Stimulation of orthopaedic implant fixation

Investigations of osteogenic growth factors and topical delivery systems

PhD thesis

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Preface

This thesis is based on studies performed october 2003 through september 2006 at the Orthopaedic Research laboratory of the University hospital of Aarhus. During this time I was employed by the Clinical Institute and the Interdisciplinary Nanoscience Center at the University of Aarhus.

Professor Kjeld Søballe M.D., D.M.Sc. was my main supervisor.

Brian Elmengaard MD, PhD was my project supervisor.

Flemming Besenbacher, Dr. Scient, Head of the Interdisciplinary Nanoscience Center at University of Aarhus (iNANO), was associated supervisor.

The surgery for study I was performed at the University of Aarhus Clinical Institute. The surgeries for studies II and III were performed at the Midwest Orthopaedic Research Facility at Hennepin County Medical Center, Minneapolis, Minnesota, USA, where Joan E. Bechtold, PhD, headed the indefatigable effort of the surgical and veterinarian team.

Gerhard Schmidmaier Dr. Med, and Britt Wildemann PhD, from the Center for Musculoskeletal Surgery, Charité University Medicine, Berlin, Germany taught us the coating technique used in studies I and II.

The preparation of chitosan scaffolds for study III were made in close collaboration with Morten Foss PhD, and Mads Bruun Hovgaard from the interdisciplinary Nanoscience Center at the University of Aarhus.

Preparation for analysis, and mechanical and histomorphometric analysis were performed at the Orthopaedic Research Laboratory at the University Hospital of Aarhus, under the competent supervision and assistance of laboratory technicians Anette Milton, Jane Pauli, Feng Ya Mei and Anette Baattrup.

Niels Trolle Andersen from the Department of Biostatistics, University of Aarhus, provided invaluable help on the statistics.

Friendships and professional relations which I appreciate immensely have been established during my time as a PhD-student. I wish to express my gratitude to all the wonderful, talented people whose help and company I have enjoyed during this period.

Behind every successful man stands a woman; surprised - wondering when that big boy morphs into a man after he kisses her goodbye and goes to work.
Thank you Anna - my beloved wife, for taking care of me and our family while I was pursuing academic enlightenment.

Acknowledgements

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List of papers

This thesis is based on three experimental animal studies:

Paper I: Locally delivered TGF-β1 and IGF-1 enhances the fixation of titanium implants. A study in dogs. (Acta Orthopaedica 2006; (77): 799-805)

Paper II: Combination of TGF-β1 and IGF-1 incorporated in a biodegradable poly(D,L-lactide) coating equals the fixation of hydroxyapatite coating. A paired study in dogs. (submitted to Acta Orthopaedica February 6th 2007)

Paper III: Chitosan scaffold coated on orthopedic implants in vivo. A study in dogs. (submitted to Acta Orthopaedica February 28th 2007)

Definitions

In the field of implant- and related research, definitions often vary between authors. The following is not an attempt to re-define these terms; it is merely the way they are used in this thesis.

Biodegradation is the process by which substances are broken down in living organisms.
An **implant** is an artificial device made to replace and act as a missing biological structure. A **gap** refers to the void between the implant surface and the host bone.
In surgery, a **biocompatible material** (or **biomaterial**) is a synthetic or natural material used to replace part of a living system or to function in intimate contact with living tissue.
Osseointegration is the direct structural and functional connection between living bone and the surface of an artificial implant. **Ongrowth** is the direct contact between a tissue and the implant surface, as seen in light microscopy. **Gap healing** refers to the volume percentage of bone in the gap.

Abstract

The long term survival of cementless orthopedic implants relies on a rapid bony osseointegration, as early implant migration has shown to be a strong predictor of early loosening. The challenge of facilitating a solid bony fixation of an implant is greatest in settings where bone stock is diminished and healing potential reduced. Since adjuvant therapies for implant fixation using osteogenic growth factors have been introduced, applications employing one growth factor have been extensively investigated. Applications combining two or more growth factors may be more favorable due to additive or synergistic effects on bone. We investigated the combination of Transforming Growth Factor beta one (TGF-β1) and Insulin-Like Growth factor one (IGF-1) as those two growth factors are highly expressed during bone growth. We investigated the growth factor combination administered topically to the implant surface by a biodegradable polymer. Furthermore we investigated the possibility of using chitosan, a ubiquitous polymer found in the exoskeletons of crustaceans, insects, fungal cell walls and plankton, as a drug carrier for implant applications.

This thesis is based on three papers. Studies I and II investigate the effect of the combination of TGF-β1 and IGF-1 coated onto the implant surface in Poly (D,L-lactide)(PDLLA), a biodegradable carrier. Study III investigates the effect of chitosan applied to an implant as a scaffold.

In study I the growth factor coating was applied on the surface of a porous coated titanium alloy implant and compared with a porous coated titanium alloy implant not coated with the growth factors. The implant model was an unloaded gap model, and situated in the proximal humerus of mature dogs. The observation period was 4 weeks, and the bone-implant specimens were evaluated by mechanical and histomorphometric tests. The fixation of the growth factor coated implants was two-fold higher than the fixation of the control implants without the growth factor coating. There was 2,5 times more bone on the surface of the growth factor implants than the controls, a 30 % median raise in inner zone gap healing and a 2-fold raise in outer zone gap healing. No fibrous tissue was found on the growth factor treated implants.

In study II the growth factor coated implants were used in a loaded gap model in the distal femurs of mature dogs. Similar implants with an additional hydroxyapatite coating was used as controls. As in study I, the observation period was 4 weeks, and the bone-implant specimens were evaluated by

mechanical and histomorphometric tests. The mechanical test found all implants to be well fixed without any statistically significant differences in any of the three mechanical parameters. In contrast to the push-out test, the histomorphometric analysis found differences in tissue distribution around the implants. Bone ongrowth to the implants was 3 fold higher for HA coated implants. There was a little fibrous tissue ongrowth in 4 of the 10 HA coated implants, and no fibrous tissue on the growth factor coated implants. In the inner half of the gap, the bone volume fraction was 26% higher in the growth factor coated implant group. In the outer half of the gap, the bone volume fraction was 28% higher in the growth factor coated implant group. There were no signs of residual polymer on the implant surfaces.

In study III a porous chitosan scaffold was produced on titanium alloy implants. A standard porous coated titanium alloy implant without chitosan was used as control. The implants were inserted in the proximal tibia of dogs for 4 weeks, in an unloaded gap model. We evaluated the effect by mechanical push out test and histomorphometry. The chitosan scaffold was converted to a thick fibrous membrane that caused the implants to be fixated very poorly compared with the uncoated controls. There was 3-fold more bone around the chitosan coated implants outside of the fibrous membrane compared with the controls.

In conclusion the combination of TGF-β1 and IGF-1 coated onto the implant surface in a biodegradable carrier facilitated a markedly improved fixation and osseointegration of the porous coated titanium implants. The results with the growth factor coating are very encouraging, especially for situations were the host bone and healing potential is compromised. Further investigations are warranted to determine the best use of this promising adjuvant therapy.

The chitosan scaffold coated on the titanium implants proved to be useless for implant applications in the present form. There was however a great increase in gap healing of bone in the outer gap of the chitosan implants, and this justifies further investigation to establish whether chitosan has a potential for implant drug delivery applications.

