Department of Clinical and Experimental Medicine,
Materials in Medicine,
Section of Orthopaedics and Sports Medicine,
Faculty of Health Sciences,
Linköping, Sweden

Following the mevalonate pathway to bone heal alley

Björn Skoglund

Linköping University

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Contact address

Björn Skoglund, AT-läkare
Centrum för Klinisk forskning
Centrallasarettet i Västerås
SE-721 89 Västerås
Sweden
E-mail: bjorn.skoglund@ltv.se

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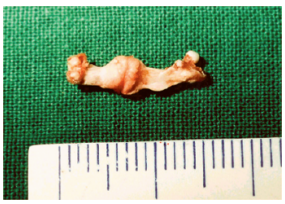
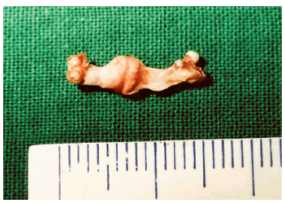
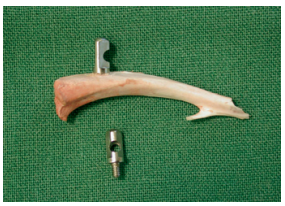
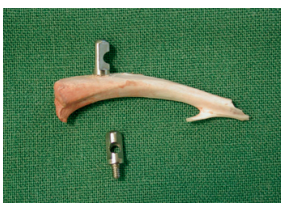
Thesis at a glance

Hypothesis	Species	Model	Hypothesis falcified	Comment
Simvastatin, a HMG-CoA reductase inhibitor, can improve fracture healing when given orally	Mouse	Long bone fracture 	No	Simvastatin, in sufficient doses, can enhance healing of long bone fractures
Simvastatin can be administered locally to improve fracture healing	Mouse	Long bone fracture 	No	Give simvastatin directly to the healing fracture
Ibandronate, a bisphosphonate, can improve early implant fixation	Rat	Tibial screw 	No	Bisphosphonates might enable the orthopaedic surgeon to improve implant fixation, also in healthy bone
N-bisphosphonates can succesfully be administered locally by coating the implant	Rat	Tibial screw 	No	Metal implants can be pre-treated with bisphosphonates

List of Papers

This thesis is based on the following papers:

I. Björn Skoglund, Carina Forslund and Per Aspenberg. Simvastatin improves fracture healing in mice. Journal of Bone and Mineral Research 2002; 17(11): 2004-2008.

II. Björn Skoglund and Per Aspenberg. Locally applied simvastatin improves fracture healing in mice. BMC Musculoskeletal Disorders, 2007; 8(1): 98 [Epub ahead of print].

III. Björn Skoglund, Jonas Holmertz and Per Aspenberg. Systemic and local ibandronate enhance screw fixation. Journal of Orthopaedic Research 2004; 22(5): 1108-1113.

IV. Pentti Tengvall, Björn Skoglund, Agneta Askendal, Per Aspenberg. Surface immobilized bisphosphonate improves stainless-steel screw fixation in rats. Biomaterials 2004; 25(11): 2133-2138.

Introduction

The mevalonate pathway

The mevalonate pathway is a crucial biosynthetic pathway, which can be found in all cells of virtually all known pro- as well as eukaryotic organisms. The end products are hydrophobic molecules involved in tasks such as cell maintenance, hormone regulation and protein anchoring (Righetti et al. 1976, Aguilera et al. 1984, Smit and Mushegian 2000, Takeda et al. 2001, Wanke et al. 2001, Begley et al. 2004, Schwartz et al. 2004, Rohdich et al. 2005, Dunford et al. 2006, Buhaescu and Izzedine 2007) (Figure 1). This thesis is an investigation into the use of two drugs, originally developed for different applications, but both affecting the mevalonate pathway, in two models of fracture repair.

Statins

Another end product of the pathway is cholesterol, a fact which led to the search for and subsequent development of the statins, or HMG-CoA reductase inhibitors, denoting the fact that they inhibit the rate limiting step in the pathway. First found in fungi of the genera penicillin (just as another important medical discovery), the statins were isolated, and later more potent synthetically produced analogues were developed (Endo 1988, 2004). Statins are, with the exception of pravastatin, administered in the form of lactone pro-drugs, which undergo cleavage and activation in the cells of the target organ, the liver. By acting on the rate-limiting step of the pathway, and by the fact that their primary target organ is the liver, less cholesterol is produced and so the hepatocytes upregulate the receptors for LDL-particles to recover cholesterol from the blood stream. The end result, then, is a lowering of total plasma cholesterol and most crucially a lowering of the colloquially named "bad" cholesterol LDL (Endo 1988).

Since these discoveries in the early seventies and the subsequent development of commercially available drugs in the eighties and nineties, the statins have gone on to become a widely prescribed and highly successful group of drugs, and is today the drug of choice for lowering cholesterol. It has also

