Neogenesis of Hyaline Cartilage by Stimulation of Mesenchymal Stem Cells in TGF-β1-Coated 3-D Scaffolds

A Research Year Thesis

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Preface and acknowledgements

The aim of this research year project has been the establishment of a method suited for tissue engineering and the production of a scientific paper qualified for publication in an international journal.
Since the results from this project have not all been analyzed this edition of my paper seems to take more the characteristics of a report. I need to evaluate several results and carry out further studies before the desired content for my paper are ready.
This report is built on the shape of a standard scientific paper but several places it lacks the quality required for a publication. Areas that need to be revised are the missing results such as RT-PCR and an in vivo study.

The studies were carried out at the Orthopaedic Research Laboratory at Aarhus University Hospital in Århus.
It has been a great experience working in the excellent facilities and the extraordinary skilled technicians have been a great inspiration.
I would like to thank everybody at the lab. especially the technicians: Anette Baatrup, Anna Bay Nielsen, Jane Pauli and Anette Milton. Thank you for sharing your experience and also for being willing to help with any thing any time.
I would also like to thank the other research year students, phD students, post doctors and everybody else involved in this unique research group. It is a very motivating and inspiring environment.

I was lucky to have the opportunity to work with human primary cells provided by the Stem Cell Lab. at Aalborg University. I would like to thank Linda, Pia and Helle in Aalborg tfor making my study possible.

Furthermore my acknowledgements go to Carsten Stengaard who helped me getting the project started and for always providing useable guidelines when needed.

And to Michael Ulrich-Winther: A big thank for sharing experience and knowledge, and for the inspiration along with all the help provided during the project.
Finally I would like to thank Kjeld Søballe who was my initial contact to the project.

During my research year I have attended and completed the following Ph. D. courses:

- ”Introduction to Litterature Searching”
- ”Introduction to Medical Scientific Research”
- ”Introduction to Litterature Searching and the use of Reference Manager”
- “Nanobiocompstibility”
- “Introduction to Animal Testing”

These courses equal 7 ECTS-points in total.

The project was funded by FSS (Forskningsfonden for Sundhed og Sygdom), Gigtforeningen, Korning Fonden and Dansk Ortopædisk Selskab. Thank you for the support. Without it, this work would not be possible.

Abstract

INTRODUCTION: Articular cartilage injury remains a serious clinical problem. The human body has limited ability to respond to the damage, resulting in pain and reduced mobility of the patient. Therapeutic options of reconstructive surgery and articular chondrocyte implantation have not shown satisfying results, for which reason the artificial development of articular cartilage with satisfying properties remains.
Tissue engineering offers good potential in this matter. Combining a matrix with cells and growth factors could improve the care of patients.
We suggest stimulating mesenchymal stem cells(MSCs) differentiation into chondrocytes, seeded in tissue engineered constructs suited for implantation into cartilage defects.

MATERIALS AND METHODS: Influence of specific growth factors is essential to the differentiation of MSCs and furthermore the cellular synthesis of mature hyaline cartilage depends on culturing in a 3-D environment.
We suggest stimulating the MSCs with transforming growth factor-β1(TGF-β1), an essential inducer of chondrogenic differentiation.
The MSCs are cultured in a 3-D scaffold, made from biodegradable polymeric chitosan, which is coated with TGF-β1 to secure a local delivery and effect of the TGF-β1. Once coated and seeded the differentiated MSCs will synthesize the mature hyaline cartilage matrix.

RESULTS: The TGF-β1 were released from the scaffold in a bifasic way with an initial boost followed by a smaller but continued release.
The MSCs stimulated with TGF-β1 proliferated and differentiated in the 3-D environment of the scaffold producing GAGs and significantly more Collagen-II than the MSCs not stimulated by the growth factor.

CONCLUSION: 3D porous chitosan scaffolds for tissue-engineering can be coated with TGF-β1.
TGF-β1 is released in a bi-phasic release-profile.
TGF-β1 coating of 3D scaffolds carrying hAT-MSC results in:

1) Increased proliferation and differentiation of hAT-MSC into chondrocyte lineage.
2) Increased cellular synthesis of Collagen-II.

Introduction

Background

Articular cartilage injury remains a serious clinical problem and is among the most prevalent diseases that affect humans. Once the cartilage is disrupted the body has only limited ability to respond to the damage[1]. Currently there is no treatment, which can restore the genuine hyaline articular cartilage, and eliminate the pain and reduced mobility of the patients.

Most hope has been applied to an advanced option to treating cartilage injury by autologous implantation of chondrocytes (ACI) [2]. Despite initial optimism, the method has been proven to be laborious, expensive and not able to regenerate hyaline articular cartilage [3]. The method is based on retrieving of healthy articular chondrocytes from a non-weight-bearing articular surface in the patient. After weeks of culturing, these cells are injected under a collagen flap covering the traumatic cartilage defect. Numerous problems are attached to this approach: Importantly, the drawback of inflicting a new cartilage injury limits the amount of obtainable chondrocytes. In order to expand the number of cells, researchers have tried culturing the chondrocytes in vitro before reimplantation. Unfortunately, chondrocytes are not well suited for this. After a few divisions they dedifferentiate and turn into fibroblastlike cells with poor chondrogenic potential[1]. Furthermore, application of a low viscous suspension of cells under a fibrin glued collagen flap is not ideal.

We suggest recruitment of new chondrocytes from large source of autologous potent cells; the mesenchymal stem cells (MSCs). It has been proven that MSCs have the ability to differentiate into chondrocytes and combined with their replicative potential of more than 20 population doublings this makes large scale production of chondrocytes possible[4]. The human MSCs (hMSCs) can easily be isolated and characterized from the patient's bone marrow. Our research group has laboratory facilities and know-how to do this.