Introduction

Total hip replacement (THR) is the end stage treatment of hip osteoarthritis and other conditions that destroy the hip joint. The Danish Hip Arthroplasty Register (DHR) registered 7.244 primary THR and 1048 revision hip replacements in 2005. Since the DHR started its registrations in 1995, the incidence of primary hip arthroplasties has been rising, while the incidence of revision arthroplasties has been constant during the registration period. It has been estimated that the incidence will increase between 22 and 210 % by the year 2020.[1]
Approximately one half to two thirds of first time revisions are due to aseptic loosening in the Scandinavian countries [1-3]. The overall 11-year prosthesis survival was approx. 92%. Cementless components are used in approximately half of the THRs in Denmark.

In the 1960's there was a vast progression in the quality of the joint replacement therapy, as bone cement was introduced. The cementing techniques improved, and to this day no alternative has proven to outlive a well cemented prosthesis in an elderly person. It was obvious though, that younger patients wore out their artificial joints much faster than elderly patients, and the cementless prostheses were reintroduced. In the beginning the cementless prostheses were associated with a high rate of failures. Many of the patients presenting with thigh pain and limping had fibrous tissue fixation of the prosthesis.

The long term survival of cementless orthopedic implants relies on a rapid bony osseointegration and it has been shown that early implant migration is a strong predictor of early loosening [4;5]. The challenge of facilitating a solid bony fixation of an implant is greater in the revision setting where bone stock is diminished and healing potential reduced compared with the primary surgery[6-10]

Implants with a hydroxyapatite (HA) coating stimulate bone ingrowth to implants. The highly osteoconductive nature of HA coatings has been demonstrated experimentally[11-13] and excellent longevity has been demonstrated clinically[2;14-17] Recent studies indicate that the initial problems with the porous coated cementless prostheses may in part have been due inadequate design. Follow up studies (up to 10 years) using modern prostheses with and without hydroxyapatite, have not been able to demonstrate the same differences in implant survival as previous studies [18-21].

Bone contains several growth factors, including bone morphogenetic proteins (BMPs), transforming growth factor beta (TGF-b), insulin-like growth factors I and II (IGF-I and IGF-II), and many others. Several of these growth factors have proven able to stimulate bone growth [22;23]. A growing interest has developed towards these osteogenic growth factors, as they have become commercially available. Applications employing one growth factor have been extensively investigated[24-30]
The growth factors OP-1(BMP-7) and BMP-2 are available for clinical use. The clinical and experimental results with these growth factors are diverging. In a prospective, controlled, randomized study of 450 patients recombinant human BMP-2 was investigated for the treatment of open tibial fractures. The study showed that BMP2 can accelerate fracture healing and reduce the infection rate [31]. OP-1 was investigated as adjuvant therapy in revision surgery where it was mixed with bone graft. The study was terminated before scheduled because there was a lack of improvement of fixation, and a high rate of revision surgery[32]. In a paired study in dogs with compacted allograft soaked with rhBMP-2, the implant fixation was reduced compared with allograft without the growth factor [33].

Combinations of growth factors have been investigated as well, and the results are promising. The combination of growth factors often yields an additive or even synergistic effect on the stimulation of bone growth [34-37]. The combination of Transforming Growth Factor beta one (TGF-β1) and Insulin-Like Growth factor one (IGF-1) is particularly interesting as those two growth factors are highly expressed during bone growth[10;38-42]. Intermittently administered parathyroid hormone caused a time and dose-dependent increase in the bone mineral density of the lumbar spine of the treated rats. This anabolic effect on bone mass was accompanied by progressive increases in bone matrix-associated IGF-I and TGF- β1[43]

Many osteogenic growth factors may have effects in other organ systems which are not related to bone. They typically have a half life of few minutes, which make them unsuitable for systemic administration. Much research is focused on finding appropriate ways to deliver the growth factors topically in the optimal concentration and with an optimal release profile. Local growth factor delivery applications previously investigated include growth factor adsorption to calcium phosphates, mixtures of calcium phosphates and collagen, collagen sponges, hydrogels, growth factor immobilization on implant surfaces, and various polymers[44-50]

Lactide based polymers such as Poly (D, L-Lactide) (PDLLA) have been used clinically as material for screws and plates for decades, and it has successfully been used as a drug carrier [6;48;51]. The material is generally well tolerated. Few cases of inflammatory reactions have been seen, but only with bulky design PDLLA implants. The reactions are believed to be caused by the corresponding pH-lowering effect of a large polymer presentation[52].

Chitosan is an alternative drug delivery system which has unique characteristics. It is used in bone tissue engineering as it has been shown to promote growth and mineral rich matrix deposition by osteoblasts [53;54]. Chitosan can be molded in various forms, and it possesses ability to form porous structures, which are generated by freezing and lyophilizing[55;56] Chitosan-based implants yield a minimal foreign body reaction, with little or no fibrous encapsulation[57] Chitosan is suggested to have an immunomodulatory effect that stimulates the integration of an implanted material by the host [58]. Chitosan also has intrinsic antibacterial activity. It has been shown that Chitosan can reduce the infection rate of experimentally induced osteomyelitis by Staphylococcus aureus in rabbits [54]

Aim

The purpose of the studies presented in this thesis was to evaluate the effect of locally delivered TGFB1 and IGF1 in poly(D,L-lactide) on the mechanical fixation and osseointegration of experimental implants. We compared the effect of this growth factor combination both to an untreated porous coated titanium implant and a hydroxyapatite coated implant. In addition we evaluated chitosan as an alternative biodegradable carrier for growth factor delivery. We used established experimental models that attempt to expose the implants to clinically relevant conditions, and the implants were evaluated by a mechanical test and histomorphometry.

Hypotheses:

Study I: We hypothesized that the combination of TGF-β1 and IGF-1 in PDLLA would enhance the osseointegration and mechanical fixation of unloaded cylindrical porous coated titanium implants surrounded by a gap.

Study II: We hypothesized that the combination of TGF-β1 and IGF-1 in PDLLA would result in better mechanical fixation and quantitatively more bone on and around the implant when compared with a hydroxyapatite coating in a loaded gap model.

Study III: We hypothesized that chitosan scaffold would result in improved fixation and osseointegration of the implant compared with the control implant.

Materials

Titanium

The implants used in the studies in this thesis were made of titanium alloy (Ti-6Al-4V). It is widely used in cementless prosthesis because it has an elastic modulus closer to that of bone than pure titanium, and it is highly biocompatible. This biocompatibility may be caused by the current density of the titanium. It has been showed that from a variety of corrosion-resistant implant metals and alloys, gold showed the highest current densities, followed by the stainless steel, the cobalt-based alloy, and the Titanium-alloy. The pure metals titanium, niobium, and tantalum showed the lowest values. This could be explained by the stable oxide layer on these base metals, preventing an exchange of electrons and thus any redox reaction. This rating of metallic implant materials based on in vitro measurements of current densities is in good accordance with their biocompatibility rating reported from in vivo experiences[59].

Porous coating

The implants used in studies I and II were all plasma sprayed to obtain a porous coating. Plasma spraying is a materials processing technique for producing coatings using a plasma jet. Deposits having thickness from micrometers to several millimetres can be produced from a variety of materials - metals, ceramics, polymers and composites. The deposits consist of a multitude of pancake-like lamellae called 'splats', formed by flattening of the liquid droplets. As the feedstock powders typically have sizes from micrometers to above 100 micrometers, the lamellae have thickness in the micrometer range and lateral dimension from several to hundreds of micrometers. Between these lamellae, there are small voids, such as pores, cracks and regions of incomplete bonding. A pore size between 50 and 400microns has been suggested to be the optimum for osseointegration[60]. The pore size and roughness was not measured for the implants used in these studies. Measurements of implant roughness of implants from the same manufacturer have varied from 26 to 47μm in earlier studies from our institution[11;61].

