Natural coral and hydroxyapatite as bone substitutes

An experimental and clinical study

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Academic dissertation

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LIST OF ORIGINAL PUBLICATIONS

The present study is based on the following articles, which will be referred to in the text by their Roman numerals.

I Vuola J, Göransson H, Böhling T, Asko-Seljavaara S.
Bone marrow induced osteogenesis in hydroxyapatite and calcium carbonate implants. Biomaterials **17**(18):1761-6, 1996.

II Vuola J, Taurio R, Göransson H, Asko-Seljavaara S.
Compressive strength of calcium carbonate and hydroxyapatite implants after bone-marrow-induced osteogenesis. Biomaterials **19**(1-3):223-7, 1998.

III Vuola J, Böhling T, Göransson H, Puolakkainen P.
TGF-ß1 released from natural coral implant enhances bone growth at calvarium of mature rat. Submitted.

IV Vuola J, Böhling T, Kinnunen J, Hirvensalo E, Asko-Seljavaara S.
Natural coral as bone defect filling material. J Biomed Mater Res **51**(1):117-22, 2000.

ABBREVIATIONS

BMP	bone morphogenetic protein
CC (NC)	calcium carbonate (natural coral)
CSD	critical size defect
GIA	growth inhibition assay
HA	hydroxyapatite
TCP	tricalciumphosphate
TGF-ß	transforming growth factor beta

INTRODUCTION

The development of new methods in trauma and reconstructive surgery has created new problems. Large traumatic bone defects can be covered by soft tissues but reconstruction of the bone itself may be difficult. The use of autogenous bone has remained the golden standard in restoring bone defects, but it is not always possible to obtain enough bone. The patient may have undergone previous bone grafting procedures and thus have poor donorsites or the amount of bone needed may exceed that available. In addition, harvesting is always a secondary operation and implies a certain amount of morbidity (Banwart et al. 1995).

To avoid these problems, allograft bone can be stored in bone banks for later use. Unfortunately this does not solve all the problems, as there is always a risk of being transmitted by viruses or bacteria, when allograft bone is used (Khan et al. 1998). Moreover, the immunogenic responses may impair the results (Aho et al. 1998).

Biomaterials may provide a solution to these problems. An ideal bone substitute should be tolerated by the host tissue without any adverse reaction; it should promote bone formation, have appropriate mechanical strength, be malleable and resorb after it has fullfilled its function. Porous calcium ceramics have proved to be biocompatible bone substitutes. The material most investigated in this group is hydroxyapatite (HA). It can be manufactured from natural reef building coral skeleton by a hydrothermal exchange reaction, where the trabecular, bone imitating structure of the coral remains unchanged and the calcium carbonate (CC) skeleton is converted to calcium phosphate, the main inorganic salt of bone (Roy and Linnehan 1974).

The original coral skeleton, consisting of CC, can also serve as bone substitute. The main difference between these structurally identical materials is that biodegradation takes place much more slowly with HA than with CC. Biodegradation should not occur before the implant has filled with bone. Very short resorption times, even a few weeks, have been reported for CC (Guillemin et al. 1989). This accentuates the importance of fast bone ingrowth into the implant.

However, both HA and CC lack the capacity to induce bone growth. Adding bone marrow to porous calcium ceramics induces bone formation, even in extraosseal sites (Okumura et al. 1991; Ohgushi et al. 1992). This would make it possible to prefabricate implants, e.g. inside muscle tissue and to

transfer a composite muscle-ceramic-implant as a vascularized graft to the defect area.

Another way to induce bone growth into porous ceramics is to add growth factors to the implant. Transforming growth factor beta (TGF-ß) is an important regulator of bone development, induction, repair and remodelling. Not only does it have strong mitogenic activity on osteoblasts but it also enhances bone matrix collagen production (Robey et al. 1987).

In 1991, Beck and coworkers showed in rabbits that a single application of TGF-ß1 leads to new bone formation (Beck et al. 1991). Since then many different materials, natural coral among them, have been investigated for the delivery of TGF-ß to bone (Arnaud et al. 1994). Recently a preliminary clinical report was published on the use of TGF-ß in humans (Arnaud et al. 1998). Although the results are promising, they must be interpreted with caution due to the complex effects of TGF-ß.

A bone implant with osteoinductive capacity would solve many of the problems associated with bone grafting. This study was designed to investigate the properties of natural coral and hydroxyapatite to serve as bone substitutes in a clinical human study and in animal experiments. Bone ingrowth into the implant was induced by bone marrow or by TGF-ß.

REVIEW OF THE LITERATURE

Structure of bone

Bone consists of a collagenous framework upon which calcium salts are deposited mainly as hydroxyapatite. The mature bone is lamellar, its collagenous fibres building regular patterns. In the cancellous bone the collagen bundles lie parallel to the long axis of the trabecula and in the compact (cortical) bone the fibres are disposed in concentric rings around the vascular spaces.

Bone can also be considered as consisting of cells and extracellular matrix, with 35% of the matrix being composed of organic and 65% of inorganic ones (Martin et al. 1988). The inorganic part is formed of calcium salts and the organic componenents of collagen and noncollagenous proteins. The noncollagenous proteins form 10% of the organic material. They modulate matrix organization, bind calcium and, like the bone growth factors, regulate bone formation and resorption (Sandberg 1991).