We also suggest production of 3-dimensional tissue ready for implantation as an alternative way of treating cartilage injuries. It is well known that cellular synthesis of mature hyaline cartilage matrix (Type II collagen and aggrecan) is dependent on culturing chondrocytes in 3 dimensional scaffolds [5-7]. Furthermore, various growth factors play an essential role[8]. Among these Transforming Growth Factor-β1 (TGF-β1) seems to be a key factor [8]. We have succeeded in coating 3 dimensional polymers with growth factors for enhancing formation of cartilage matrix [9].

In summary, we suggest stimulating MSC differentiation into chondrocytes seeded in tissue engineered constructs suited for implantation into cartilage defects. In this study mesenchymal stem cell constructs will be created using 3 dimensional polymers coated with TGF-β1. Cell viability, differentiation, recovery, synthesis of extra-cellular matrix and immunohistology will be determined in vitro.

Previous experience:

This projekt is an extension of 5 years of ongoing research in field of molecular orthopaedics in The Orthopaedic Research Group at Aarhus University Hospital.

The research concerning regeneration of cartilage using tissue engineering is performed in an ongoing cooperation with the Regenerative Medicine Institute (REMEDI), National University of Ireland, Galway.

Articular cartilage

Cartilage belongs to a small group of avascular tissue. Instead of being fed with oxygen through red blood cells in the vessels cartilage is supplied with oxygen via diffusion through the surrounding tissue or synovium. As a consequence the oxygen tension is considerably lower than elsewhere in the body. The low need of oxygen corresponds to the low cell density in the tissue with chondrocytes occupying less than 10%[10].

Instead of the main substance being cells, extracellular matrix and water takes up 75-80% of the space. The extracellular matrix is made solid by collagen II that make up a half to two thirds of the extracellular matrix. Anotherhighly represented constituent is proteoglycan.

Articular cartilage is found on the joining parts of the bones. The composition of extracellular matrix is essential to provide low friction movements. As well as the ability to deform when exposed to direct loads during motion especially in the weight bearing joint in the lower limbs.

Collagen
The tensile strength of articular cartilage is especially supplied by collagen II (coll-II) which is also one of the matrix proteins that help shape this specialized tissue[11]. Common to all types of collagen is their helical structure formed by 3 α-chains. In a physiologic environment these trimers will spontaneously arrange themselves as fibrils and further combinations form the microfibril. Different types of microfibrils can aggregate and form collagen fibrils but only type I, II and III will form fibres that are microscopically visual[12]. Other types of collagen in articular cartilage are IX, X and XI these are all present in considerably lower amounts, but the combination of the different types is essential to the structure and strength.
Thin fibers of collagen type II form the basic mesh and to this the other types form cross bonds thus stabilizing the structure and leaving space for the interposed proteoglycans.

Proteoglycans
In articular cartilage the proteoglycans are predominantly organized as aggrecan[10]. This is characterized by a core protein to which glycosaminoglycans (GAGs) are attached. The core protein can carry up to 300 GAGs and these all contain a large amount of negative charges in one end.
These opposed negative poles attract water and cat ions and because of that keep the proteoglycans distended. The swelling is limited by the interactions of the surrounding collagen fibres.
Because of an osmotic pressure created by the large amounts of water the collagen is distended and held tight in its mesh structure thus limiting the space and water uptake of the proteoglycans. This organization explains the viscoelastic properties of articular cartilage[12].

Tissue Engineering
Tissue engineering is the field where engineering and life sciences are joined in order to develop a biological substitute that can restore, maintain or improve tissue function[13]. In other words the aim of tissue engineering is to create a tissue that can re-establish the natural structure and function of lost tissue.

In order to achieve this three basic building blocks are essential:

A framework on which to grow the cells
A solid framework will provide the 3-D environment needed for growth and differentiation. Using a scaffold as framework you can control the attachment of cells and and their migration at a specific site.
Furthermore a scaffold can function as a local drug delivery system by applying a polymeric coat containing biochemical signals e.g. growth factors.

Cells to produce tissue components
In order to create a new tissue with structure and function similar to a natural tissue the cells chosen must have the potential for certain cellular functions.
In the case of cartilage the cells must take over the role of extra cellular matrix production and remodelling along with integration with the surrounding tissue[14].

Biochemical signals to guide the cells
In order to stimulate and direct the cellular respond to the desired proliferation and differentiation. Such signal molecules can be cytokines with anabolic effect on cells like growth factors, e.g. TGF-β1.

Furthermore cartilage is an ideal choice for tissue engineering because of the absence of vascularisation in this hypoxic tissue[15].

The aim is to create tissue for implantation in patients so there is a risk of immunological rejection. Using biocompatible and perhaps biodegradable materials along with autologous cells can minimize this risk.

Scaffolds

Chitosan
Chitosan is a polysaccharide made from chitin derived from crap shells.

It can be made into a porous 3-Dimensional structure. Different types of chitosan are available with following different pore sizes. The chosen chitosan for this study has a pore size of 100-200 μm which is ideal for chondrogenic differentiation of MSCs.

The polysaccharide is biocompatible and biodegradable by hydrolytic cleavage. It does not trig an immunological or inflammatory response.

The degradation time in the body depends of the mass of material used. The scaffolds in this project will be degraded within months leaving behind only the new tissue with cells and extra cellular matrix.

Coating
The desired coat should be a biodegradable polymer containing growth factor molecules.

The polymer of choice was Poly D-L-Lactic Acid (PDLLA) a widely used polymer used in drug delivery systems. PDLLA is degraded in the body by hydrolytic cleavage into lactic acid which enters the citric acid cycle ending up with H_2O and CO_2.

The solvent used to dissolve the polymer was ethyl acetate, also a widely used solvent in different industries. Ethyl acetate was chosen because of its rapid evaporation to enhance the distribution of the coat.

The growth factor was dissolved into the ethyl acetate-PDLLA solution making it ready to apply to the lyophilized scaffolds.