Hydroxyapatite

Hydroxyapatite is the main mineral component of dental enamel, dentin, and bone. Seventy percent of bone is made up hydroxyapatite. It is a naturally occurring form of calcium apatite with the formula Ca5(PO4)3(OH), but is usually written Ca10(PO4)6(OH)2 to denote that the crystal unit

cell comprises two molecules. The crystals are platelets or rods. The bioactivity of hydroxyapatite may be due to its binding to serum proteins and growth factors, promoting osteoblast adhesion and proliferation [62].

Poly (D,L-lactide)

Polylactic acid or Polylactide (PLA) is a biodegradable, thermoplastic, aliphatic polyester derived from lactic acid. It is easy to produce in a high molecular weight form through ring-opening polymerization. Due to the chiral nature of lactic acid several distinct forms of polylactide exists: poly-L-lactide (PLLA) is the product resulting from polymerization of lactic acid in the L form. PLLA has crystallinity around 37%. The polymerization of a mixture of both L and D forms of lactic acid leads to the synthesis of poly-DL-lactide (PDLLA) which is not crystalline but amorphous.

Lactide based polymers undergo hydrolytic bond cleavage to form water-soluble degradation products that can dissolve in an aqueous environment, resulting in polymer erosion. In drug delivery applications, a surface-eroding polymer can provide constant and easily controllable drug release rates and protect the drug from in vivo degradation, whereas bulk erosion may give increasing release rates and provides less protection for the drug from body fluids. The relationship between degradation and erosion of PDLLA is poorly elucidated. Most polymers display bulk erosion characteristics, as surface erosion is difficult to achieve[63]. Water penetrates and degrades the interior of the material faster than the surface can erode. A copolymer approach is attractive because it imparts the capability to tailor the polymer's properties by simply changing the monomer ratio. In the case of PDLLA each cleavage by hydrolysis generates a carboxyl acid group. The consequent lowering of pH-value accelerates degradation by augmenting further hydrolysis [64]. In the second phase, the implant loses its form and breaks physically into particles, which are attacked by macrophages. Depending on the size and chemistry of the particulates, they are phagocytosed and the by-products enters metabolism or are excreted by the kidneys and lungs.

Transforming growth factor beta 1 (TGF-β1)

TGF-β1 is the founding molecule for the TGF-β superfamily. 90% of TGF-β in bone is the TGF-β1 isoform. The transforming growth factor beta superfamily of ligands includes inhibins, activin, anti-müllerian hormone, bone morphogenetic protein, decapentaplegic and Vg-1[65]. It is a secreted protein that performs many cellular functions, including: cell growth, cell proliferation, cell differentiation and apoptosis. Many cells synthesize TGF-β and almost all of them have specific

receptors for this peptide. TGF-β1 will only induce new bone formation when injected in close proximity to bone. Unlike BMPs, TGF-β1 will not produce bone when injected into ectopic sites[66].

Insulin-like growth factor 1 (IGF-1)

Insulin-like growth factor 1 (IGF-1) is mainly secreted by the liver as a result of stimulation by growth hormone (hGH). It is a polypeptide protein hormone similar in molecular structure to insulin, and it is abundant in the bone microenvironment. Almost every cell in the human body is affected by IGF-1. In addition to the insulin-like effects, IGF-1 can also regulate cell growth and development, as well as cellular DNA synthesis.[65]. IGF-1 is important for both the regulation of normal physiology, as well as a number of pathological states. It is produced by bone cells and released from bone matrix to act as autocrine/paracrine regulators of bone formation[65]. It plays an important role in childhood growth and continues to have anabolic effects in adults. The highest rates of IGF-1 production occur during the pubertal growth spurt. The lowest levels occur in infancy and old age[67].

Chitosan

is a linear polysaccharide composed of randomly distributed ß-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It is produced by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (crabs, shrimp, etc.). The DA in commercial chitosans is 60-100 %, and can be determined by NMR spectroscopy. Chitosan is used in bone tissue engineering as it has been shown to promote growth and mineral rich matrix deposition by osteoblasts in [53;54]. Chitosan can be molded in various forms, and it possesses ability to form porous structures, which are generated by freezing and lyophilizing[55;56] Chitosan is cationic of nature and thus electrostatically interacting with anionic glycosaminoglycans (GAG), proteoglycans and other negatively charged molecules. A large number of cytokines such as growth factors are linked to GAG, and a scaffold incorporating a Chitosan–GAG complex may retain and concentrate growth factors secreted by colonizing cells [68] Chitosan-based implants yield a minimal foreign body reaction, with little or no fibrous encapsulation[57] Chitosan is suggested to have an immunomodulatory effect that stimulates the integration of an implanted material by the host [58]. Chitosan also has intrinsic antibacterial activity. It has been shown that Chitosan can reduce the infection rate of experimentally induced osteomyelitis by Staphylococcus aureus in rabbits [54]

Methods

Implant models

The basic design of the models is the cylindrical implant of 10mm of length and 6mm diameter (Figure 1a).

In Study I and III, we used a 1mm unloaded gap model. This model allows bone regeneration within the gap, without the influence of loading or joint fluid (Figure 1b).

In study II we used a 0,75mm loaded gap model. In this model the implant is under the influence of joint fluid passing by the surface as well as loading during each gait cycle (Figure 1c).

Figure 1: The left image shows the basic structure – the 6x10 mm porous coated implant. The middle image illustrates the unloaded gap model. The right image illustrates the loaded gap model.

The two models are basically quite similar. In the canine gap model, there will be limited bone regeneration around porous coated titanium implants when the gap exceeds 1mm, even in the unloaded model[69-71;71;72]. This allows us to detect a difference between the effects of different surface modifications. This difference is accentuated considerably in the loaded gap model, where the "regular" porous coated titanium implant will be very poorly fixed. The surface and gap will predominantly be occupied by fibrous tissue. For a corresponding implant coated with hydroxyapatite this will look completely different, with good fixation and good osseointegration. There seems to be a threshold for fixation in the loaded model that might expose a difference between surface treatments in a clinically relevant way, more than the unloaded gap model.

These models are designed to detect a difference between two surface modalities. If we do not find a statistically significant difference we cannot conclude that the effect of the modalities is equal. Though it might be lesser than that of other species, there is considerable inter-individual variation in the dogs. The paired design of these studies compensate for that. We base our conclusions on the

rank of the data pairs, rather than the actual numbers. This in turn makes it impossible to use earlier studies in the same models for direct comparisons.

The choice of observation period is based mostly on experience in our institution. We want to test the initial fixation and osseointegration. We believe the initial few weeks after implantation of the prosthesis to be critical in the race for the surface. If the process of osseointegration is slow, too much fibrous tissue forms. This leads to a fixation of the prosthesis based on fibrous tissue rather than bone, which in turn leads to early loosening through continued micromotion of the prosthesis[4;8;17;73].

These models are well suited as screening methods for differences between treatments. We have chosen to examine the effect after a short observation period, so we are restricted to conclude or speculate on predictability of prevention of early loosening.