Bone healing

Bone healing has been studied at length in enchondral, tubular bones but much is still to be learned of the healing mechanisms of intramembranous bone, i.e. bone formed without the cartilage phase. Such bone is found mainly in the cranio-facial area.

In tubular bone trauma, osteoprogenitor cells are recruited to the trauma site and are differentiated to bone forming cells. Multipotential cells from bone marrow and primitive fibroblasts from adjacent soft tissues may also be recruited. Biochemical, mechanical and biophysical factors are involved in this process, in which periosteal cells are activated for the repair process and the multipotential stem cells and prefibroblasts are induced to differentiate into osteoblasts.

The bone forming cells need a template to attain the proper three-dimensional structure. This is formed by the collagen network on which the hydroxyapatite crystals are deposited by the osteoblasts. This phenomenon, called osteoconduction, helps to bridge large segmental defects (Cornell and Lane 1992).

Fracture repair proceeds in three physiologically and histologically characteristic but overlapping phases: 1) acute inflammation, 2) reparation and 3) adaptation (Urist and Johnson 1941; Sevitt 1981). Different tissue types are represented in the sequence: haematoma,

granulation tissue, fibrous tissue, cartilage and bone.

In the acute inflammation phase the haematoma forms, bone necrosis occurs and cell death releases byproducts into the fracture site. Inflammation begins within 48 hours and lasts untill cartilage and bone appear. The inflammation activates the cellular mechanisms necessary for the repair (Sevitt 1981).

In the reparative phase the fracture gap becomes highly cellular, vascularizes and forms soft callus. Osteoblasti and chondroblasts begin to appear in the fracture gap and to replace the fibrovascular stroma (McKibbin 1978). The soft callus is converted by enchondral ossification to woven bone, where the collagen fibres are randomly oriented. Ossification can, however, happen without previous cartilage formation.

The last phase is adaptation (modelling and remodelling), starting early in the reparative phase (Sevitt 1981). The woven bone is transformed to lamellar bone with organized collagen matrix. During this process the bone shape is restored and the medullary canal is reconstructed. This process, which may take from months to years appears to be governed by Wolff's law (Wolff 1892).

Bone grafting

History

Various materials, from gold to allografts have been used to fill bony defects (Habal and Reddi 1994). Despite the range of materials available today, the standard procedure for the repair of bony defects is still autogenous bone grafting.

Even if autogenous bone grafts had been used before, Leopold Ollier was the first to study bone transplantation systemically (Ollier 1858). He also pointed out the difference between auto-, allo- and xenografts. In 1914 Phemister described clinically the healing of autogenous bone grafts and stressed the importance of vascularization, the various tissue components involved in bone healing and creeping substitution *(schleichender Ersatz)* (Phemister 1914). After them numerous investigators contributed to the research into bone grafting, in particular after World War II, when there were a great number of victims with severe bone defects, especially in the craniofacial area.

Autografts

The autogenous bone graft has several advantages over allo- or xenografts. It has greater osteogenic capacity and it is biocompatible.

As the autograft resorbs, revascularization recruits mesenchymal-type cells, which differentiate into osteogenic, chondrogenic or other cell lines (Brown and Cruess 1982).

The high osteogenic potential of **cancellous bone** derives from the bone marrow it contains, and the marrow part as such can be used to induce bone growth into different porous materials (Nade et al. 1983).

The cancellous graft is mouldable and resistant to infection; it vascularizes fast and can be obtained quite easily, usually as "chips" from the iliac crest (Burwell 1966). Unfortunately the amount of cancellous bone is limited. Moreover, it cannot be used in stress bearing areas, and the harvesting causes morbidity, such as pain, haematoma, infection and nerve injury (Banwart et al. 1995) or even iliac hernia and ureteral injury (Challis et al. 1975; Escalas and DeWald 1977). The major complication rate amounts to 8.6% and the minor complication rate to an additional 20.6% (Younger and Chapman 1989).

Cortical (compact) bone is used when mechanical support is needed. Common donor sites used to be fibula, ribs and iliac crest. Unfortunately the biological properties of compact bone are poorer than those of cancellous bone, and the more compact the transplant the slower the vascular invasion. The bone grafts are replaced by local tissue through "creeping substitution", in which the graft acts as a scaffold for growing fibrovascular tissue (Phemister 1914). Compact bone is probably never fully replaced or invaded by the graft site tissue; the outcome is therefore inferior integration and infections. It also has the tendency to resorb, making it a unreliable material (Burchardt 1983; Banwart et al. 1995).

Intramembranous grafts have been considered to resist resorption better than enchondral grafts and their biological behaviour seems to be different. Cortical (and cortico-cancellous) grafts harvested from the calvaria (skull) are commonly used in craniofacial surgery (Tessier 1982).

The cortico-cancellous graft offers stability and osteogenic capacity. It can be used in weight-loaded areas, often in spinal fusions or in mandibular reconstructions. Vascularization is, however slow and large defects cannot be reconstructed due to the same complications as with cortical grafts.