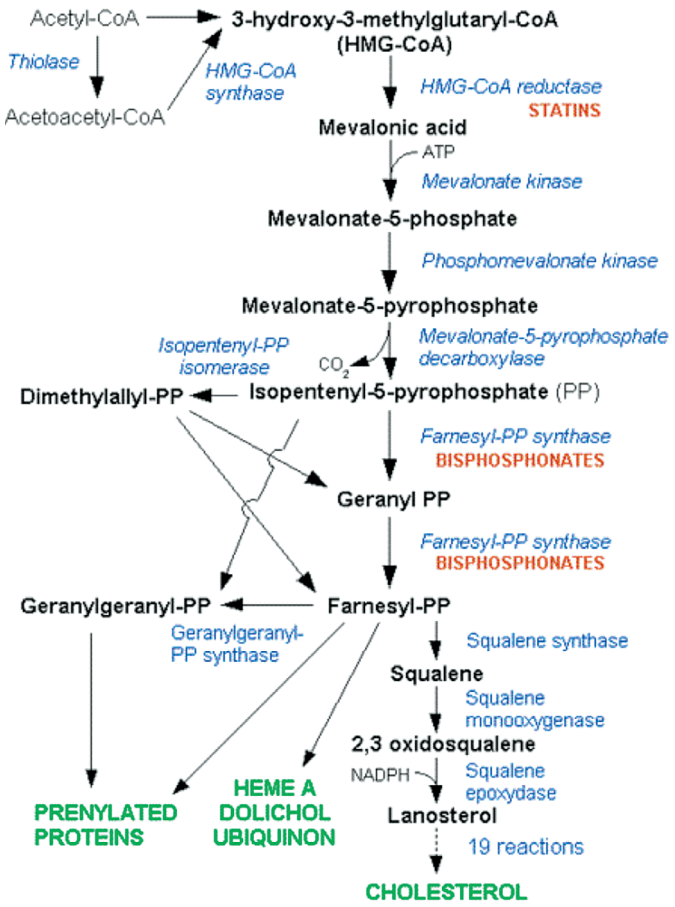


Figure 1. The mevalonate pathway. Adapted from Wikipedia.

been demonstrated that statins, and in particular simvastatin, exert beneficial effects beyond their ability of lowering cholesterol. For instance, in the 4S study, simvastatin was shown to be efficacious in secondary prevention of ischemic heart disease, an effect not satisfactorily accounted for by solely a lowering of cholesterol (Pedersen 1998, Gotto and Grundy 1999, Hay et al. 1999, Jonsson et al. 1999, Pedersen 1999, Velasco 1999, Forrester et al. 2000, Pedersen et al. 2000, Doggrell 2001, Tonkin 2001, Wilhelmsen et al. 2001, Liao 2002, Ong 2002, Pedersen and Tobert 2004). This and other findings have spurred research into what other effects statins can effectuate on the body. Since the mevalonate pathway is ubiquitous in nature and involved in so many crucial cellular reactions, it perhaps to no surprise that effects have been demonstrated in models ranging from angiogenesis, inflammatory reactions, neural glial cell development and neoplastic cell cultures to osteoporosis (Davignon and Leiter 2005, Ahmed et al. 2006; Campbell et al. 2006, Crisby 2006, de Souza Neto et al. 2006, Dirks and Jones 2006, Iwata et al. 2006, Poli and Pujia 2006, Ray et al. 2006, Steffens and Mach 2006, Weber et al. 2006, Acheampong et al. 2007, Akdim et al. 2007, Alegret and Silvestre 2007, Beaudry et al. 2007, Campese and Park 2007, Cimino et al. 2007, Devaraj et al. 2007, Ghittoni et al. 2007, Lee et al. 2007, Okuyama et al. 2007, Shirai et al. 2007, Tousoulis et al. 2007, von Tresckow et al. 2007). It is also well known that statins, although usually well tolerated, do carry with them the risk for the potentially lethal side effect of rhabdomyalysis, a breakdown of muscular tissue, especially when combined with other cholesterol lowering drugs, as demonstrated by the recent withdrawal of the newer and more potent drug cerivastatin from the market.

A complete review of all the effects that statins have been shown to elicit is beyond the scope of this thesis, which focuses on the effects on healing bone. The first evidence that statins could be beneficial to bone came in 1999, when Gregory Mundy et al. published a study in Science, which demonstrated that the statins lovastatin and simvastatin both in vitro and in vivo could stimulate bone matrix production by acting primarily on osteoblasts via the local growth factor BMP-2. Further, in an established model for non-steroid induced osteoporosis, orally administered simvastatin could halt and alleviate bone loss (Mundy et al. 1999). Since the effect Mundy and associates could see seemed to be the result of an enhanced transcription of BMP-2 and subsequent increase in proliferation and differentiation of osteoblast progenitors, in essence an anabolic effect on bone in a model for osteoporosis, the findings caused quite a bit of excitement. Osteoporosis is a major health care problem, at least in the developed world, afflicting millions and bringing with it much suffering and premature deaths by the associated fractures. To date, and with the exception of the hugely expensive recombinant human parathyroid hormone (rhPTH), there has been no really successful anabolic treatment for osteoporosis. This means that what physicians can offer to the majority of patients presenting with osteoporotic fractures, then often at an at least somewhat advanced stage, since the fracture had already occurred, is treatment to lessen the pace of the deterioration; in a word anti-catabolic drugs such as bisphosphonates (more on these later) and to some extent vitamin D3 and calcium supplements. Hence, the promise of a cheap drug, widely prescribed for a decade, and so well known and usually well tolerated, able to offer anabolic benefits was naturally greeted with enthusiasm by most. Not long after the study by Mundy, epidemiological studies into the effects on osteoporosis by statin treatment started to appear. The first studies showed promise, with several reporting a lower incidence of fractures. However, other studies have followed, many showing little or no effect on fracture incidence (Visseren et al. 2000, McFarlane et al. 2002, LaCroix et al. 2003, Gonyeau 2005, Rutishauser 2006, van der Sande et al. 2006, Uzzan et al. 2007). Likewise, when looking at bone turn-over markers, some have found evidence which point to an anabolic effect, others have demonstrated an effect more similar to that of bisphosphonates, and others still have failed to demonstrate any positive effect. Interestingly, and in perfect harmony with other mysterious findings about the effect of statins, a recently published study put forward the hypothesis that statins might affect people differently at different ages, with an effect on elderly osteoporotic patients, but not on younger patients (Braatvedt et al. 2004). Perhaps this could explain at least some of the confusion

concerning the clinical effect on bone of statins already available in the market. Nonetheless, the verdict is certainly still out and the issue will not be sufficiently answered without a properly conducted prospective study, which also has to take into account the findings of the experimental work. For instance, one of the statins, pravastatin, is a variant which is in its original form is hydrophilic, unlike the other statins which are highly hydrophobic and so can traverse the bi-lipid cell membrane of all cells with relative ease. Pravastatin, on the other hand has to be, and is, actively transported across the cell membrane of hepatocytes. Consistent with this, pravastatin has not been shown to be effective in in vitro or in vivo experiments on bone metabolism. Another issue, which has to be addressed, is the fact that orally administered statins to a large extent are confined to their target organ: the liver. The majority of what we ingest is digested and absorbed through the duodenum. The ingested material is then transported via the portal vein through the liver and out in the systemic circulation. In the case of the statins, the liver is very good at extracting the drug from the vein in question: As only about 5% of ingested simvastatin reaches the systemic circulation. Since the effect on bone seems to be a local phenomenon, with increased BMP-2 transcription and proliferation and differentiation of osteoblasts, or inhibition of osteoclasts, whichever the case might be, sufficient locally available levels would probably be difficult to achieve with oral treatment.