The evaporation of solvent leaves the polymer on the surface of the scaffold containing growth factor molecules homogeneously distributed in the coat.

Once incubated in a media or implanted into an animal or human the PDLLA will degrade because of hydrolysis releasing the growth factor molecules to the surface of the scaffold delivering the growth factor locally and directly to the cells seeded on the surface of the scaffold.

Growth factors in Chondrogenesis

The TGF-β superfamily is a group of polypeptides of which many are essential to tissue growth and differentiation. Among several other proteins in this superfamily are TGF-β1-5.

TGF-β1 has a number of functions in normal cells including effects on proliferation, cellular differentiation or apoptosis[16].

The differentiation of MSCs into chondrocyte lineage is a complex interaction between the extra cellular matrix and locally produced proteins. Among these TGF-β1 is an essential and necessary growth factor in chondrogenic differentiation. TGF-β1 is found in normal articular cartilage as a homeostatic factor making it an obvious choice for this study.

Recent studies suggest an additive effect of insulin-like growth factor (IGF) when used together with TGF-β1[17]. However we only embedded TGF-β1 in the polymeric coating in order to simplify the study.

Mesenchymal Stem Cells

As mentioned above ACI has been target for research for some years without achieving satisfying results. The largest problem seems to be recruiting a sufficient amount of mature chondrocytes for an implant. The mature cells lose their differentiated status and turn into fibroblast like cells[1].

On the contrary adult mesenchymal stem cells have the potential of more than 20 population doublings and still maintain their potential for chondrogenic differentiation. This way you are able to proliferate MSCs to the amount needed and then stimulate them to chondrogenic differentiation.

Adult mesenchymal stem cells are found in the connective tissues of the body. Mostly known is the bone marrow but also muscle and adipose tissue contains MSCs.

Thanks to a great cooperation with the stem cell lab. at Aalborg University we had the opportunity to use human adipose tissue-derived adult mesenchymal stem cells(hAT-MSCs). It has been shown that hAT-MSCs possess the same potential of chondrogenic differentiation as bone marrow-MSCs.

An advantage using hAT-MSCs is the easy accessible reservoir by liposuction which makes autologous implantation possible.
And the invasive procedures of ACI are reduced to a single operation in the knee, lowering risks of complications.

Aim of the study

The overall aim of the study was to investigate the possibility of using a TGF-β1-coated chitosan scaffold for tissue engineering hyaline cartilage by, investigating the release properties of the PDLLA-TGF-β1-coat on chitosan and potential chondrogenic differentiation of hAT-MSCs in the TGF-β1 coated 3-D scaffold model.

Hypothesis

1 TGF-β1 is slowly released from TGF-β1-coated polymers.

2 MSCs can be uniformly distributed and grown in 3-Dimensional polymer scaffold.

3 Addition of TGF-β1 to a 3-Dimensional polymer scaffold will enhance the differentiation and chondrogenic potential of MSCs.

Materials and Methods

Release Kinetics of TGF-β1-coated chitosan scaffolds:

Production of chitosan scaffolds
The recipe used was inspired by Nettles et al:

1. Chitosan powder (DCMF) was dissolved in 0,2M Acetic acid in a glass beaker at a concentration of 20mg/mL (2% w/v chitosan/acid solution).
2. The solution was mixed for 48 h to ensure complete dissolution, using a magnetic stirrer and non-stick stir bar. After 48 h pH was measured to 4,4.
3. The solution was then poured into 5mL non-stick eppendorf tubes.
4. The tubes were transfered to a styrofoam container and placed inside a -20˚C freezer for at least 24 h.
5. The styrofoam container was then transferred to a -80˚C freezer for at least 24 h.
6. The tubes were then lyophylized at -40˚C for at least 24 h or till completely dry.
7. The lyophylized samples could now be placed in a dessicator until rehydration could be carried out.
8. Before rehydration the chitosan samples were cut in slices of 1 mm using a costum made scaffold-microtom and microtom knife.
9. The chitosan slices were then cut using a 6mm derma biopsy punch to cut out the scaffold from the slice. Leaving a scaffold of the desired 1mm x 6 mm diameter.
10. Next step was neutralizing the acetic acid on the scaffolds by rehydration. This was carried out by putting the scaffolds into a graded series of ethanol. Starting with 99,9% ethanol and proceeding to 96%, 80% and 70%. Each solution was equilibrated twice for at least 30 min. (e.g. 2 x 30 min).
11. After incubating the scaffolds for a least 2 x 30 min. in 70% ethanol the scaffolds were washed with PBS and stored in PBS overnight.
12. Before Re-dehydrating the scaffolds they were washed with fresh PBS and then incubated in the inversed graded ethanol series starting with 70% ethanol and proceeding to 80%, 96% and 99,9%. Each solution was equilibrated for at least 30 min.
13. Then the scaffolds were air dried for an hour before re-lyophylization for at least 24 h.
14. Scaffolds were now ready for coating.

Groups
The study consisted of 6 groups and a positive control:

Group	PDLLA (%-(W/v))	TGF-β1 (ng/scaffold	N
I	3%	500	10
II	3%	50	10
III	6%	500	10
IV	6%	50	10
V	18%	500	10
VI	18%	50	10

The control group consisted of 10 wells with 25 µL of EA containing 100 ng TGF-β1 incubated in 500 µL Isotonic saline, 0,9% NaCl.

Coating solution
The coating solution was made from PDLLA and human recombinant TGF-β1, carrier free, dissolved in ethyl acetate.

-Two different concentrations of TGF-β1 was used: 50 ng/scaffold and 500 ng/scaffold

-3 different concentrations of PDLLA was used: 3%, 6% and 18% (w/v).