Microvascular grafts have helped to overcome some of the drawbacks associated with the reconstruction of large defects. Large cortico-cancellous grafts can be harvested with their nutricient vascular pedicle, allowing the vessels to be anastomosed to suitable artery and vein in the recipient site. The healing

takes place in the interface of the graft and recipient bone as in a normal bone fracture situation (Weiland et al. 1984). The donor site complications may, however be more severe, because the grafts used tend to be very large.

The periosteum contains osteoprogenitor cells and can as such or as an osteo-periosteal graft be used to enhance bone formation (Ritsilä et al. 1976). This method has not gained much popularity though.

Allografts

The known limitations of autografting, e.g. secondary operation, limited availability of bone and operation morbidity, have encouraged the search for other options. The natural choice is allograft bone, human bone, usually harvested from a dead person or obtained in a hip fracture operation. The basic concept underlying allograft bone use was established in the early 1900s, when Baschirzev and Petrov showed that most of the cell components in the graft die after transplantation and that bone regeneration starts from the host bed (Baschirzev and Petrov 1912).

Allografts demonstrate a lower osteogenic capacity, higher resorption rate and larger immunogenic response and, finally, less revascularization of the graft than autografts (Chase and Herndon 1955; Friedlaender et al. 1978).

Despite of these drawbacks, allograft bone offers a useful adjunct to the range of bone graft materials. Bone can be minced and mixed with autogenous grafts in spinal fusions or hip prosthesis operations (Burwell 1966). Allograft bone can even be used for large grafts comprising whole joints in tumour surgery. The results, however, are somewhat contradictory (Aho et al. 1998).

To maintain the availability of allograft bone, a well-organized bone bank is needed (Tomford et al. 1987). The possibility of transmitting viruses or bacteria may limit the use of allografts (Buck and Malinin 1994; Khan et al. 1998).

Xenografts

The xenogenic bone graft, that is, a graft made with bone from another species, presents similar problems to the allograft. It elicits an acute antigenic response with a high failure rate. Partial deproteination and defatting have been demonstrated to decrease the antigenic response (Kiel bone) but at the cost of the osteoinductive capacity. Xenografts are indeed rarely used (Heiple et al. 1967).

Demineralized bone

Demineralized (decalcified) bone was first studied in the late 19th century, when it was

mainly used for filling cavities in osteomyelitis operations (Senn 1889). It is manufactured in a process whereby first the bone marrow is removed, then the bone is defatted and finally the mineral contents are decalcified with hydrogen chloride, leaving the collagen matrix intact. Demineralized bone can be used in powder form, in chips or in corticocancellous blocks. Urist and coworkers noted the osteoinductive capacity of demineralized bone, and later attributed it to the influence of morphogenetic protein (Urist and McLean 1941). Clinically demineralized bone has been used primarily for craniomaxillofacial reconstructions (Mulliken et al. 1981).

Mechanical properties of bone

Mature bone can be divided into cancellous (trabecular) or compact bone, depending of the degree of bone porosity. Compact bone has a porosity of 5-30% and cancellous bone of approximately 30-90%, which is the proportion of the volume occupied by non-mineralized tissue (Carter and Hayes 1977). The diaphyses of long (tubular) bones are composed mainly of compact bone whereas the epiphyses and methaphyses consist of cancellous bone that is continuous with the inner surface of the cortical shell and exists as a three-dimensional, sponge-like lattice composed of plates and columns of bone. The trabeculae divide the interior volume of bone into intercommunicating pores of different dimensions. The composition and true densities of compact and trabecular bone are thought to be similar (Galante et al. 1970) as are the microscopic material properties (McElhaney et al. 1970).

A key requirement in bone is compressive strenght, and the most important factor in compressive strength is the degree of mineralization. Loss of mineralization results in increased risk of fracture (Wright and Hayes 1977). A collagen and hydoxyapatite composite is advantageous from a mechanical standpoint. Mineralized tissue can be considered as a a porous, two-phase composite consisting of hydroxyapatite crystals embedded in collagen matrix (Lees and Davidson 1977). Increasing collagen intermolecular cross-linking is associated with increasing mineralization. The resulting composite structure is much stronger and stiffer due not only to the higher mineral content but also to the stiffening of the collagen matrix caused by the greater cross-link density (Carter and Spengler 1978; Mammone and Hudson 1993). It has been suggested that the longitudinal strength and stiffness of mineralized bone tissue are ap-

proximately proportional to the strain rate raised to the 0.06 power.

Bone can also be considered as a composite of both a solid and a fluid phase. The solid phase consists of mineralized bone tissue and the fluid phase of blood vessels, blood and marrow, nerve tissue, miscellaneous cells and interstitial fluid (McElhaney et al. 1970).

The role of marrow, intertrabecular fluids and soft tissues does not affect the compressive strength under modererate, physiological conditions such as walking (Swanson and Freeman 1966). The results at higher strain rates, 10.0 per second suggested that the presence of marrow during severe, traumatic, compressive loading *in vivo* may serve to absorb considerable energy. Increasing the strain rate from 0.001 to 10.0 per second increases the strength and stiffnes of bone by a factor of approximately 2 (Carter and Hayes 1977).