In the laboratory, the results have also been mixed. In vitro, the results seem reasonably clear, with most published studies pointing to an anabolic effect (Hoffman et al. 1983, Mundy et al. 1999, Garrett et al. 2001, Llevadot et al. 2001, Phillips et al. 2001, Garrett and Mundy 2002, Bauer 2003, Hatano et al. 2003, Staal et al. 2003, Hwang et al. 2004, Lee et al. 2004, Maeda et al. 2004, Whang et al. 2005, Benoit et al. 2006). Furthermore, the effect on bone turnover seems to be closely correlated with the effectiveness to inhibit the mevalonate pathway, and as of yet the most probable direct mechanism seems to be an increased transcription of BMP-2. However, the results have not been unanimous, and some researchers have not found effects consistent with this, but instead effects on neuroglial cell differentiation (Lee et al. 2004) and others have pointed to a mechanism not involved with BMP-transcription . In vivo, the effects are even more contradictory. Although there are studies which agree with the original findings of Mundy et al (Garrett et al. 2001, Oxlund et al. 2001, Sugiyama and Kawai 2001, Garrett and Mundy 2002, Kajinami et al. 2003, Staal et al. 2003, Wong and Rabie 2003, Kawane et al. 2004, Stein et al. 2005, Gutierrez et al. 2006, Garrett et al. 2007), some very conscientiously designed studies have not been able to demonstrate any such effects (Maritz et al. 2001, von Stechow et al. 2003, Yao et al. 2006).

At the time that I started work on what would become this thesis, all published studies had considered statins as drugs to be used in the treatment of osteoporosis, trying to prevent fractures from occurring in the first place. But what if you had a fracture to begin with? In relation to the present thesis, we read the study published by Mundy et al and the relatively few subsequently published studies in 1999 and 2000, and became interested to know how statins would affect another scenario of bone, the healing fracture. We hypothesized that, if statins affected bone growth in a scenario, with what I like to call (although not technically correct), “resting”, or “undisturbed” bone, then the effect could perhaps be similar but more pronounced in a state of frenetic activity. In a fractured bone in the reparative/ regenerative phases lots of osteoblasts and pre-osteoblasts are right there to be stimulated. We therefore administered oral simvastatin to rats in a model for bone ingrowth in an implanted bone conduction chamber previously used to study the effects of Osteogenic Protein-1 (OP-1), another local growth factor. With this experimental setup, we could not demonstrate any effect of the simvastatin. Disappointed, but not despondent, we decided to instead introduce the simvastatin to a fracture model we had developed to study the healing of long bones. We started by administering the simvastatin systemically and subsequently moved on to local administration. It is this model, described in subsequent sections and in the individual papers, which forms the basis for one of the thesis’ legs (this thesis being a bipod—metaphorically.)

Bisphosphonates

The other class of drugs, which has been the focus of this investigation is drugs developed and used for application on bone metabolism from the start. These are the bisphosphonates, organic analogues to inorganic pyrophosphates (P-O-P in chemical lingo) and were discovered to have biological effects by the group of Fleisch et al. in and around 1968. They are characterised by two P-C bonds, expressed in the P-C-P configuration for the bisphosphonates used clinically. The P-C-P basic structure is what gives the molecule a great affinity for the mineral moiety of bone, in essence the calcium ions, and means that the drug, systemically administered, will almost exclusively end up in areas of bone remodelling, and there affect preferentially osteoclasts (Rogers 2003). The drug does not have specificity to osteoclasts, but simply happens to be around in high concentrations in a way, which targets osteoclasts. Basically: if you administer a bisphosphonate systemically, it will end up at exposed bone and stay there for years until ingested by osteoclasts. In this way, it can be said to follow the good old orthopaedic maxim of "get to the bone and stay there". The biological effect, however, is associated with the side chains.

It is only recently that the cellular mechanism of the bisphosphonate effect has become elucidated. There seems to be two ways in which different bisphosphonates affect osteoclasts. The older group of drugs, called non-nitrogen containing bisphosphonates since they do not contain an amino side chain in one of the R-places, become metabolised into non-hydrolysable analogues of ATP. These then wreak havoc with the internal machinery of the osteoclast, eventually leading to a diminished function and eventual apoptosis of the cell. The newer, more potent, group of drugs is called nitrogen-containing bisphosphonates, because they do have an amino group. These drugs have been shown to inhibit the mevalonate pathway. They do so at a step further down than the previously mentioned statins (see Figure 1). This leads to an inhibition of protein prenylation and hence an eventual lack of such crucial proteins as GTPases Ras and Rho. The end result for the osteoclast seems to be the same, namely reduced performance and eventually programmed cell death. There has also been published evidence for an in vivo and in vitro effect on osteoblasts (Fromigue and Body 2002, Horie et al. 2003, Itoh et al. 2003). This effect is of increased survival and so the bisphosphonates would work differently on osteoblasts and osteoclasts. As of yet, this effect is still of unclear significance, but might be a consequence of different internal concentrations of either the pathway molecules or the drug itself. This is pure conjecture at this point and I expect it will so remain until further research has spread some light on the issue.

Interestingly, there have now been published results, which seem to point to the mevalonate pathway being involved also in the production of ATP-analogues. Either way, the bisphosphonates, which were used in this study, ibandronate and pamidronate, belong to the nitrogen-containing group of bisphosphonates.