Implant coating

Recombinant human Transforming Growth Factor beta-1 (rhTGF-β1) and recombinant human Insulin-like Growth Factor 1 (rhIGF-1) (R&D Systems, UK)(Figure 2) were dissolved in a Poly (D, L-lactide) (PDLLA) - Resomer 203 (Boehringer Ingelheim GmBH,Germany) and ethyl acetate solution resulting in a 1% w/w ratio of TGF- β1 and 5% w/w ratio of IGF-1. The implants were dipped in the solution and air-dried, all under sterile conditions. The thickness of the coating was approximately 15-20 μm[51]. Based on coating experiments performed at Dr. Schmidmaiers laboratory, the estimated amount of growth factors on each of our implants was 140 μg IGF-1 and 28 μg TGF-β1. We did not control the coating thickness or the actual amount of growth factors around the implants; however, due to the nature of the coating process some variation in the coating thickness and amount of growth factors is predictable (Figure 2).

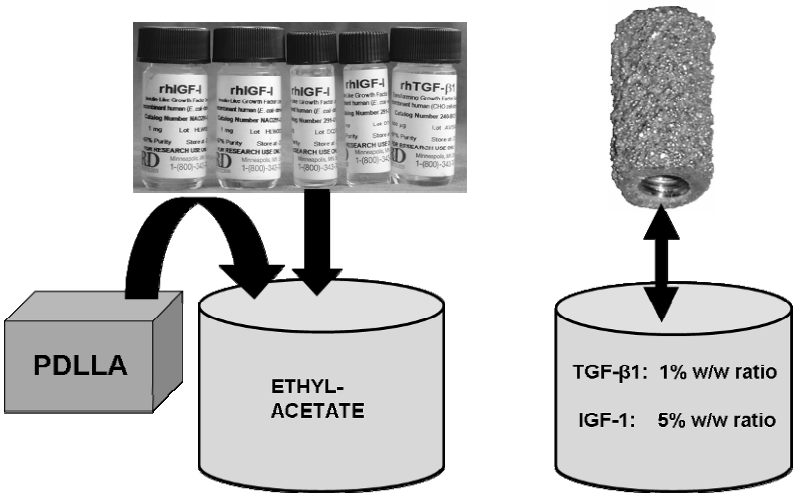


Figure 2: The coating procedure

For the production of a chitosan scaffold we used sterile chitosan with a Degree of Acetylation (DA) less than 20, molecular weight 50-1000kD (average 500kD)(Bioneer A/S, Hørsholm, Denmark). It was mixed at a concentration of 20 mg/mL with 0.2M acetic acid under continuous stirring for 24 hours, eventually forming a thick, homogenous, and highly viscous solution.
For the coating procedure wells made of polypropylene were produced in different sizes stepping 100µm in diameter to fit the largest outer diameter of each of the porous coated titanium implants (Fig 3a). The wells were filled with the chitosan solution and subsequently loaded with the implants, frozen to - 50 ˚C, and freeze dried for 48 hours (Figure 3b). The implants were rehydrated in an ethanol-water series of varying ethanol concentrations to remove remaining acetic acid from the chitosan scaffold, kept for 12 hours in a phosphate buffer solution, and dehydrated by a reverse ethanol-water series. Finally the implants were freeze dried for another 48 hours, and sterilized using ethylene oxide. The scaffold coating was examined with scanning electron microscopy (SEM) (Fig 3c)

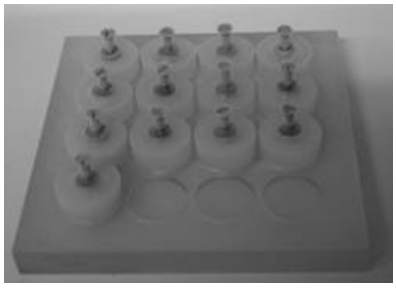
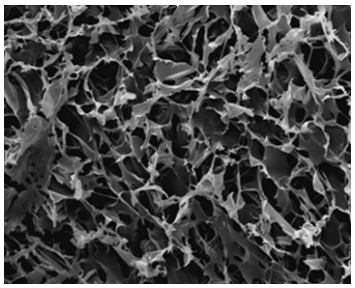
3a  3b  3c 

Figure 3: 3a shows the wells holding the implants. The screws are inserted in the thread for handling during preparation. 3b shows the freeze drying setup. 3c is a SEM image of the resulting scaffold on the implant surface.

Specimen preparation

Pending preparation the bone-implant specimens were stored at –20° C. The specimens were cut on a water-cooled diamond band saw (Exact Appartebau, Germany) leaving two transverse sections. The outermost section of approximately 3.5 mm was used for mechanical testing. The remaining section of 6,5mm was used for histomorphometric analysis. The histomorphometry specimens were dehydrated in graded ethanol (70-100%) containing 0.4% basic fuchsine, and embedded in methylmethacrylate. The embedded specimens were randomly rotated around the vertical axis of the implant. In the central part of the implants 4 serial sections of 15-20 µm were produced using a Leiden microtome (Leiden, Holland),[74] (Figure 4).

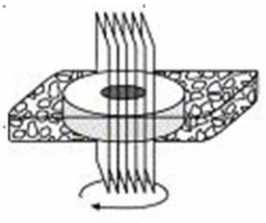


Figure 4: The principle of random sectioning. The embedded implant is rotated, and the direction of the sectioning is chosen based on where the rotation stops.

After sectioning, the specimens were counterstained with 2 % light green. This preparation method allows distinction between mineralized bone, fibrous tissue and bone marrow [75].

Mechanical testing

The bone-implant specimens were tested to failure by a push-out test on an Instron Universal Test Machine (Model 4302, Instron, UK). The specimens were placed on a metal support jig with a 7.4 mm circular opening. A clearance between the implant surface and the support jig of at least 0.7mm has been recommended to get a uniform interface stress distribution[76]. A preload of 2 N was applied, to ensure contact with implant. The displacement rate was 5.0 mm/minute. (Figure 5)

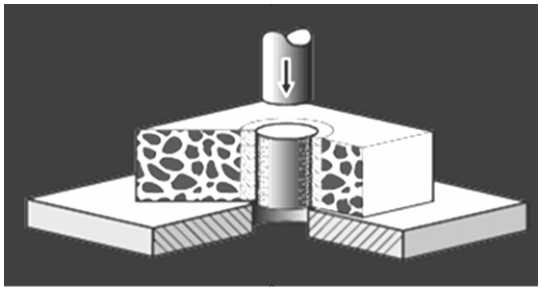


Figure 5: the mechanical push-out test.

The output from this test was load-displacement data (Figure 6). The surface area of the sections was used for the calculation of the mechanical parameters, to compensate for the variability in both length and diameter.

implant surface area (m^2) = π x implant diameter(m) x implant length(m)

This is the area measure of a smooth surface with such dimensions. As the implants were porous coated and thus had a larger surface area of unknown magnitude, the presented parameters are an overestimation of the true value. The mechanical parameters were calculated with the following formulas:

$$\text{shear strength in Pascal(Pa)} = \frac{\text{force(N)}}{\text{implant surface area } (\text{m}^2)}$$

$$\text{shear stiffness (MPa/mm)} = \frac{\Delta \text{ shear strength}}{\Delta \text{ displacement (mm)}}$$

$$\text{energy to failure}(\text{J/m}^2) = \frac{\sum(\Delta \text{ displacement (m) x shear strength})}{2}$$

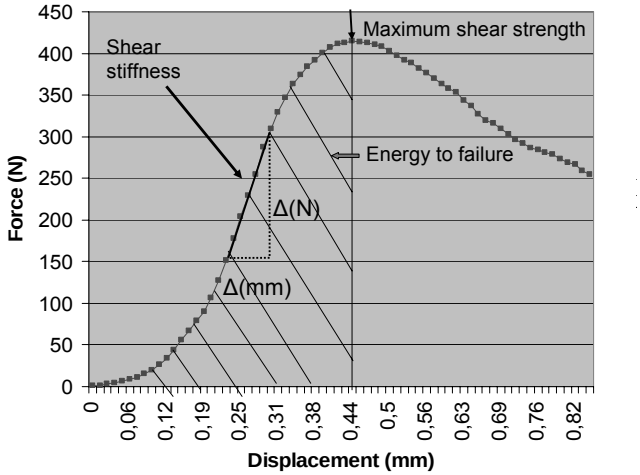


Figure 6: The load-displacement curve obtained with the mechanical push-out test. The parameters calculated from the data are illustrated in the figure.