The bone can be loaded by compression (or tension), shear, bending or torsion. The load F acting on the specimen can be measured with a testing instrument. The strength (maximal stress at breaking point) can be calculated from the load (stress)-strain curve by the ratio of the ultimate load to the cross-sectional area of the specimen (A).

strength = F/A

When compressive strength is measured, the displacement per unit is called strain. A stress-strain curve of the compressive test is shown in Figure 1. At low levels of strain, stress is proportional to strain. Stress-strain curves in tension and compression consist of an initial elastic region that is nearly linear. It is followed by yielding and considerable nonelastic deformation until the breakpoint has been reached. This part of the the curve reflects the irreversible microdamage of the bone structure.

The yield point represents the onset of plastic deformation, which occurs at strain levels of around 1% in ceramics (Crowninshield and Pope 1974). In the zone of plastic deformation, a composite is able to absorb energy via matrix-filler debonding before ulti-

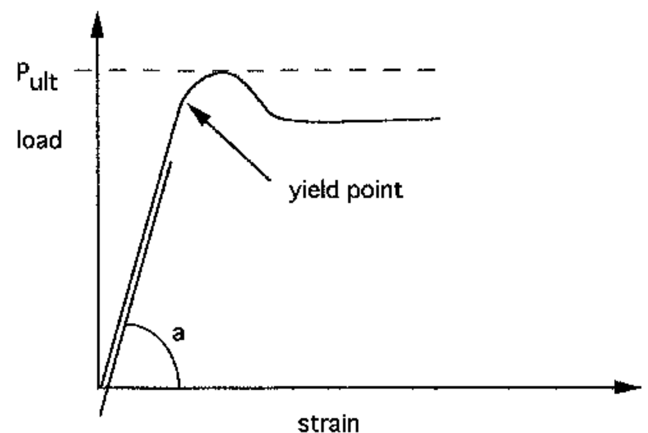

Figure 1. Stress-strain curve. P_{ult} is the ultimate compressive load before the final collapse of the specimen.
Tan(a)=elastic modulus.

mate failure (Mammone and Hudson 1993).

The elastic compressive modulus is the slope of the load strain-curve in the most linear portion. Called Young´s modulus when tension or compression are studied (Carter and Spengler 1978), it reflects the stiffness of bone.

The compressive strength of cortical bone varies, being in humans around 200 MPa (megapascals) for the femur; the elastic compressive modulus is around 17 GPa (Reilly et al. 1974; Reilly and Burstein 1975). Cancellous bone is much weaker and the results obtained have varied, depending on the location of the bone (Goldstein 1987). Compressive strengths of 0.15-27 MPa and elastic modulus from 50 to 350 MPa have been reported for cancellous bone (Table 1) (Schoenfeld et al. 1974; Carter and Hayes 1977; Manninen 1993).

Biomaterials

A biomaterial can be defined as “a material intended to interface with biological systems to evaluate, treat, augment, or replace any tissue, organ, or function of the body” (Williams et al. 1992). There are three classes of biomaterial: metals, polymers and ceramics. The following looks briefly at some of the biomaterials used in clinical practice.

Metals

The main metals in clinical use are titanium, vitallium, aluminium and stainless steel, all of them inert and biocompatible (Mofid et al. 1997). Metal implants are used for load bearing purposes such as joint prostheses, and screws and plates. They may undergo corrosion over time due to galvanic corrosion produced by two different types of metal. Fretting corrosion may occur when the oxide film on the metal is damaged by, say, a screw in a plate hole (Gosain and Persing 1999).

The integration of metal prostheses to the host bone can been promoted by coating them with a bioactive ceramic such as hydroxyapatite. Coating with plasma-sprayed apatite leads to the formation of a strong bond between bone and metal implant (Geesink et al. 1988). This is particularly advantageous in hip arthroplasty, where implants have a tendency to detach with time.

Table 1. Studies of compressive strength on cancellous bone (adapted from Manninen 1993)

Authors (year of publishing)	Bone studied	Storage	Ultimate Strength (MPa)	Modulus (MPa)
Sonoda (1992)	Human whole lumbar vertebral bodies	Fresh	6.3	-
Weaver and Chalmers (1966)	Human -3rd lumbar vertebra -calcaneus	Fresh frozen	<50 yrs:4.2 >50yrs: 2.5 <50 yrs: 3.9 >50 yrs: 3.5	- - - -
Shoenfeld et al. (1970)	Human femoral head	Fresh	0.15-13.5	340
Lindahl (1976)	Human tibia	Dried, defatted	Males 3.9 Females 2.2	35 23
Carter and Hayes (1977)	Human tibial plateaus	Fresh frozen	Marrow in situ 27.0 Without marrow 5.9	 210 54
Stone et al. (1983)	Bovine humerus	Fresh frozen	8.3	-
Hvid and Jensen (1984)	Human proximal tibia	Fresh frozen	1.6 7.7	- -
Kaplan et al. (1985)	Bovine proximal humerus		12.4	
Wixson et al. (1989)	Human distal femur and proximal tibia	Dried, soft tissue removed	5.6	150

Polymers

Polymers comprise a large group of materials of heterogenous origin. They are composed of the macromolecules that are typically formed by the bonding of one or a small number of types of subunits repeated along the length of the polymer (Gosain and Persing 1999). A polymer widely used in traumatic skull defects is methyl methacrylate. It is esy to mould, cheap and nonresorbable and is even stronger than calvarial bone. The infection rate is considered to be comparable to that in bone grafting procedures but its use is not recommended for patients who previously had infections in the cranioplasty site. The use of methyl methacrylate requires good quality of the overlying soft tissues (Manson et al. 1986).

