels shrunk to less than 20 % of their original size in two weeks. In order to circumvent this problems there are some possible measures. To create a harder gel that resist degradation better some modifications of the fibrin component can be made. Factor XIII is a protein that augments the covalent cross linking in the fibrin network and creates a harder gel. This is one possible way to decrease the degradation rate of the gel. Another way could be to increase the amount of aprotinin in the fibrinogen component or to add other protease inhibitors to the gel.

Chondrocytes are less metabolically active than BMSC. The gels containing chondrocytes showed no signs of differentiation after two weeks induction. This could be due to either a too short time in the matrix or that they have to be closer to each other in order to have the proper

cell-cell and cell-matrix contacts needed for differentiation. Another observation was that chondrocytes did not degrade the matrix at all in two weeks. This is probably because of their low metabolic activity. Either the BMSC proliferated more than the chondrocytes did, or they degraded the matrix more.

The literature states that it is the microenvironment that decides the fate of BMSC, and not intrinsic properties of the cell itself.[1] This hypothesis seems to be in line with the results given here, since the gels consisting of hyaluronic acid appear to further encourage the cells to differentiate, compared to the collagen-containing ones. However the cells differentiate also in the collagen gel, but not at the same speed and with the same amount of deposited matrix as in the hyaluronic acid based one.

It would be interesting to grow the cells in a gel with higher concentration of hyaluronic acid than 20 mg/ml, to see if they produce even more matrix, but one has to consider the handling of the gel. From a clinical point of view, the gel must be easy to handle in a surgical situation, and hyaluronic acid of higher concentrations is very viscous and it would be practically impossible to inject the fluid without damaging the cells.

Next step in this area is to make comparative studies with chondrocytes. They are easier to work with in regard to chondrogenesis, since they are "programmed" to become cartilage, while BMSC can become many types of cell lines. Also a growth curve of the cells, both BMSC and chondrocytes, in the gel has to be established to see their proliferation rate.

Future undertakings could be an *"in vivo experiment in vitro"*, meaning that the gel can be tested to be placed in a disc of cartilage in which a lesion is created. The disc will be kept in medium to keep the cartilage alive, thus mimicking an *in vivo* environment. This approach is advantageous since it does not induce an immunologic response and the entire course of events can be studied. This is an intermediate step before animal testing and later on, hopefully, clinical trials.

Regarding the matrix, a closer examination should be made, in order to match the modulus of the gel with that of the tissue. Also the degradation rate must be lowered, since the gel must fill the lesion until the cells are differentiated. This proceeding takes approximately 8 weeks in the body, and the gel must keep its size throughout this time.[38]

The hyaluronic acid showed preferable characteristics for chondrogenesis, and this implies that a gel consisting of only hyaluronic acid would be advantageous. However, hyaluronic acid in its natural form is water-soluble and needs to be chemically modified in order to form a gel with the right mechanical properties for an implant.

7. Conclusions

BMSC and chondrocytes need a three dimensional matrix in order to differentiate into cartilage. This work presents an investigation of injectable gels consisting of hyaluronic acid, fibrin or collagen for cartilage formation. The number of cells needed as well as preferable matrix composition was investigated. The histology results showed that the cells differentiated in all the gels tested, but with different amount of deposited matrix. 800 000 cells per 200 µl matrix showed more tissue-like appearance than lower cell concentrations, according to the histology. Gels based on hyaluronic acid were inducing chondrogenesis more efficiently compared to gels based on fibrin or collagen and the higher concentration of hyaluronic acid, the more deposited matrix could be seen. However, the gels were degraded very fast, and future work must be directed towards creating a gel with appropriate degradation rate as well as a matching of the modulus to that of the tissue.

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