The coating solutions were as follows:
TGF-β1 was dissolved in ethyl acetate in a vial and 3% (w/v) PDLLA was added resulting in a solution (A) of 20 ng TGF-β1/mL with 3%(w/v) PDLLA.
25 µL of A were then applied to each of the ten scaffolds in group I.
Then 25 µL of A were transferred to another vial and ethyl acetate was added resulting in the next solution of 2 ng TGF-β1/mL with 3%(w/v) PDLLA and group II was coated with 25 µL each.

More PDLLA was now added to A resulting in the solution (B) with 20 ng TGF-β1/mL with 6%(w/v) PDLLA and group III was coated.
25 µL of B were transferred to another vial and ethyl acetate was added resulting in the next solution of 2 ng TGF-β1/mL with 6%(w/v) PDLLA and group IV was coated with 25 µL each.

In the same manor the next solution (C) was mixed to a concentration of 20 ng TGF-β1/mL with 18%(w/v) PDLLA and group V was coated.
25 µL of C were then transferred to another vial and ethyl acetate was added resulting in the last solution of 2 ng TGF-β1/mL with 18% PDLLA and group VI was coated.

Coating procedure
1. To ensure maximum evaporation area on the scaffold aech one was placed on a needle in the custom made scaffold holder.
2. This holder is placed in a small glass dessicator.
3. 25µL of coating solution was then applied by pipette to each scaffold.
4. Quickly after application the small dessicator was connected to the big dessicator, in which a vacuum pump was preserving a pressure of -980 mbar.
5. The pressure was surveyed on a gauge.
6. The solvent, ethylacetate, evaporated within seconds of connecting the small dessicator to the vaccum system.
7. To ensure complete evaporation the scaffolds were kept in vacuum for 10 minutes and the scaffold was ready to use.

Incubation
The coated scaffolds were placed in a low attachment 24-wells plate and then pre-soaked for a couple of minutes with 100µL Isotonic saline 0,9%NaCl.

When thorougly wet 925 µL of isotonic saline was added to each well and the time was started as the wells were incubated in 37˚C and 5% CO_2.

Isotonic Saline, 0,9% NaCl, was used as incubation media to imitate the physiological environment of the body.

Samples
Were taken at the timepoints: 1h, 12h, 24h, 2, 4, 7, 14, 21, 28 and 35 days. Each sample consisted of 100 µL of 0,9 % isotonic saline.
Backup samples were taken, also 100 µL.
The total sample volume, 200 µL, was replaced by 200 µL fresh 0,9 % isotonic saline.

Due to the tendency of TGF-β1 to stick to all surfaces only silicone coated non-stick vials and pipette tips were used.

Evaluation
Samples were evaluated using a standard ELISA-kit specific to TGF-β1 in order to measure the amount of TGF-β1 released from the coating to the media at every timepoint.

AT-MSCs incubated in chitosan scaffolds

Scaffolds
Chitosan scaffolds used were 1 mm in thickness and 6 mm diameter. Produced as described above.

Groups
The composition of PDLLA and TGF-β1 was varied into 4 groups as displayed below:

Group	PDLLA	TGF-β1	N
I	**-**	**-**	**10**
II	**+**	**-**	**10**
III	**-**	**+**	**10**
IV	**+**	**+**	**10**

Group I served as control group and group IV as intervention group. Group II and III were

used as references regarding the effects og PDLLA and TGF-β1 respectively.

Coating
The procedure was carried out as described above.
Group I was not coated.
Group II and III contained 6% (w/v) PDLLA in the coat.
Group III and IV contained 100 ng TGF-β1 in the coat.

Cells
hAT-MSCs were isolated from liposuctions at the stem cell lab at Aalborg University following their standard isolation procedure.

After isolation the hAT-MSCs were instantly frozen in liquid N_2 and transported to the orthopaedic research lab in Aarhus at a temperature of -80˚C.

In our lab $8x10^6$ hAT-MSCs were proliferated to $30x10^6$ cells and suspended in 1 mL DMEM equal to 0,75 $x10^6$ cells/25 µL.

Seeding procedure
Each scaffold was pre-soaked with 25µL DMEM. When thoroughly wet 25µL of cell suspension was applied on top of the scaffold. Holding the scaffold with a forceps the cells stayed on top until a piece of filter paper touched the scaffold underneath. The filter paper would suck up the DMEM from the scaffold creating a hydro dynamic pull dragging the cells into the scaffold distributing them evenly throughout the 3-D structure.

In order to avoid sucking up cell suspension as well as DMEM the filter papers were cut into a size capable of sucking only 25µL of liquid.

Incubation
The seeded scaffolds were placed in agarose-coated low attachment 24-wells plate and incubated at 37˚C, 5% CO_2 for 24 hours. Allowing the cells to attach properly to the scaffold.

After 24 hours the scaffolds were moved to a new low attachment 24-wells plate and incubated in these plates for the rest of the time.

Chondrogenic induction media (CIM) was used as incubation media. 500 µL were distributed to each well.

Samples
Samples of 500 µL media were collected from each well every 2 days and stored at -80˚C. Samples were collected the following days: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 and 28 days.

Every week 2 scaffolds from each group were fixated by instant freezing in liquid N_2 and stored at -80˚C. Resulting in 2 samples from each group at 1, 2 and 3 weeks and 4 samples from week 4.

Evaluation
Samples from the media were evaluated using a standard ELISA-kit specific for TGF-β1 in order to measure the amount of TGF-β1 released from the coating to the media at every timepoint.
Furthermore the media was analyzed using biochemical assays to measure Collagen-II and GAGs released to media.

Histologically the scaffolds were embedded in Technovit and stained with Alcian Blue in order to analyze the proliferation and morphological differentiation of the cells.
To measure the number of cells in each scaffold DNA was extracted.
In order to investigate the differentiation of the cells RNA was extracted for RT-PCR analysis.

Results

The following graphics are shown with standard deviations.