Polyhydroxyethylmethacrylate with calcium hydroxide coating (Hard Tissue Replacement, HTR®, Walter Lorenz Surgical, Inc., U.S.A), is another popular polymer composite. It is a nonabsorbable porous material allowing tissue ingrowth into pores of 150 to 350μm. It can be prefabricated in custom shapes and is mainly used in facial augmentations (Guyuron 1990).

Porous high-density polyethylene (Medpor®, Porex Surgical, Inc., U.S.A.) is used in facial augmentation surgery, in ear and orbital reconstruction and to fill empty eyeball sockets after enucleation or evisceration. The implants are fixed to bone or soft tissue by fibrous tissue ingrowth. The material is not resorbable, which is an advantage in augmentation surgery. The complication rate does not seem to differ from that of bone graft procedures. Among the disadvantages of polyethylene are its rigidity and the difficulty of contouring it to the surface of complex skeletal structures (Guyuron 1990; Frodel and Lee 1998). Another polymer material is a synthetic porous composite of polyethylene polymer and aluminium (Proplast®). Although it is light, porous, resilient, malleable and easy to shape the material is currently little used due to the high complication rate associated with its application in recent yeas (Whear et al. 1993).

Polyglycolide and polylactide are hard synthetic crystalline polymers used as fixation material in orthopedic and craniofacial surgery. In living tissue they degrade to glycolide and lactide mainly by hydrolysis. Their unique mechanical properties are such that they can be used for manufacturing screws, pins and plates that absorb with time (Rokkanen 1998), thus eliminating the need for a secondary operation of removing the fixation material. In recent years the possibility of

polyglycolide and polylactide acting as delivery material for growth factors has been investigated (Gombotz et al. 1994).

Ceramics

Ceramics consist of crystalline metallic oxides, carbides, nitrides and borides fused by the high temperature process known as sintering. They are brittle, have low electrical and heat conductivity and elicit very little tissue reaction (Mears and Rothwell 1979). Different glass ceramics are typical representatives of this group of biomaterials. In the 1970s, certain glass compositions were found to be able to bond chemically to bone, a property called bioactivity. The first bioactive glass, Bioglass®, was composed of SiO_2, Na_2O, CaO and PO_5. Bioactive glasses are manufactured by conventional glass manufacturing methods and even minor changes in their composition change the character of the bone-material bonding and resorption (Hench et al. 1975). Granular and solid forms of bioactive glasses have been used clinically to reconstruct orbital walls and facial bone defects and to obliterate frontal sinuses. Bioactive glasses are osteocompatible and the infection rate is low. The resorption rate may, however, be too high and the migration of granules can cause problems (Suominen and Kinnunen 1996).

Another important group of ceramics comprises materials deriving from calcium. Such ceramics are composed of calcium sulphate, phosphate and carbonate derivates and their mixtures in dense, porous and granular forms. The following sections examine these materials in greater detail.

Calcium ceramics

Calcium sulphate

Plaster of Paris (calcium sulphate) was one of the first materials to serve as bone substitute. In 1892, Dreesman used it to fill bony defects in eight patients with nine defect sites. Six defects healed well and three remained unhealed (Dreesman 1892). Since then calcium sulphate has proved to be biocompatible and adsorbable but not osteoconductive. Resorption occurs in weeks and may be too fast for the bone formation process (Calhoun et al. 1967). This, together with its poor mechanical properties, has limited the use of calcium sulphate as bone substitute.

Fast resorption may, however, be an advantage. In 1928, plaster of Paris was investigated by Petrova as a delivery material for antiseptics, and in the 1950s cylinders containing penicillin and sulphonamide were examined clinically (Peltier 1961). Recently the

properties of calcium sulphate were suggested to be suitable for a carrier of bone morphogenetic protein in a mouse model (Yamazaki et al. 1988).

Calcium phosphate

Calcium phosphate biomaterials are polycrystalline ceramics deriving from individual crystals of a highly oxidized substance that have been fused together (Jarcho 1981). The two most important are tricalcium phosphate $Ca_3(PO_4)_2$, or ß-whitlockite, and hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$. Both materials are known to be biocompatible and osteoconductive and to bond directly to bone. The main difference between these two materials is that tricalcium phosphate (TCP) degrades much faster than HA (Jarcho 1981; Shimazaki and Mooney 1985). The chemical structure of calcium ceramics resembles that of bone. Hydroxyapatite is the main inorganic salt of bone and the synthetic form has been shown to be chemically and crystallographically similar, but not identical, to naturally occurring HA (LeGeros et al. 1988).