So, with the exception of a few researchers, the consensus seems to be that at least the predominant in vivo effect of bisphosphonates is that of inducing apoptosis in osteoclasts. It was this effect which we were interested to see if we could use to our advantage when put into use in a model for screw fixation. This model, described in more detail in the next section, consists of drilling a hole in a rat tibia and then inserting a screw. After a specified amount of time you can then by various methods test the strength of the bone-implant module. This model can also be considered a model for fracture healing, and then a fracture healing in a stable environment, leading to the formation of bone by predominantly direct osteogenesis. After a trauma to bone, the injured bone needs to be replaced by new vital bone. It does this by the action of osteoclasts. However, it is possible that the osteoclast-osteoblast coupling which is normally very tightly and closely regulated for normal bone turnover, in this situation loses some of this closely regulated nature. It is even likely, we hypothesised, that bone resorption at least initially should outpace the laying down of new bone around an implant. If this were true, the introduction of an osteoclast inhibitor should improve initial fixation of an implant. The second part (or other leg) of this thesis, then, consists of experiments where we manipulate the mevalonate pathway by introducing bisphosphonates first systemically and then by local administration to achieve better bone strength.

Aims

The main aim of this thesis was to investigate the possibility of affecting the mevalonate pathway as a way of improving fracture healing. The specific aims, as relating to the individual papers were:

1. To see whether, by inhibiting the mevalonate pathway at the rate-limiting step, we could stimulate fracture healing of long bones.
2. To investigate the possibility of using local treatment to stimulate fracture healing of long bones.
3. To test whether we could improve upon early screw fixation by inhibiting the mevalonate pathway at the level of farnesyl-PP-synthase in osteoclasts.
4. To test if we could administer the bisphosphonates as a one time administration.
5. To test a novel way of coating implants with bisphosphonates and see if we could achieve improvement in this way.

Methods

The mouse fracture model (Papers I and II)

The fracture model we used in the papers I and II was developed to study the impact of various drugs on the healing of long bones. It is stabilised by an intramedullary pin and so is not rigidly fixed. This leads to a situation of a certain amount of instability and so healing occurs both by indirect and direct osteogenesis and with a sizeable callus.

Surgical procedure

All surgical equipment was sterilized in an autoclave. Sterile gowns, gloves, surgical masks and theatre caps were used. The leg on the operated side was shaved and the entire mouse was placed inside a sterile surgical glove. Subsequently, a hole was cut out of the glove and the leg pulled out through the hole using tweezers. The foot was then clothed in sterile adhesive plastic and the leg washed with chloro-hexidine. A lateral incision was made along the distal femur. The patella was dislocated medially with the blunt side of the scalpel, so that the femoral condyles were exposed. Using an intercondylar approach, a hole was drilled through the medullary canal of the femur using a cannula (diameter 0.4 mm). A wider cannula (0.6 mm) was then inserted into the canal made. The sharp end of the cannula was blunted in order not to penetrate through to the hip. The cannula was cut off at the other end, so that the remaining bit was inside the bone and no end extended beyond the bone. A specially made pair of scissors with semi-lunar cutting edges was then slid along the bone to mid-diaphysis and the femur cut to produce a fracture (Figure 2). Finally, the patella was repositioned over the knee-joint and the muscles and skin sutured separately.

Simvastatin administration

For paper I, the simvastatin was administered mixed in the feed, the diet consumed was measured every 3 days, and the mice weighed at surgery and at time of death, so that an average dose of simvastatin per mouse could be established. For paper II, simvastatin was administered in three different ways; daily systemic subcutaneous injections, subcutaneously implanted mini-pumps yielding a continous systemic release, and finally a silicone tube was subcutaneously led from implanted osmotic mini-pump to the fracture area, in this way achieving continuous local treatment.

Evaluation

The mice were killed at 8, 14 or 21 days in the case of paper I, and 14 days for paper II. At these time points, bilateral femurs were harvested, the marrow-nail extracted and the maximal sagittal and transverse diameters of the callus and the mid-

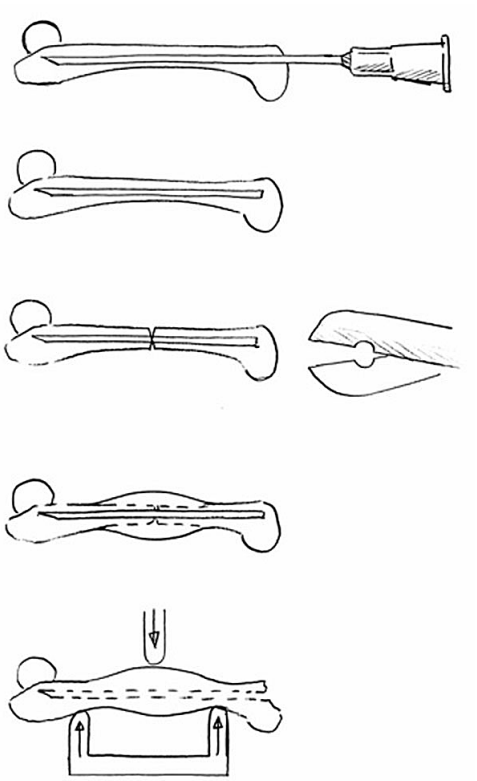


Figure 2. Overview of experimental set-up. After insertion of the intramedullary cannula a specially made pair of scissors with semi-lunar cutting edges was slid along the bone to approximately mid-diaphysis and the femur cut.