Release profile for TGF-β1:

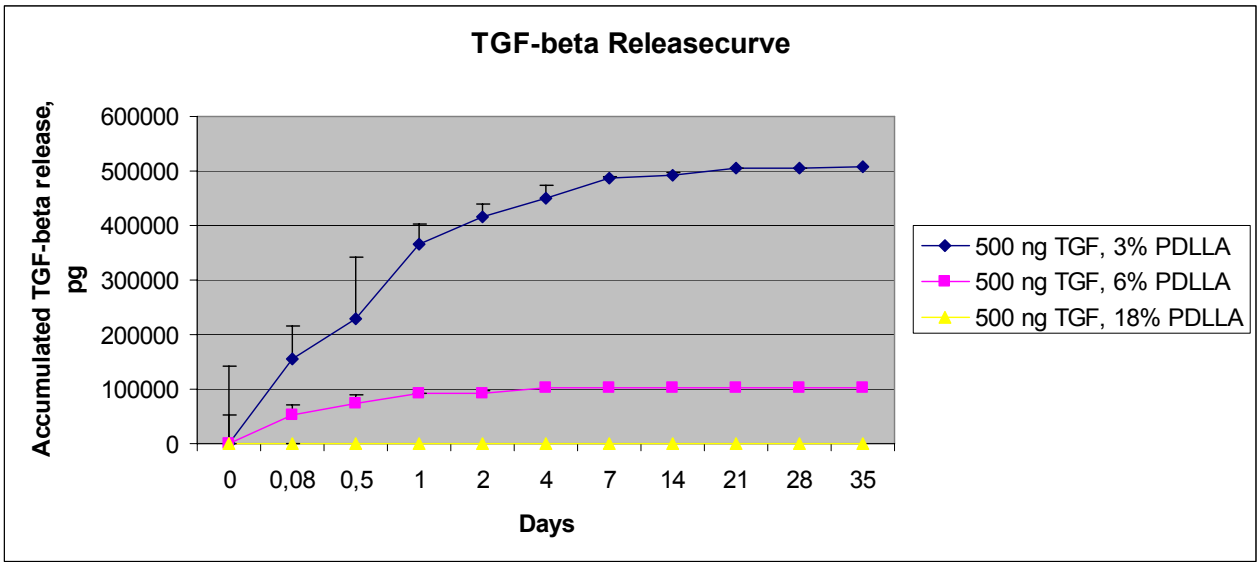

Figure 1. The profile showing TGF-β1 released bi-phasic from the PDLLA-coat on chitosan.

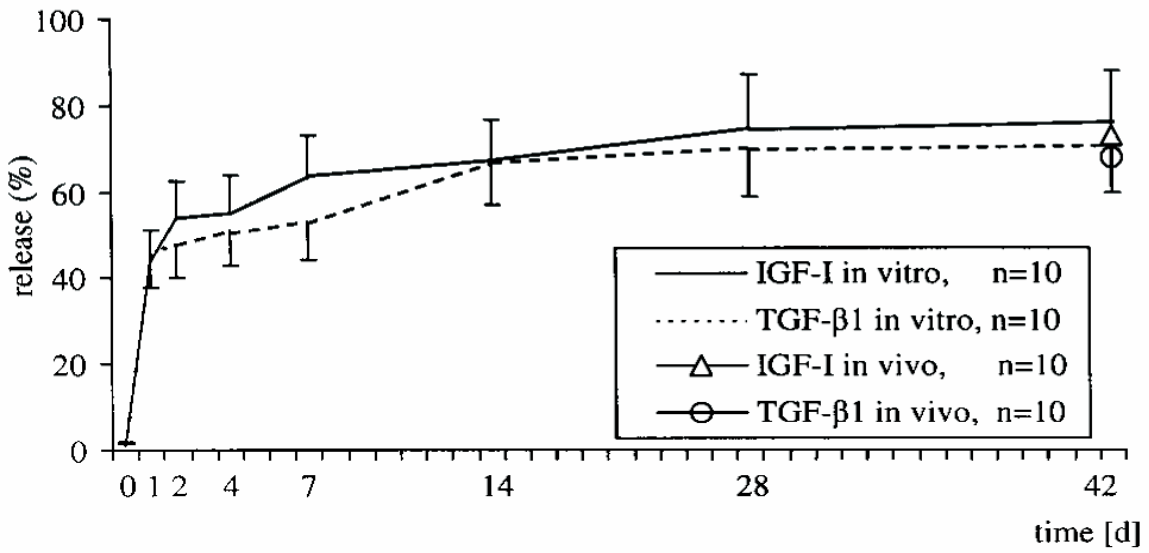

Figure 2. From Schmidmaier et al.[19] The bi-phasic release of TGF-β1.