HA is the most studied calcium phosphate material with clinical experience of its use going back to the 1970s (Hulbert et al. 1970). Offering better integration to bone, porous HA has now replaced the dense form. When HA has been placed into bony defects, bone growth into pores has ranged from 18% to 74% (new bone area compared to total implant area) (Holmes et al. 1988; Martin et al. 1993). The entire porous space of the implant is probably never completely filled with bone (Rosen and McFarland 1990).

Porous HA can be manufactured in several ways. Homogenizing calcium phosphate powder with appropriately sized naphthalene particles results in macroporous material after the naphtalen has been removed. The final form is achieved after sintering at high temperatures (1100-1300°C). Another method relies on the decomposition of hydrogen peroxide to generate a pore-filled structure.

The porous structure achieved in these methods is not, however, consistent. To avoid this problem, a completely different approach was developed in the early 1970s as a joint investigation by the Materials Research Laboratory at The Pennsylvania State University and the Orthopaedic Research Laboratory of the Upstate Medical Center at Syracuse, both in the U.S.A.

The idea is based on the finding that the structure of certain reef-building coral species resembles osteon evacuated bone (Holmes 1979). The coral pore size is consistent and varies very little (Figure 2). A pore size of

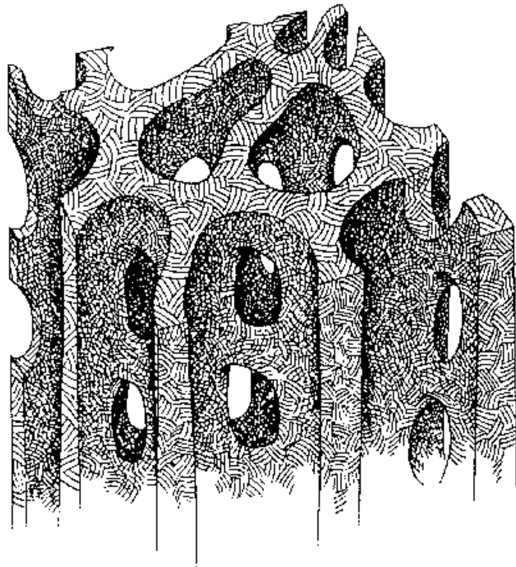

Figure 2. Drawing of a bone graft substitute replicated from *Porites*. Channels of osteonic diameter and channel wall fenestration mimic the interstitial matrix of cortical bone (after Holmes 1988).

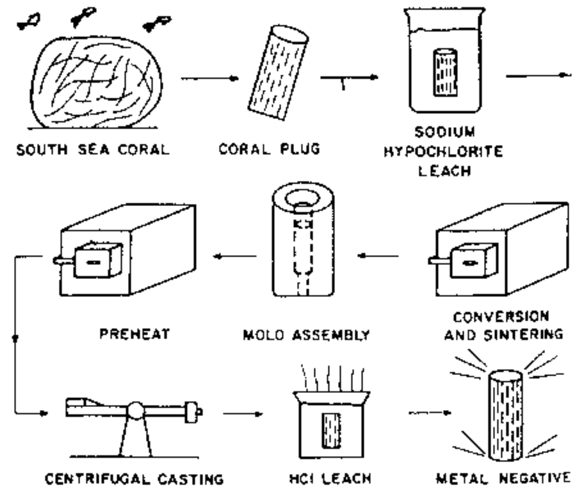

Figure 3. Schematic representation of the replamineform process. The coral is shaped for treatment, as shown, to yield porous implants in a variety of materials (after Chiroff 1975)

over 100μm was previously found to allow fibrovascular and bone tissue ingrowth (Hulbert et al. 1970). With pore diameters of 140-160μm, the reef-building coral genus *Porites* meets the structural requirements for bone substitute (Chiroff et al. 1975).

This structure can be preserved in the "replamineform process" (meaning the replication of the life forms) in which the chemical composition of the coral is changed (Figure 3) (White et al. 1972). In 1974 Roy and Linneham refined the replamineform process and succeeded in changing the calcium carbonate ($CaCO_3$) skeleton of coral into hydroxyapatite in the "hydrothermal exchange reaction". This resulted in a hydroxyapatite implant with controlled porosity but with a chemical composition differing from that of the original coral material.

Several studies have shown that porous hydroxyapatite is osteoconductive, biocompatible and very inert (Chiroff et al. 1975; Holmes 1979; Jarcho 1981). It resorbs with time but the degradation rate is very slow (White and Shors 1986). HA has been used clinically in dental, craniofacial and orthopaedic surgery, mainly in granular form (Meffert et al. 1985; Bucholz et al. 1989; Salyer and Hall 1989; Rosen and McFarland 1990; Uchida et al.

1990; Byrd et al. 1993; Kamegaya et al. 1994). Recently a hydroxyapatite cement was developed for cranial defects. It is in paste form and can be moulded into the desired shape. The material integrates to the host bone and is claimed to be replaced by bone, at least to some extent (Costantino et al. 1992). The high cost of the material has, however, restricted its wider use clinically.

Porous HA is brittle and can be used only in non-loading sites. Its compressive strength is enhanced by bone ingrowth but it is only comparable to that of cancellous bone (Martin et al. 1993).