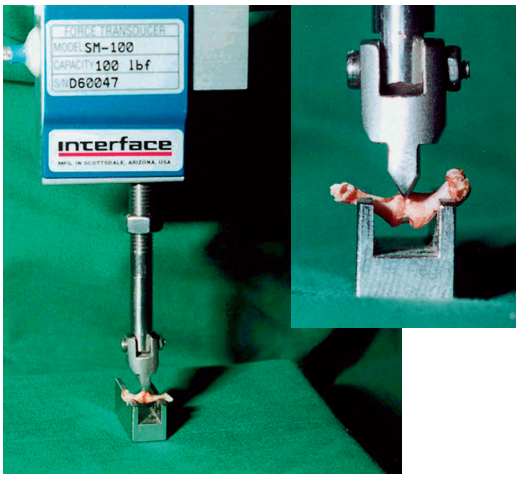


Figure 3. The materials testing machine. Photographs taken at evaluation to illustrate the three-point bending.

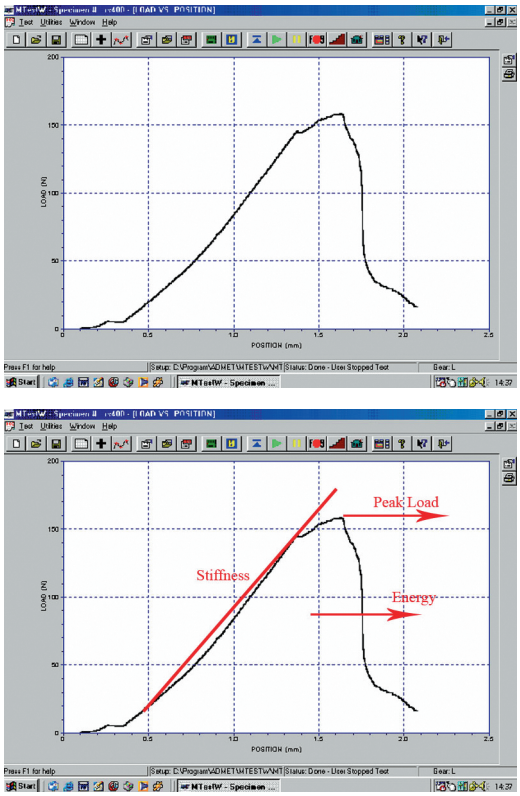
diaphysis of the unoperated femurs were measured with a digital calliper.

Biomechanical evaluation

The harvested femurs were put in a custom made cradle which yielded a beam length of 6 mm and subjected to a sagittally applied bending force, achieved by a materials testing machine connected to a computer (100R, DDL Inc., Eden Praire, Mn, USA, Figure 3).

The result was a load-deformation curve plotted in real time on a computer screen (Figure 4). From this load-deformation curve was derived the analysed biomechanical parameters of force at failure, energy at 10% droop of the curve and stiffness, from which one can calculate the modulus of elasticity for the femur fracture model. Briefly, force at failure represented the maximum measured force on the load-deformation curve expressed in Newtons, energy represented the integral of the curve (N/mm) and stiffness the slope (Nmm) (Figure 4). The modulus of elasticity, or Young's modulus, represents an object's tendency to deform along an axis when subjected to forces along that axis and is defined as the stress to strain ratio. For our experiments, the value for the modulus mostly reflects the material properties near the periosteal surface.

To arrive at Young's modulus, the callus and unbroken femurs were approximated to ellipses and stiffness incorporated with the diameter measurements, using the formula



Figures 4. The load-deformation curve was plotted in real time (upper) and then used to evaluate the biomechanical properties (lower).

$$e = \frac{\Delta F \times L^3 \times 64}{\Delta f \times \pi \times B \times H^3}$$

where ΔF/Δf was stiffness, L equals the beam length, B the breadth and H the height of the specimen.

Biomechanical evaluation was performed in the above manner for the experiments in papers I and II. Paper I also attempted to evaluate the effect of simvastatin histologically.

Histological evaluation

Histological sections were cut parallell to the long axis of the callus, prepared using standard techniques and stained with hematoxylin and eosin. All measurements were blinded as to treatment and time, and the histology was evaluated in a light microscope at 40 times magnification. The slides were scanned for cartilage and bone in the overbridging callus. In an attempt to evaluate the stage of healing in the callus, an arbitrary grading

Table 1. Arbitrary scaling system for histological analysis

Histological appearance of callus	Grade
No cartilage	1
Some cartilage	2
Mostly cartilage, no bone	3
Cartilage and bone	4
Bone only	5

system was used, in which the tissue in the fracture gap was graded according to prevalence of fibrous tissue, cartilage and bone (Table 1).

Statistical analysis

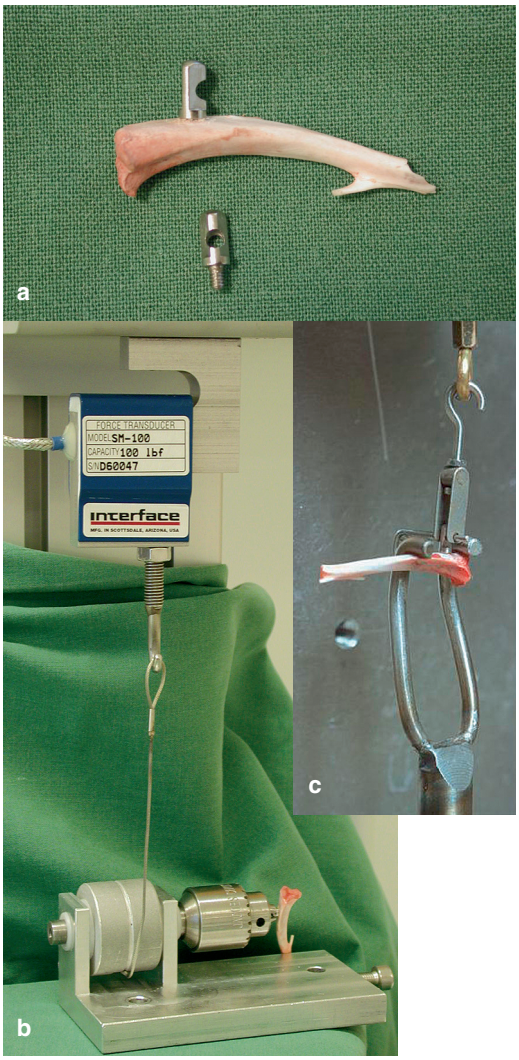
In paper I, the biomechanical data was analysed by ANOVA followed by Scheffés post-hoc analysis for comparison between groups. Histological data was analysed using Mann-Whitney's U-test. For biomechanical data of paper II, Student's unpaired t-test was used.