Histomorphometry

Histomorphometry was performed in a blinded fashion using an image-analysis system (C.A.S.T-Grid; Olympus, Denmark). The quantification of tissue was performed applying stereological principles. Stereological methods are precise tools for obtaining quantitative information about three-dimensional, microscopic structures, based on observations made on sections. Two-dimensional sections contain quantitative information about three-dimensional structures in a statistical sense. For this statistical information to be "true" or unbiased a few requirements must be fulfilled about the sections and the way they are made. The tissue must intrinsically possess an identifiable directional (vertical) axis, or the observer must generate such a direction. The long axis of the implant will naturally be chosen for the cylindrical implants. The vertical sections must be parallel to the vertical, i.e. normal to the horizontal, and the vertical direction must be identified in each section. Relative to the common horizontal plane, the vertical sections must have random

positions and random orientations. This is obtained by random rotation of the specimen before sectioning (Figure 4). On the vertical section, a test line is given a weight proportional to the sine of the angle between the test line and the vertical direction [77]. By using this unbiased method of quantification, adequate precision is obtained with only three to four sections per specimen. This is because the biological variation is far greater than the variation in repeated sampling with this method [74]

Tissue ongrowth was defined as tissue in direct contact with the implant surface, and was determined using cycloid intercepting technique in study I. In study II and III we used line intercept technique to quantify the ongrowth. The number of intersections with tissue in contact with the implant surface was counted in successive adjacent fields at the tissue-implant interface.

The gaps were divided into inner and outer zones. Tissue volumes in the two zones were determined by point counting technique.

Data are presented as area percentage for the surface measurements, and volume percentage for the gap healing. This is calculated by dividing the number of hits per parameter (bone, fibrous tissue or marrow) with the total number of hits per implant. To achieve adequate precision, measuring a minimum of 100 hits per tissue of interest is recommended[74]. This was virtually impossible to achieve with the cycloid intercepting technique. The maximum number of cycloids per field of view often failed to produce the adequate number of hits, even in implants with decent amounts of the tissue of interest. As a consequence of this, the ongrowth in study II and III were quantified with line intercept technique. The line intercept technique is not principally perfect, like the cycloid intercept technique, in correcting for the anisotropy of the cylinder. The sine weight of the intercepting lines however, makes this method adequate for our analysis.

The staining technique used gives a fairly accurate estimate of the bone area or volume, as it is a surface staining coloring bone mineral green to a depth of a few micrometers. For the other tissues and cell types, quantification is not as accurate. Fibrous tissue is stained by the basic fuchsine, and thus stained through the entire thickness of the section (15-20 micrometers. This means that fibrous tissue will be overestimated compared with bone (Figure 7).

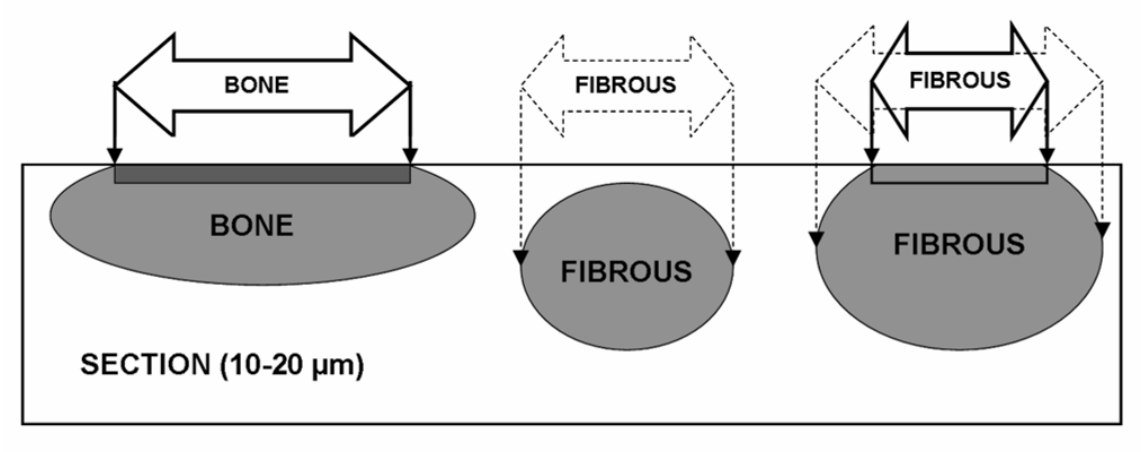


Figure7: Illustration of the overestimation of fibrous tissue. The solid lines and arrows represent the true value. The dotted lines and arrows represent the tissue overestimation. The bone is stained green in the surface of the section, while the fibrous tissue, as well as the bone under the surface remains red. The red bone will not be mistaken for fibrous tissue because of its easily recognizable structure, but it is practically impossible to differentiate which fibrous tissue is on the surface and which is below.

Sample size

The sample sizes of our studies (I-III) are based on the assumption that SD is 50% of the mean, and the clinically interesting difference (MIREDIF) is 50%. With an error of the first kind chosen to be 5% (2α) and the second kind 20% (β), we needed 8 animals in each study. In order to allow for exclusions due to animal deaths, mistakes in preparation of specimens and other unforeseen incidents, this number was increased.

Statistics

The data were tested for normality in a normal q-plot. The data that showed a normal distribution were analyzed with student's t-test for paired comparisons, and presented as mean and 95% confidence intervals or standard deviation. Data that did not follow a normal distribution were tested with Wilcoxons signed ranks matched pairs test, and presented as median and range.

Reproducibility

Double measurements were performed to assess intra-observer agreement. The measurements were performed months apart. The coefficient of variance (CV) was calculated for the double measurement, estimating the intraobserver variance with this formula:

$$CV = \frac{s}{\bar{x}} \quad \text{and} \quad S^2 = \frac{\sum_1^k d^2}{2k}$$

where k is the number of samples, d^2 is the sum of squared deviations and $\bar{x}$ is the mean of the sampled values [78].

The CV ranged from 0,03 to 0,06 for all double measurements of bone. The fibrous tissue double measurements had CV's from 0,19 to 0,33. A CV less than 0,10 is considered acceptable [79].

Choice of animal

There is a long tradition for using dogs at our institution. The dogs have several qualities that make them suited for this kind of research. They are just large enough to fit the implant models we use. They are skeletally mature at a relatively young age (approximately one year), and they have sufficient amounts of cancellous bone in the epiphyses to support our models. A study on bone composition of growth factors, density concluded that dogs' bone properties came closest to the human bone's properties.(monkeys were not included in this study)[80].