Slow resorption is advantageous in certain clinical situations. If used as an onlay graft as in alveolar ridge augmentation, degradation is not desired. However, an ideal bone substitute should be osteoconductive, osteoinductive, biocompatible and easy to shape; it should also biodegrade after it has been occupied with new bone and be a reasonable price. Its mechanical properties should be comparable to those of cortical bone. HA has many of these qualities but it resorbs very slowly and is brittle.

Calcium carbonate

Calcium carbonate ($CaCO_3$) resembles hydroxyapatite in many respects. The material is biocompatible and osteoconductive but, like HA, has no osteoinductive properties (Guillemin et al. 1987). The main difference to HA is the resorption rate. Resorption seems to be clinically unimportant with HA, but animal experiments have shown resorption rates of only a few weeks, when CC is used (Guillemin et al. 1989).

Certain coral species form a structure that resembles the matrix or bone. Each species builds a structurally and geometrically typical calcium carbonate skeleton. Choice of the appropiate species therefore enables a desired and constant implant structure to be achieved.

Coral reefs are formed by colonies of polyps ranging in size from one millimetre to several centimetres, depending on the species.

Coral polyps live in symbiosis with unicellular algae, which photosynthesize compounds essential to the polyps. The outer layer of the polyp is capable of secreting a substance that calcifies in the seawater milieu and serves as matrix for the coral skeleton. The coral polyp lives only in the upper part of the skeleton, moving slowly upwards, leaving an empty skeleton behind. Coral reefs play an important role in maintaining the equilibrium of carbon dioxide, which is absorbed from the seawater in the building process.

More than 2000 coral species have been described from the intertropical area and, of these, fourteen Scleractinian corals have been studied as possible bone substitutes.The following genera have already been used as bone grafts: Pocillopora, Acropora, Montipora, Porites, Goniopora, Fungia, Polyphyllia, Favites, Acanthastrea, Lobophyllia and Turbinaria (Bouchon et al. 1995). The most promising is *Porites astreoides*, which forms massive colonies and is common throughout the Caribbean area, including the Bahamas, Bermuda and Brazil. The porosity of the skeleton is around 50% and the mean size of the pore is 150µm, the pores interconnecting with each other.

The harvested coral is purified physically and chemically and the final implant material contains no proteins and less than 0.1% amino acids. The manufacturer of the commerciably available coral implant (Biocoral®) guarantees the following chemical composition of the product:

calcium carbonate	>97%
trace elements	0.5-1%
magnesium	0.05-0.2%
sodium	<1%
potassium	<0.03%
phosphorus	<0.05%
water	<0.5%

Experimental studies on CC started in the early 1970s. Chiroff and coworkers placed CC in cancellous defects in dogs for 8 weeks and found that the material was biocompatible and that new bone could fill the pores. Some implants were left for 1 year and were observed to be almost completely resorbed (Chiroff et al. 1975). The favourable results were confirmed when other animals, e.g. monkeys (Souyris et al. 1984) and sheep and pigs (Guillemin et al. 1989), were used.

The first clinical reports were published in France by the Institut de Recherches Orthopédiques, Université René-Descartes Paris V in 1980 (Patel et al. 1980). Since then CC has been used clinically in maxillofacial surgery to correct periodontal defects (Issahakian and Ouhayoun 1989; Mora and Ouhayoun 1995) and to fill and reconstruct bony defects in cranial surgery (Roux et al. 1988; Mercier et al. 1996; Soost et al. 1998). The craniofacial bones can be augmented by the granular form of CC (Marchac and Sandor 1994). In orthopaedic surgery CC has been used as a filler in tibial osteotomies (Kenesi et al. 1997), in bone tumour surgery (Rouvillain et al. 1997) and in lower limb metaphyseal fractures to support articular surfaces (de Peretti et al. 1996). The possibly most appropriate indication at the moment is spinal fusion, where CC can be

used to diminish the amount of bone grafts in conjunction with autogenous bone (Pouliquen et al. 1989; Kehr et al. 1995).

Little is known about bone ingrowth into the CC implants. Bone and fibrous tissue do grow into the pores but exact information is scarce. This may be due to the difficulty of measuring the already partially resorbed implant. The same difficulty applies to measurements of the mechanical properties, which, according to the manufacturer, are better than in cancellous bone.

Very little exact information exists also on the resorption time of CC. It seems to depend on the animal species used. When the implant was placed in the cortex of the femur in pigs, 64% of the CC blocks were resorbed after 1 month, whereas in sheep the figure was 93% (Guillemin et al. 1989). The granular form has been observed to resorb completely at 24 weeks in a connective tissue site in pigs but, in humans, the same material placed in subcutis can still be found after several years (Marchac and Sandor 1994; Naaman et al. 1994). Roux and coworkers reported almost complete resorption after 1 year in 50% of cases when coral was used to fill craniotomy burrholes in humans (Roux et al. 1988). Larger blocks used in humans have still been x-ray positive after 4 years (de Peretti et al. 1996).