The rat screw model (Papers III and IV)

The rat screw model was initially developed to evaluate the in vivo effect of intermittent PTH injections (Skripitz and Aspenberg 2001a and 2001b). In this setup, you drill a hole in the proximal tibia and insert a stainless steel screw. Since the healing bone is in an essentially unloaded or stable environment, the healing which ensues around the implant is mainly achieved by direct osteogenesis, and so can be said to be both a model for implant fixation and also to a certain degree a model for the healing of a rigidly fixed fracture.

The screw

Custom made stainless steel screws, with threads measuring 1.7 mm in diameter (type M 1.7) and 3 mm in length were used (Figure 5a). The screws had a hole in the head, so that they could be fastened to a hook suspended in the materials testing machine mentioned above (Figure 5b). Before autoclave sterilisation, the screws were cleaned in ultrasonic baths with formic acid and sodium formiate followed by trichloroethylene, and finally 99% alcohol.



Figures 5. The stainless steel screws (a) and test setup (b). Like with the femur fracture experiments, the screws were fastened to the materials testing machine (b and c) and a load-deformation curve plotted.

Surgical procedure

Implants and surgical equipment were sterilized in an autoclave. Sterile gowns, gloves, surgical masks and theatre caps were used. The rat leg on the operated side was shaved and the entire rat was put into a sterile surgical glove. Subsequently, a hole was cut out of the glove and the leg pulled out through the hole using tweezers. The foot was then clothed in sterile adhesive plastic and the leg washed with chloro-hexidine alcohol.

The screws were inserted in the predominantly cancellous bone of the proximal tibial metaphy-

ses approximately 3 mm from the physes for the systemically treated rats. In the experiments with local treatment, the screws were inserted further distally in predominantly cortical bone. The reasoning behind this particular placement was that we presumed that with local treatment there would be a larger bone area available for treatment when inserted in compact bone. With the proximal position of the screws, the fixation is more dependant on the surrounding cancellous bone, which is easy to treat systemically, but more difficult to reach with local treatment. For both placements, a 5–6 mm longitudinal incision was made along the medial aspect of the rat tibia. The periosteum was reflected proximally to the physis. Then, a hole with a diameter of 1.2 mm and length of 2 mm was drilled through one of the cortices with a custom made hand held drill and the screw inserted. Finally, the skin was sutured over the screw.

Bisphosphonate administration

For paper III, ibandronate was administered in two ways; by daily subcutaneous injections and by applying it directly to the drilled insertion hole at time of surgery. For paper IV, the bisphosphonates pamidronate and ibandronate were administered by surface immobilisation in collaboration with Professor Pentti Tengvall at The Section for Applied Physics at Linköping University, more closely related in the following section.

Surface immobilisation

First, the surface was roughened by etching in hydrogen fluoride. Next, the surface was washed in a hydrogen peroxide solution and prepared by baking it in a silane solution. The screws were then cleaned in xylene and incubated in glutardialdehyde, after which they were rinsed in Tris buffer. Subsequently, a matrix consisting of ten layers of covalently bound fibrinogen was coated to the screws. Finally, into this matrix two amino-bisphosphonates were bound, namely pamidronate and ibandronate. Pamidronate was immobilized to the fibrinogen matrix using peptide bonds, while ibandronate was spontaneously adsorbed and so much more loosely associated to the matrix, presumably leading to a much quicker release.

Evaluation

The rats were killed at 14 days post-operative and the tibiae harvested. Evaluation was performed by biomechanical measurements and histology in paper III and biomechanical measurements only in paper IV.

Biomechanical evaluation

In paper III, biomechanical evaluation was performed in two ways. For systemically treated tibiae, the screws were pulled out and a load-deformation curve plotted in real time on a computer screen as above. The biomechanical data thus collected were force at failure, stiffness and energy at 10% droop of the curve. Locally treated tibiae were either subjected to pull-out strength measurements as above, or to torque measurements. These latter were performed using a specially constructed device. The data thus collected were processed using the formulas

Torque moment at failure = force (N) × radius of device (r)

Maximum torque moment = force (N) × radius of device (r)

The stiffness derived from the linear curve (N/mm) was transformed to delta torque moment per angular degree = [slope of curve (N/mm) × r (mm)] /29 (mm^{-1}) where 29 is the angle corresponding to 1 mm excursion.

In this way, we arrived at torque-moment at failure, defined as the moment at which the moment deformation curve reached its first peak, stiffness per angular degree of the elastic slope, maximum friction torque-moment, defined as the highest moment measurements recorded after the screws had started to turn and energy, defined as the area under the curve for one quarter of a revolution.

In paper IV, the biomechanical evaluation was performed by measuring pull-out strength.

Histological evaluation

Specimens were sectioned parallel to the axis of the screw through the middle of the hole, and stained with haematoxylin and eosin. Evaluation was performed in two ways. Firstly, the specimens were analysed using a computerised video system

attached to a light microscope at a 25 × 1.25 magnification. The evaluation criterion for this analysis was bone-implant contact and was done by measuring the length of bone contact, divided by the total length in contact with the threads. In a second analysis, Merz's grid was used to evaluate the density of the bone in contact with the threads. The grid was laid over the thread contours at a magnification which yielded a grid size of 0.17 × 0.17 mm and points counted. Then, the number of points at bone was divided by the total possible points. 4 grids were used for each histological slide.

Statistical analysis

In papers III and IV, data was analysed with Student's unpaired t-test.

Summary of Papers

Paper I: Simvastatin improves fracture healing in mice

Can we improve the healing of a long bone fracture by oral treatment with simvastatin?