Ethical considerations

The studies were approved by the Danish Control Board for Animal research, and the Animal Care and Use Committee of the Minneapolis Medical Research Foundation. The purpose bred dogs were treated according to local rules and regulations.

In deciding to spend animal lives for research purposes it is important to be careful when designing a study. The study should produce new knowledge, and one should expect this new knowledge to be useful either in the understanding of a specific problem, or to help clarify the potential of the substance/subject investigated. A carefully designed and competently conducted animal study is justified in the opinion of this author.

Results

In study I, 8 of 9 treated implants had higher values in the mechanical parameters compared with their controls. The effect of the growth factors on fixation was relatively larger in animals were the controls were poorly fixated.

There was a 2.5 fold median raise in bone ongrowth, a 30 % median raise in inner zone gap healing, and a 2-fold median raise in outer zone gap healing. There was practically no fibrous tissue on the growth factor coated implants. There were no signs of residual polymer on the implant surfaces.

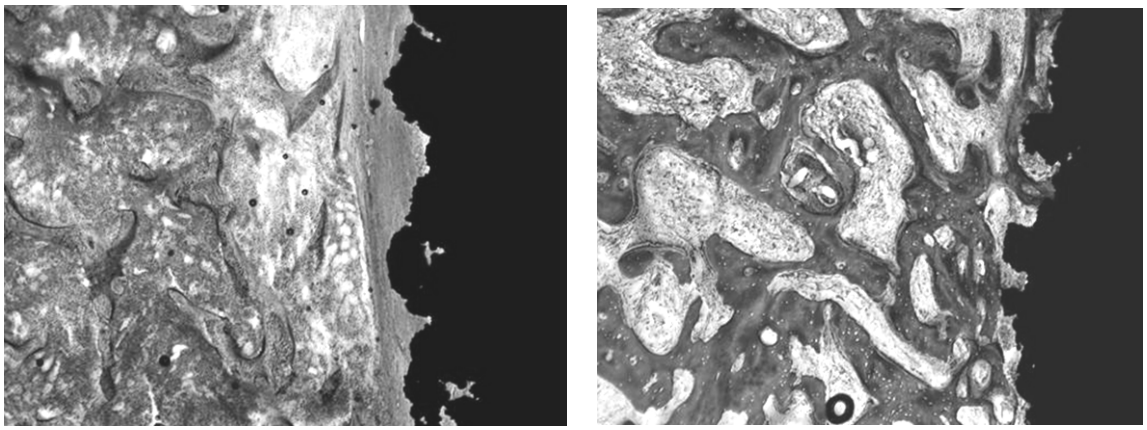


Figure 8: Images illustrating the results in study I: The image on the left is the control implant with scattered bone in the gap, and plenty fibrous tissue on the surface of the implant. The image to the right is a growth factor treated implant with plenty bone in the gap and on the implant surface, and no fibrous tissue.

In study II the mechanical push-out test found all implants to be well fixed with no statistically significant differences in any of the three mechanical parameters. In contrast, the histomorphometric analysis found differences in tissue distribution around the implants. Bone ongrowth to the implants was 3 fold higher for HA coated implants. There was fibrous tissue ongrowth in 4 of the 10 HA coated implants (up to 8%), and no fibrous tissue on the growth factor coated implants. In the inner half of the gap, the bone volume fraction was 26% higher in the growth factor coated implant group. In the outer half of the gap, the bone volume fraction was 28% higher in the growth factor coated implant. There were no signs of residual polymer on the implant surfaces.

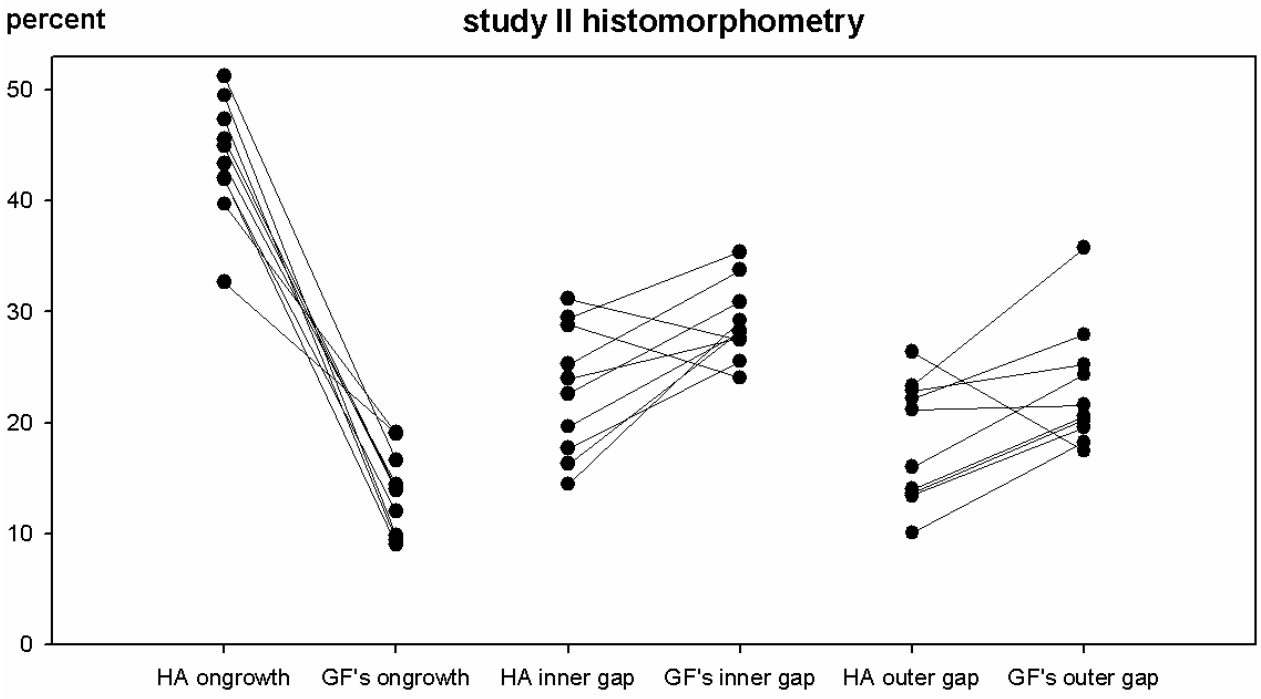


Figure 9: Graphs from study II; There was no difference in mechanical fixation despite the differences in osseointegration. Hydroxyapatite (HA) produced 3-fold more ongrowth, and the growth factors (GF's) produced more bone in the gap.

In study III the chitosan coated implants had significantly poorer mechanical fixation compared with the uncoated titanium control implants. Four of the ten chitosan coated implants failed to resist the preload of 2N in the push-out test.

The histomorphometric quantification of tissue supported the results of the push-out test. All chitosan coated implants were entirely covered by a thick fibrous membrane. This membrane also dominated the inner gap. Outside of the fibrous membrane there was almost 3-fold more bone around the chitosan implants than around the control implants. Fibrous tissue was present in the control implants, but in lesser amounts and with markedly larger variance than in the chitosan implants. Only scattered small fragments of the chitosan scaffold remained.