In vitro study: hAT-MSCs incubated in chitosan scaffolds

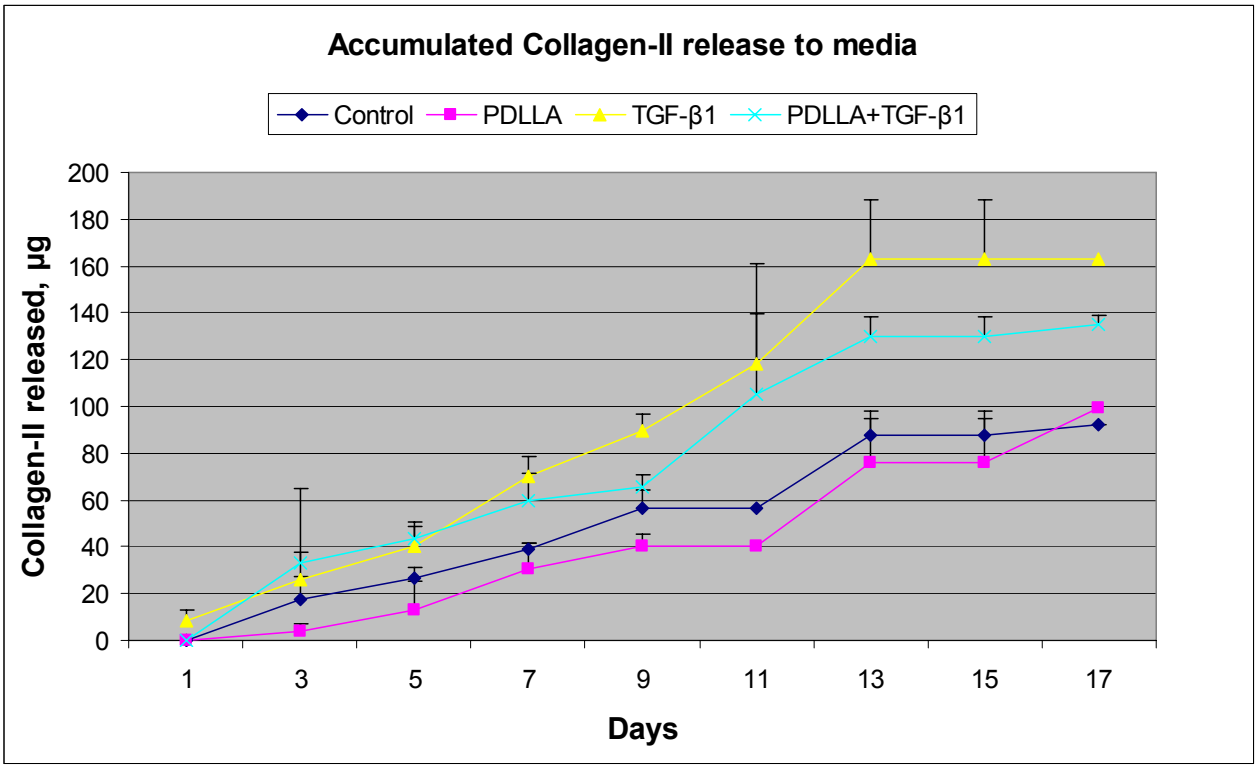

Figure 3. Shows the accumulated cellular synthesized Collagen-II released to the media.

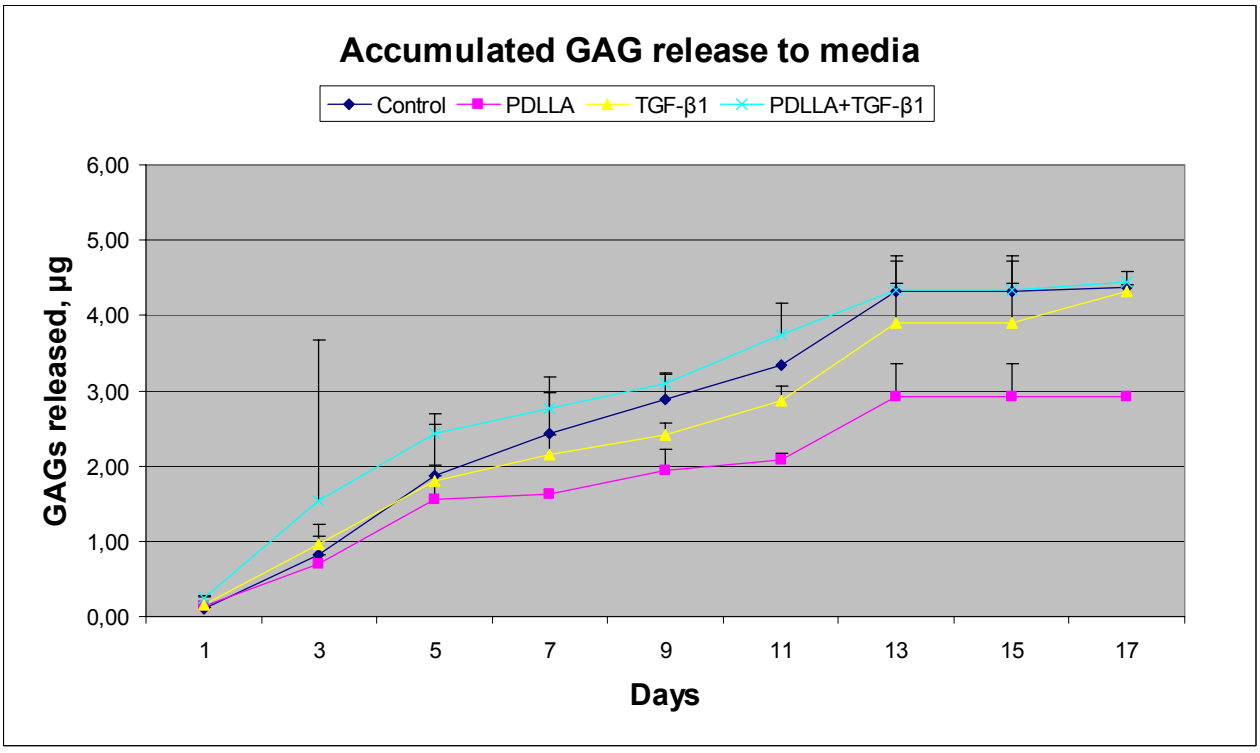

Figure 4. Shows the accumulated cellular synthesized GAGs released to the media.