Femur fractures were produced in 81 mature male Balb-C mice and stabilized with marrow-nailing. 41 mice were given a diet prepared with simvastatin, so that each mouse received an approximate dose of 120 mg per kg of body weight per day. The remaining mice received the same diet with the exception of the simvastatin. Bilateral femurs were harvested at 8, 14 and 21 days post-operative, the marrow-nail extracted and diameters measured. Biomechanical tests were performed on 36 mice, by way of three-point bending. Histological specimens were prepared using standard techniques.

At 8 days, the fracture callus was too soft for meaningful biomechanical testing. At 14 days, the callus of the simvastatin treated mice had a 53% larger transverse area than controls (p=0.001), the force required to break the bone was 63% greater (p=0.001) and the energy uptake was increased by 150% (p=0.0008) (Table 2). Stiffness and modulus of elasticity were not significantly affected. At 21 days, the fractures were histologically healed and the mechanical differences had disappeared. The contra-lateral unbroken bone showed a slight increase in transverse area as a result of the simvastatin treatment, but no significant effect on the force required to break the bone, or on energy uptake (Table 3). No significant difference could be demonstrated as regards to maturity in the histological specimens (Table 4).

Table 2. Three-point bending (beam length 6 mm) of healing mice femur fractures treated with systemic simvastatin

Treatment	Days	n	Transverse area (mm^2)		Force at failure (N)		Energy uptake (Nmm)	
			mean	SD	mean	SD	mean	SD
Simvastatin	14	12	15	1.8	7.3	1.6	5.7	3.9
Control	14	11	10	1.9	4.8	0.9	2.2	1.0
Simvastatin	21	9	7.3	0.8	7.9	1.2	2.2	0.7
Control	21	10	6.4	1.5	8.4	2.3	2.2	0.8

Table 3. Three-point bending (beam length 6 mm) of undisturbed contralateral femur treated with simvastatin

Treatment	Days	n	Transverse area (mm^2)		Force at failure (N)		Energy uptake (Nmm)		Modulus of elasticity (Pa x 10^5)	
			mean	SD	mean	SD	mean	SD	mean	SD
Simvastatin	14	10	1.70	0.20	14.46	1.24	3.46	0.92	1.21	0.43
Control	14	9	1.23	0.45	16.62	2.29	4.03	1.92	2.46	0.55
Simvastatin	21	10	1.55	0.06	16.74	1.62	3.05	0.81	1.16	0.34
Control	21	10	1.63	0.14	16.89	1.37	4.12	1.04	1.14	0.25

Table 4. Arbitrary histological grading of healing callus

Treatment	Grade 1	2	3	4	5
Simvastatin 8 days	2	3			
Control 8 days	3	1			
Simvastatin 14 days		6	4	3	
Control 14 days		3	2	8	
Simvastatin 21 days					8
Control 21 days					10

Paper II: Locally applied simvastatin improves fracture healing in mice

Can we bypass the liver and achieve a positive effect by local administration of the simvastatin?

Femur fractures were produced in 70 mature male Balb-C mice and stabilized with marrow-nailing. 20 mice received daily subcutaneous injections of either simvastatin (20 mg/kg) or vehicle. 30 mice were divided into three groups of 10 mice receiving continuous subcutaneous delivery of the vehicle substance, the vehicle with 5 mg or with 10 mg of simvastatin per kg bodyweight per day. Finally, 20 mice received the simvastatin locally via a silicone tube led from an osmotic mini-pump to the fracture area. In this way, 10 mice received an approximate local dose of simvastatin of 0.1 mg per kg per day for the duration of the experiment and 10 mice received the vehicle compound. All treatments lasted until the end of the experiment. Bilateral femurs were harvested 14 days post-operative. Biomechanical tests were performed by way of three-point bending.

With daily simvastatin injections, no effects could be demonstrated for any of the parameters examined. Continuous systemic delivery resulted in a 160% increase in force at failure (Table 5). Continuous local delivery of simvastatin resulted in a 150% increase in the force at failure as well as a 200% increase of energy uptake (Table 6).

Table 5. Three-point bending (beam length 6 mm) of healing mice femur fractures at 14 days treated with continuous local administration of simvastatin

Treatment	n	Force at failure (F) mean	SD	Energy uptake (Nmm) mean	SD	Area (mm^2) mean	SD	Young's modulus (MPa) mean	SD
Simvastatin	9	7.2	2.0	3.3	1.0	11.2	1.7	635.7	647.1
Control	10	4.5	1.3	1.7	0.9	10.4	1.2	278.4	130.8
P-value		0.003		0.002		0.24		0.1	

Table 6. Three-point bending (beam length 6 mm) of healing mice femur fractures at 14 days treated with continuous subcutaneous administration of simvastatin

Treatment	n	Force at failure (F) mean	SD	Energy uptake (Nmm) mean	SD	Area (mm^2) mean	SD	Young's modulus (MPa) mean	SD
Simvastatin									
5 mg	8	5.1	1.5	2.4	0.9	11.2	1.5	201.8	128.8
10 mg	8	4.1	1.4	1.7	0.7	10.9	1.7	231.3	221.2
Control	10	3.2	1.6	1.9	1.1	9.8	2.3	163.5	67.3
P-value [a]		0.04		0.6		0.3		0.9	

[a] Signifies the p value for Scheffe's post-hoc test when comparing simvastatin 5 mg with controls. ANOVA showed a significant difference between groups (p = 0.04)

Table 7. Systemic treatment, pull-out

Mechanical data				Percent increase by ibandronate (95%CI)			
Treatment	n	mean	SD	min	mean	max	P-value
Force at failure (N)							
Control	7	53	10				
Ibandronate	8	69	15	6	30	55	0.04
Stiffness (N/mm)							
Control	7	27	10				
Ibandronate	8	44	15	0.2	63	124	0.07
Energy (Nmm)							
Control	7	33	9				
Ibandronate	8	37	8	-15	11	38	0.45

Table 8. Local treatment, pull-out

Mechanical data				Percent increase by ibandronate (95%CI)			
Treatment	n	mean	SD	min	mean	max	P-value
Force at failure (N)							
Control	9	68	4				
Ibandronate	9	79	10	4	15	26	0.02
Stiffness (N/mm)							
Control	9	59	12				
Ibandronate	9	76	12	9	28	46	0.01
Energy (Nmm)							
Control	9	47	6				
Ibandronate	9	46	8	-15	-1	13	0.90

Paper III: Systemic and local ibandronate enhance screw fixation

Can we improve initial screw fixation in a rat model by repeated systemic as well as a one time local treatment with a bisphosphonate?