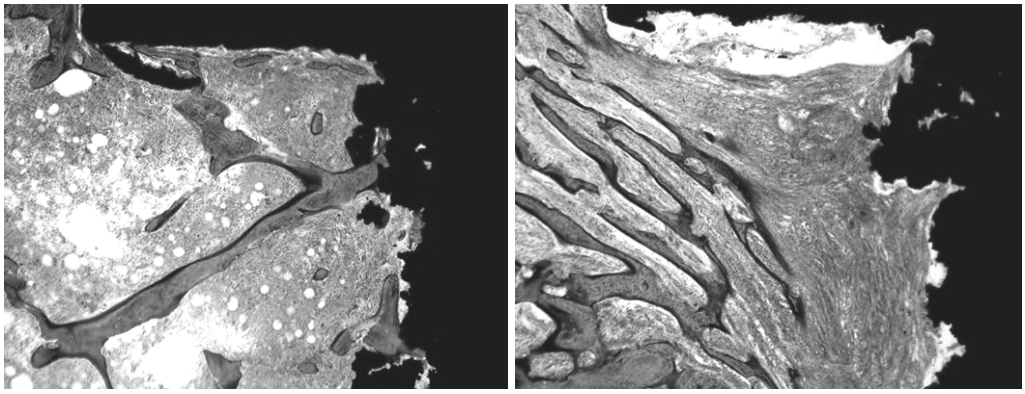


Figure 10: Images illustrating the results in study III: The image on the left is the control implant with scattered bone in the gap, the fibrous tissue not present in this implant. The image to the right is a Chitosan coated implant with a thick fibrous tissue membrane covering the implant surface. Outside of the fibrous tissue, there is an abundance of bone.

Discussion

The purpose of the studies presented in this thesis was to evaluate the effect of locally delivered TGF-β1 and IGF1 in poly(D,L-lactide) on the mechanical fixation and osseointegration of experimental implants, and to evaluate chitosan as an alternative biodegradable carrier for growth factor delivery conditions.

Growth factors

The combination of Transforming Growth Factor Beta one (TGF-β1) and Insulin-like Growth Factor one (IGF-1) in a biodegradable Poly(D,L-lactide) coating markedly increases the fixation and osseointegration of porous coated titanium implants. In study I the increase in fixation was markedly higher for those dogs whose control implant performed poorly, while the difference was modest in the group of dogs with well fixed control implants. In study II the graph showing the fixation indicates a smaller variance in data for the growth factor coated implants than for the hydroxyapatite implants (Fig 11). This cannot be shown statistically in our studies, but it seems that the variation in results is smaller in the growth factor treated implants than in both the porous coated and the hydroxyapatite controls.

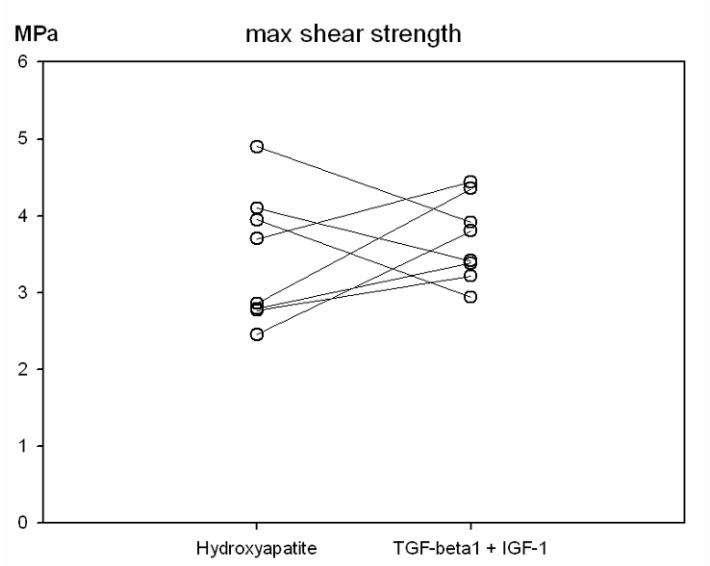


Figure 11: Graph showing data from the mechanical push-out test. IT illustrates the author's impression that the variance in data is smaller in the growth factor treated implants compared with both porous coating alone, and porous coating with hydroxyapatite on top.

The indications of lower variance by the growth factor treatment are accompanied by results obtained by other groups. A dose response study with TGF-β1 and IGF-11 in PDLLA was

performed in the sheep spinal fusion model. The best dose-response relationship was achieved with the medium growth factor dose (150 micro g IGF-I and 30 micro g TGF-beta1). This dose was obtained with the exact same concentrations of growth factors in the PDDLA as we used in our studies. With the double dose of these growth factors, no further stimulation of bone matrix formation was observed [81]. In a long term study in the rat fracture model, the short term healing is accelerated and enhanced by the growth factor combination. At the last time point, 84 days after fracture, no differences were measurable in the biomechanical testing and the callus composition between the groups [82]. It would seem that the combination of TGF-β1 and IGF-1 has an effect limited to where there is a need for stimulation of bone growth, and no excess bone resorption is caused by higher dosage. Should this prove to be the case, then it is in contrast with growth factors like BMP-2 and BMP-7 where excessive bone resorption and very poor initial fixation has been shown in some cases [32;33;83].

In study I and II there was a remarkable absence of fibrous tissue in the growth factor treated implants. The intra-observer agreement for the measurement of this tissue was higher than what is usually considered acceptable. This influences the interpretation of the data, as the only safe conclusion in study I and II is that there is fibrous tissue in the control groups, and none in the growth factor groups. We can not compare the amount of fibrous tissue in the control groups in our studies with corresponding controls in other studies, based on the specific data. In study III there is fibrous tissue in both the control and treatment group, but the difference is so gross that it is safe to conclude that there is much more fibrous tissue in the treatment group.

There were no clinical indications of systemic effects of the growth factors. We did not collect blood samples to investigate the systemic distribution of growth factors or polymer residues. It would have been desirable to have an indication of the level of circulating growth factors, as there are indications that there may be an association between dupuytren's contracture and certain cancer types, and high levels of TGF-β1 [84;85]. This should be investigated thoroughly before a decision on clinical trials is taken.

Comparison with HA

The hydroxyapatite produced a 3-fold higher ongrowth percentage than the growth factor combination. This result needs careful interpretation because the hydroyxyapatite coating process renders the actual surface area much smaller in the hydroxyapatite group (Figure 12).

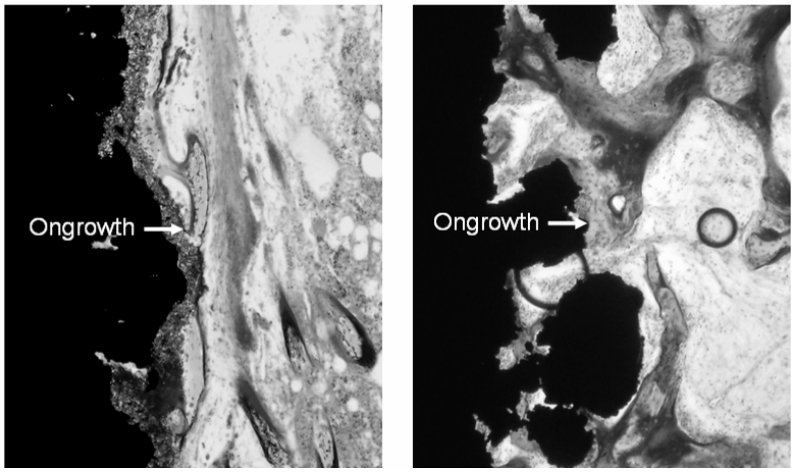


Figure 12: The hydroxyapatite coating (left image) fills the pores of the porous coating, rendering a smaller surface area for measurement of ongrowth.