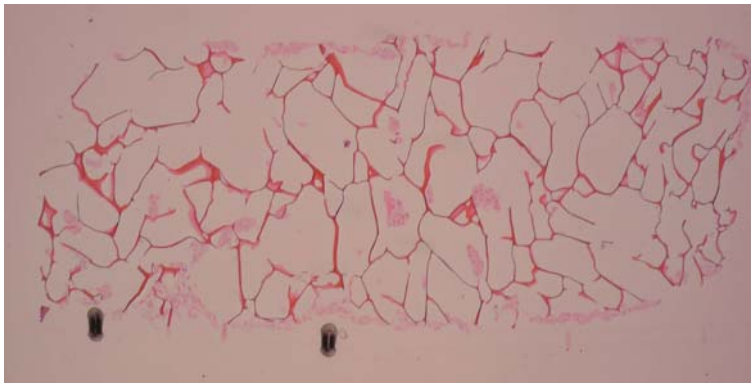

Figure 5. HE stain. Chitosan scaffold without coat, hAT-MSCs, day 14

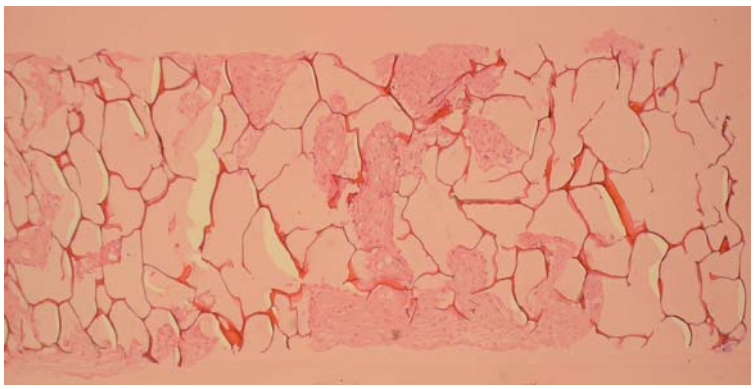

Figure 6. HE stain. Chitosan scaffold with TGF-β1/PDLLA coat, hAT-MSCs, day 14.

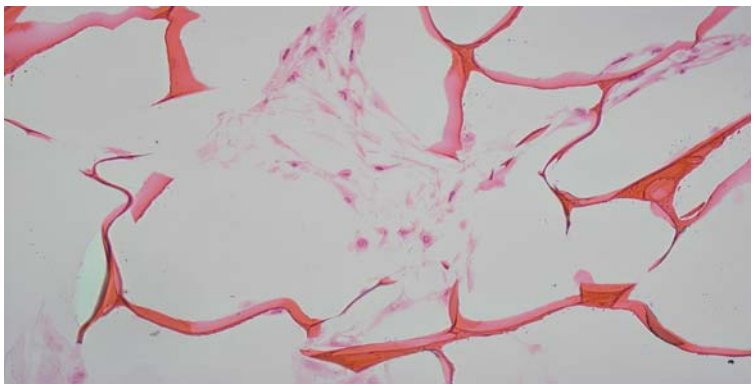

Figure 7. Close-up from figure 5. Cells don't show morphological signs of differentiation.

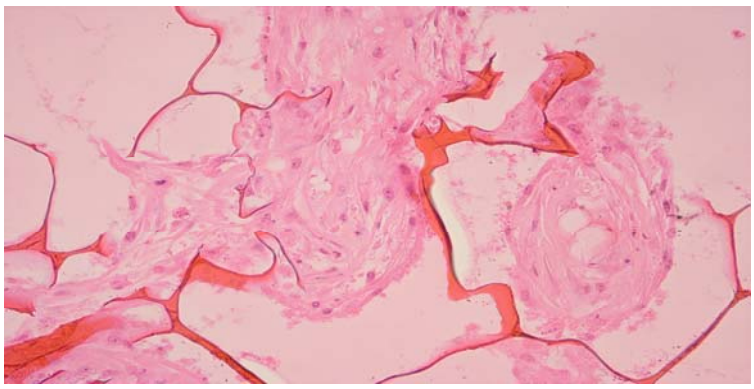

Figure 8. Close-up from figure 6. Cells show morphological signs of differentiation.

Discussion

By growing hAT-MSCs on a TGF-β1 coated chitosan scaffold we acquired a certain degree of differentiation in the direction of chondrocyte lineage measured on the cellular synthesis of coll-II after 15 days.
Looking only at coll-II we can constitute that the hAT-MSCs have undergone differentiation after stimulation by TGF-β1.
The postulate is very much supported by the morphological appearance of the cells in the histological slides.

From figure 4 we were not able to show a significant difference in cellular synthesis of GAGs between the control group and the intervention group. Yet the data presented in figure 4 only show the synthesis until day 15. It is possible that the synthesis will change at a later time point resulting in a cross over from where the cells in group IV become superior in GAGs production. The argument for this could be a lag period during induction of differentiation. Results from the later time points will have to be analyzed to examine this possibility.

To finally conclude that we have induced chondrogenic differentiation it would be necessary to look at the gene expression in the cells.
In our RT-PCR reactions we want to detect changes in the presence of our target genes: Collagen type I, Collagen type II, Aggrecan and the transcription factor SOX-9.
We will normalize the expression by detecting the 2 housekeeper genes GAPDH and RPII.
Relative gene expression is performed according to Pffafl [18].
The RT-PCR will give us a more specific indication of the differentiation status. The results from RT-PCR are expected to show higher levels of cartilage specific genes in group IV than in group I.

The histological slides also support the expectation of a clear difference in differentiation of the cells. Looking at figure 5 and 6 it is clear that the TGF-β1 stimulated cells in group IV showed a higher degree of proliferation than the unstimulated cells in group I.
In figure 7 and 8 the difference is even more obvious. The unstimulated cells in figure 7 still have the fibroblast-like appearance of undifferentiated MSCs. The cells in figure 8 show clear signs of differentiation towards chondrocyte lineage. They are rounder and vacuoles containing extra cellular matrix are beginning to appear. This is a clear sign of differentiation however they do not completely resemble mature chondrocytes.