Stainless steel screws (M 1.7) were inserted into the tibiae of 76 male Sprague-Dawley rats. Daily subcutaneous injections of ibandronate (3 µg) or saline were given to 20 rats. The remaining rats received ibandronate or saline directly applied into the drill hole before the screw was inserted. Tibiae were harvested at 14 days. Mechanical tests were performed on 50 tibiae. Systemically treated tibiae were tested for pull-out strength alone. Locally treated tibiae were tested for either pull-out or torque resistance. The remaining 18 tibiae were prepared for histology.

Systemic ibandronate increased the pull-out force at failure by 30% (p=0.04, Table 7). Local treatment increased the force at failure by 15% (p=0.02) and stiffness by 28% (p= 0.01, Table 8). In the removal torque measurements, local ibandronate increased the torque-moment at failure by 60% (p=0.04), and the maximum friction moment by 51% (p=0.04). Energy for turning the screw ¼ revolution was increased by 68% (p= 0.02, Table 9).

Paper IV: Surface immobilized bisphosphonate improves stainless steel screw fixation in rats

Can we improve initial screw fixation in a rat model by applying bisphosphonates directly coated onto the screws?

Screws were roughened and coated with immobilized and cross-linked fibrinogen. Subsequently, the bisphosphonates pamidronate and ibandronate were attached to this matrix. The so coated screws were

Table 9. Local treatment, torque

Mechanical data				Percent increase by ibandronate (95%CI)			
Treatment	n	mean	SD	min	mean	max	P-value
Torque moment at failure (Nmm)							
Control	8	23	12				
Ibandronate	9	37	13	7	56	105	0.04
Stiffness (Nmm/angular degree)							
Control	8	391	207				
Ibandronate	9	481	207	-27	23	73	0.38
Maximum friction torque moment (Nmm)							
Control	8	27	12				
Ibandronate	9	41	13	2	47	92	0.04
Energy at 1/4 revolution (Nmm)							
Control	8	15	7				
Ibandronate	9	25	8	23	68	114	0.02

then inserted into the tibiae of eighteen male Sprague-Dawley rats. Tibiae were harvested at 14 days and pull-out strength mechanical tests performed.

Pull-out force at failure was 28% higher in the bisphosphonate coated screws compared to controls. Pull-out energy was 90% higher.

Discussion

It seems to me that at least some parts of the orthopaedic community have regarded common orthopaedic problems with too much of the constructional engineer's perspective, reducing issues of fracture repair and implant fixation to matters of the best nut-and-bolt design, if you will. Although important and highly productive, the drawback of this perspective is that it rarely incorporates the hugely dynamic aspect of the bone and, more importantly, does not factor in the cells which create the plasticity. Another approach, one which has been taken in the work on this thesis, is what might be called the applied biologists viewpoint (in analogy to the applied physicist). If we can try to take what we have learned of bone cell interaction and signalling pathways in the Petri dish and apply it to living systems, the yield is potentially dramatic. Basically, do not think of the fractured femur on the operating table as you would two pieces of wood to be nailed together. Rather, think of it as a twig which has broken and which we must try to help grow back together with a little encouragement. This encouragement would be the combined effects of initial mechanical support (e.g. some sort of implant fixator material) and biological factors which induce and strengthen the healing response.

Statins are cheap and in our experiments highly effective in promoting bone repair processes. Considering how ubiquitous and apparently crucial the mevalonate pathway seems to be, it is perhaps not surprising that we may see an effect when we introduce drugs which interfere with it. The example of statins, which inhibit the pathway at the rate limiting step, illustrate this point with all desired clarity, where it seems the more you look, the more effects can be demonstrated. Indeed, the process of prenylation, where proteins gain a hydrophobic moiety, seems to be so crucial for many cellular functions, that the more research which you read about it, the more apprehensive you may well become of using such a drug, although we saw few adverse side effects in our experiments. As we have seen, statins do carry with them the potential for serious side effects, and it seems a little like chasing flies with a shotgun. Therefore, the idea of preferentially affecting the cells in the area which we want to affect by local treatment is an attractive one. By using local treatment we were able to reduce the total dose by three orders of magnitude. The resultant negligible systemic levels bring us one step closer to the feasibility of using statins to improve fracture healing in a clinical setting. A natural next step would be to deliver the simvastatin coated to the implants, as we did with bisphosphonates At least you fire the shotgun closer in towards your target, hitting fewer innocent bystanders.

Bisphosphonates are attractive in their ability to attach to bone and stay there. Moreover, and possibly because of this, they do not seem to be associated with the same potential risk of serious adverse effects. The main in vivo and clinical effect which has been clearly demonstrated for bisphosphonates is anti-resorptive, and so their use in environments where you already have a bone matrix-deficient situation might be limited.

At this point, there remains much to be made clear. For instance, the optimum dosing regimen for both drugs is something which needs to be cleared out. Further, treatment length is an unexplored area. The work of Mundy et al has indicated that a treatment length of statins for three days is sufficient to see effects long after (months).

The overriding aim of this thesis has been to investigate the potential for these two drugs to stimulate fracture repair. It seems clear that both drugs have uses in orthopaedic applications. One interesting avenue of further research would be to combine the two drugs and see if we can get the benefits while at the same time diminishing the drawbacks.

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