Our data are calculated as area fractions, and are unbiased as such. However, a large part of the porous coated surface consists of pits and pores too small to hold a bone trabecula. If our methods allowed an estimation of the total area of bone coverage in square meters, the difference would probably be smaller. It is important to keep in mind that the fixation of an implant depends more on the connection between the surface bone and the trabeculae in the gap, rather than how much bone is situated on the surface of the implant. The measured mechanical fixation is a product of the weakest link in the chain of fixation.

The studies in this thesis do not answer the question of the long term effect of the treatments. It is widely accepted that an initial bony fixation prevents early loosening, as loosening is considered to be associated with high levels of fibrous tissue healing. It is possible that the hydroxyapatite coating renders a longer lasting protection against loosening as it stays on the surface; while the growth factors are gone in instant they have exerted their function. If microfractures were to occur around the prosthesis due to trauma or excess loading of the hip joint, it is likely that the hydroxyapatite coating protects the prosthesis from loosening by stimulation of bone growth even years after the implantation.

Polymer coating

The PDLLA coating seems well suited for this application. It retains the growth factors at the implant surface for an unknown period of time, without blocking the new bone's access to the implant surface. We observed scattered giant cells on the implant surface. Our method of quantification does not allow concluding on the amount of cells. This phenomenon has been

reported earlier, and is not thought to represent a problem [86;87]. In a safety study of the combination of TGF-β1 and IGF-1 in PDLLA in rats, no indication of a foreign body reaction due to the use of the polylactide or the growth factors was found.[88] Cyst formation at the site of implantation has been reported with bulky designed polymer implants[52;81]. In both study I and II there were strong indications that all of the PDLLA was gone at the end of the observation period. If there was any polymer left on the implants we would not have been able to see it, as it is dissolved in the methylmethacrylate used for the embedding process. However, there were no signs of leftover polymer on the implants in terms of empty zones on the implant surface. There was bone, fibrous tissue or bone marrow covering the entire implant surface. This is in contrast with rat fracture studies, where only 8% of the mass of PDLLA was gone after the observation period [51]. The in vitro and in vivo observed release kinetics from the rat trials thus do not seem to apply in implant studies in dogs. Considering that the rat implant is relatively larger than the dog implant compared with the host bone, it is likely that the observed difference is caused by the difference in relative amounts of polymer, and different impairment of blood supply in the two models.

The coating method where we dip the implants in a small container with the growth factors and PDLLA dissolved in ethyl acetate is crude, and there is probably some variation in the resulting coating thickness and thus amount of growth factors. The interesting issue of this problem is the fact that this coating method still yielded experimental results with lower variance than the controls we used. This suggests that the combination of TGF-β1 and IGF-1 has a large therapeutic range in terms of the dosage.

We only investigated one composition of PDLLA, the resomer 203, with a D and L ratio of 30/70, as this polymer composition has been used with success in studies on rat fractures and spinal fusion.[51;81;89]. It is probably a prerequisite for the implant fixation that the polymer is totally degraded and leaves room on the implant surface for the bone. However we do not know how fast this has to happen to secure the fixation of the implant. It is likely that a different release profile of the polymer would alter the results one way or another.

Chitosan

We decided to investigate chitosan as an alternative drug carrier based on its alleged properties as bone stimulating, antibacterial, anti-inflammatory, low cost and easily accessible.

We expected that a scaffold structure would enhance the osseointegration. Study III clearly showed that this is not the case with the scaffold we used. The chitosan or its degradation products

presented a barrier of osteoblast invasion and subsequent mineralization. This negative effect may be due to a rapid degradation of the chitosan in vivo. The resulting thick layer of fibrous tissue completely compromised the mechanical fixation of the implants. The morphology of the scaffold may not have been optimal for implant purposes. A pore size of minimum 100µm, preferably more than 300 µm has been suggested to be optimal[90]. It is possible to produce a scaffold with interconnected pores that allows fluid to reach the implant surface[91]. We do not have a characterization of our scaffold, but given the method of production we used, the pores were not likely to have been interconnected, thus no fluid could reach the implant surface initially.
Still there was a substantial increase of bone in the gap adjacent to the fibrous tissue layer compared with the gap around the control implants. This finding indicates that chitosan is bone stimulating as others have stated[53;54;90].
The porous coating of the implant surfaces vary in pore depth and diameter, thus the thickness of the chitosan scaffold varied from almost nothing to several hundred µm within the same implant. The polymer coating in study I and II was approximately 15-20µm thick. It is possible that a chitosan coating of similar dimensions would perform different than the scaffold did, stimulating bone growth while being degraded completely and not leaving a thick layer of fibrous tissue.

Conclusion

The combination of TGF-β1 and IGF-1 in PDLLA showed some very promising results in study I and II. The presence of more bone and absence of fibrous tissue around the growth factor treated implants provides far better conditions for the continued osseointegration of the implant. The mechanical fixation obtained with local release of TGF-β1 and IGF-1 is comparable to that of a hydroxyapatite coating. The mechanical fixation was achieved in different ways as the hydroxyapatite primarily stimulated bone ongrowth while TGF -β1 and IGF-1 significantly increased gap healing.

It is necessary to keep in mind, that hydroxyapatite has had a long and successful clinical implementation, while the growth factors in clinical use may yield unexpected side effects.

The PDLLA coating proved to be suitable as a carrier for the growth factors in these implant models.

A chitosan scaffold coated on titanium implants and surrounded by a gap had a significantly negative effect on the mechanical fixation. Chitosan promoted formation of a fibrous tissue membrane, In contrast a three fold increase in bone was observed in the gap. Chitosan applied in a thin layer may still be of value as a drug carrier, but this is subject to further investigation.

Future research

The results in study I and II suggest that controlled local growth factor release has potential to be used in clinical situation where a more general stimulation of bone is needed e.g. revision arthroplasties. Given their different target of effect, a combination of the two coatings may be an interesting subject for further investigation. Applying this growth factor coating to an implant implanted with a graft material is another intriguing possibility, as one might expect the growth factors to accelerate the remodelling of the graft without impairing the mechanical fixation while doing so.

The systemic distribution of the growth factors when they are coated on an implant surface should be investigated. Attention should be directed to potential side effects if this application is going to be tested clinically, as there are indications that TGF-β1 may influence the development of Dupuytrens contracture and some cancer types.

Chitosan as a drug delivery system for implants needs to be investigated further, perhaps with minuscule amounts of chitosan. If a scaffold of similar dimensions should be investigated, it may be beneficial to make the pores interconnected thus allowing flow of fluid to the implant surface. Lesser amounts of chitosan may still stimulate bone growth, without completely blocking the new bone's access to the implant surface.

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Appendix

Paper I (page 42-48): Locally delivered TGF-β1 and IGF-1 enhances the fixation of titanium implants. A study in dogs. (Acta Orthopaedica 2006; (77): 799-805)

Paper II (page 49-65): Combination of TGF-β1 and IGF-1 incorporated in a biodegradable poly(D,L-lactide) coating equals the fixation of hydroxyapatite coating. A paired study in dogs. (submitted to Acta Orthopaedica February 6th 2007)

Paper III (page 66-78): Chitosan scaffold coated on orthopedic implants in vivo. A study in dogs. (submitted to Acta Orthopaedica February 28th 2007)