Regarding the release properties of the PDLLA/TGF-β1-coating we succeeded in making a coat with release properties similar to a previously established method.
Figure 1 shows the same bi-phasic release of TGF-β1 from PDLLA as shown in figure 2 by Schmidmaier et al. in 2001[19].
Schmidmaier et al. showed that 48% of the TGF-β1 was released within 48 h and 71% was released after 42 days.
When using 3% (w/v) PDLLA in the coating solution we showed that 80% of the TGF-β1 was released within 48h. 100% was released within the 4 weeks observation period.
Using 6% (w/v) 20% TGF-β1 was released within 48h and using 18% (w/v) only 5% TGF-β1was released within 48h.
The different appearance of the three graphs in figure 1 indicates that the amount of PDLLA in the coat is crucial to release of TGF-β1. When using more PDLLA in the coat the release of TGF-β1 is slowed down but the profile is maintained.
The evaporation of media during incubation could increase the concentration of TGF-β1 as time progressed during the study. But unbound proteins are rapidly degraded at 37˚C. Therefore we assumed that the samples

collected were a sign of present release of TGF-β1 at that time point.

In order to support our theory of chondrogenic differentiation of hAT-MSCs DNA are to be extracted from the scaffolds. This measurement will show how many cells are present at the different time points. Indicating what happens to the cells during incubation regarding proliferation.

Also underway is an in vivo study concerning implantation of PDLLA/TGF-β1 coated scaffold with and without hAT-MSCs implanted in a dorsal pouch on nude mice. Nude mice are chosen because of their lack of immunological response which enables us to use the same cells as used in vitro.

Assuming that we acquire the expected results it should be relevant to continue investigating this model for chondrogenic induction in MSCs.
An obvious target point is the biochemical signals such as growth factors. As mentioned above the possibility of combining growth factors like TGF-β1 and IGF seems relevant.
More knowledge on the cellular signalling in chondrogenic differentiation is needed. And when it has been acquired the idea of applying a bi-layered coat to the scaffold seems ideal. This way it should be possible to fine tune the release profile of different growth factors embedded in the coating.

Another very relevant issue is the hypoxia of the tissue. All studies described in this report were carried out in standard incubators at 37˚C and 5% CO_2. Yet since they were carried out our laboratory has established the necessary equipment for hypoxic incubation. This would be a decisive step towards mimicking the natural environment of articular cartilage.

Summary

We investigated the possibility of using a 3-D porous scaffold to grow MSCs for hyaline cartilage tissue engineering and found that:

1) 3-D porous chitosan scaffolds can be coated with TGF-β1.
2) TGF-β1 is released in a bi-phasic release-profile.
3) TGF-β1 coating of 3D scaffolds carrying hAT-MSC results in:
    a. Increased proliferation and differentiation of hAT-MSC into chondrocyte lineage.
    b. Increased cellular synthesis of Collagen-II.

Therefore this method is suitable for further investigation of the tissue engineering hyaline cartilage.

Reference List

1 Ulrich-Vinther M, Maloney MD, O'Keefe RJ, Schwarz EM, 2003:, Articular Cartilage Biology: J.Am.Acad.Orthop.Surg; v. 11, p. 421-430.

2 Brittberg M, Lindahl A, Nilsson A, Ohlsson C, IssaKson O, Petterson L: Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med. 1994 Oct 6;331(14):889-95.

3 Derrett S, Stokes EA, James M, Bartlett W, Bentley G: Cost and health status analysis after autologous chondrocyte implantation and mosaicplasty: a retrospective comparison. Int J Technol Assess Health Care. 2005 Summer;21(3):359-67

4 Halleux C, Sottile V, Gasser JA, Seuwen K, Multi-lineage potential of human mesenchymal stem cells following clonal expansion. J Musculoskelet Neuronal Interact. 2001 Sep;2(1):71-6.

5 Randolph MA, Anseth K, Yaremchuk MJ: Tissue engineering of cartilage. Clin Plast Surg. 2003 Oct;30(4):519-37.

6 Ushida T, Furukawa K, Toita K, Tateishi T, Three-dimensional seeding of chondrocytes encapsulated in collagen gel into PLLA scaffolds. Cell Transplant. 2002, Cell Transplant;11(5):489-94.

7 LeBaron RG, Athanasiou KA. Ex vivo synthesis of articular cartilage. Biomaterials. 2000 Dec. 21(24):2575-87.

8 Spagnoli A, Longobardi L, O'Rear L: Cartilage disorders: potential therapeutic use of mesenchymal stem cells. Endocr Dev. 2005;9:17-30.

9 Stengaard C, Elmengaard B, Soballe K, Ulrich-Vinther M: IGF-coating of polymer stimulates chondrocyte synthesis of collagen and aggrecan.

10 Buckwalter JA, Mankin HJ. Articular cartilage. Part 1: tissue design and chondrocyte-matrix interactions. (1997) J. Bone Joint Surg. 79: 600-611.

11 Eyre D. Collagen of articular cartilage. (2002) Arthritis Res. 4:30-35.

12 Genser F. Histologi. (2001) 1st edition, 2nd print, Munksgaard.

13 Langer and Vacanti, science 260, 1993

14 LeBaron RG, Athanasiou KA. Ex vivo synthesis of articular cartilage. (2000) Biomaterials 21; 2575-2587.

15 Sharma B, Elisseeff H. Engineering structurally organized cartilage and bone tissues. Ann Biomed. Eng. (2004) 32;148-159.

16 Franklin H. Epstein, NEJM

17 Longobardi L, O'Rear L, Aakula S, Johnstone B, Shimer K, Chytil A, Horton WA, Moses HL, Spagnoli A. Effect of IGF-I in the chondrogenesis of bone marrow mesenchymal stem cells in the presence or absence of TGF-beta signaling. (2006) J Bone Miner Res. 21;626-636.

18 Pfaffl, M. W. "A new mathematical model for relative quantification in real-time RT-PCR." *Nucleic Acids Res.*, May 2001, *29* (9), e45.

19 Schmidmaier, G., Wildemann, B., Stemberger, A., Haas, N. P., Raschke, M. "Biodegradable poly(D,L-lactide) coating of implants for continuous release of growth factors." *J.Biomed.Mater.Res.*, 2001, *58* (4), 449-455.