Coral resorption is most active in the bone-implant contact areas and proceeds centripetally (Braye et al. 1996). Carbonic anhydrase, an enzyme abundant in osteoclasts, plays a key role in the resorption process. Locally it lowers the pH at the osteoclast-implant interface, dissolving the CC matrix (Chétail and Fournié 1969; Gay and Mueller 1974; Guillemin et al. 1981. Resorption can be halted by the administration of the diuretic acetazolamide, a known inhibitor of carbonic anhydrase (Guillemin et al. 1981). Moreover, according to Fricain and coworkers, data suggest that both fibroblasts and macrophages dissolve the coral, and that one of the mechanisms is the intracellular degradation in phagolysosomes (Fricain et al. 1998, a). A prerequisite for the process is direct contact between these cells and the coral matrix (Fricain et al. 1998, b).

Induction of bone growth into calcium ceramics

Enhancement of bone growth

Porous calcium ceramics are osteocompatible (Jarcho 1981; Doherty et al. 1994) and osteoconductive (Chiroff et al. 1975) but they lack the capacity to induce new bone forma-

tion from determined osteogenic precursor cells, a quality known as **osteoinduction.**

Osteoinduction can be attained by harvesting bone marrow cells as such or in conjunction with autogenous bone and placing them at a site where bone formation is needed, e.g. at bony defects caused by fracture or by pseudoarthroses. Another way is to induce bone growth by the members of the TGF-beta superfamily, a group of growth factors that make an important contribution to the bone formation process.

Bone marrow

Back in 1869 Goujon observed heterotopic (extraskeletal) bone formation after red bone marrow transplantation. Bone marrow contains osteogenic precursor cells, which are capable of differentiating into osteoblasts. When marrow is placed in a heterotopic site (subcutis, muscle), bone may derive from these cells, from endosteal osteoblasts or from the host cells at the site of grafting, induced to differentiate by bone marrow (Burwell 1985). In 1971 it was found that autogenous marrow formed bone in association with various materials and that calcified matrix increased the ostegenic capacity of the marrow (Newman and Boyne 1971). In 1980 Lindholm and Urist reported enhanced bone formation in composite grafts of bone matrix and bone marrow (Lindholm and Urist 1980). A year earlier McDavid and coworkers had placed tricalcium phosphate pellets with autogenous bone marrow under the skin of rats. At 4 weeks bone was evident only in marrow-coated implants (McDavid et al. 1979). Porous aluminate, calcium aluminate, HA and TCP inserted together with marrow into the intermuscular space of rabbits were observed to allow bone formation (Nade et al. 1983).

Ohgushi and coworkers investigated porous calcium phosphate (60% HA and 40% TCP) blocks in a segmental rat-femur defect experiment. The group treated with bone marrow showed significantly better osseous or osteochondral union than did the control group without marrow (Ohgushi et al. 1989). Placing similarly treated implants into the subcutaneous pouches of rats resulted in bone ingrowth only into the implants treated with bone marrow; after 1 months the proportions of the pore area filled with bone for implanted HA and TCP were 16.9% and 15.1%, respectively. After 2 months the proportions were 34.3% and 30.9 % (Ohgushi et al. 1990). Later, CC disks (genus *Porites)* were investigated similarly and bone formation was observed after 3 weeks. No histomorphomeric analysis was, however, performed.

Transforming growth factor-beta

The data above show that bone formation can be induced into porous calcium implants by bone marrow. The next step is to prefabricate implants with already existing potential for bone formation. Members of the transforming growth factor-beta superfamily play an important role in the bone formation process and may offer a solution to the manufacture of such bone substitutes.

TGF-ßs are a group of growth regulatory peptides consisting of five isoforms (Roberts et al. 1988). They form the TGF-ß superfamily together with bone morphogenetic proteins (BMPs) and the embryonical growth factors inhibin, activin and the Müllerian substance (Massague 1990). The unifying properties of these peptides are their similarity in structure and their ability to regulate development and cellular differentiation.

The five TGF-ß isoforms are encoded by closely related genes. Three of them, TGF-ß1, ß2 and ß3, are found in mammalian bone, which is the largest reservoir of TGF-ß the concentration being 100 times as high as in other tissues. TGF-ß1-3 are very similar in their way of action and are here considered together as TGF-ß.

Platelets are the most concentrated source of TGF-ß although most cells can synthesize it (Massague 1990). TGF-ß1 was first isolated from human platelets (Assoian et al. 1983) and was later cloned from the human complementary DNA library (recombinant TGF-ß) (Derynck et al. 1985). The universality of its action is emphasized by the almost identical amino acid sequence in various mammalian and avian species (Massague 1990).

TGF-ß is a homodimeric protein of 25kDa. It is found in the form of an inactive high molecular weight complex that is activated by an acid environment, heat and enzymatic activity. Such an of environment is found close to the osteoclast. Osteoclast activity could activate TGF-ß, which then activates the osteoblasts (Brown et al. 1990) with the highest amount of TGF-ß receptors (Robey et al. 1987). This could be an important part of bone remodelling.

TGF-ß is a major regulator of bone development, induction, repair and remodelling. It has strong mitogenic activity on osteoblasts and it enhances bone matrix collagen production (Robey et al. 1987). It also plays an important role in soft tissue repair, chronic inflammatory fibrotic disorders, autoimmune diseases, and even in the repair of ischaemic cardial injury (Roberts and Sporn 1993).

In 1991 Beck and coworkers showed in rabbits that a single application of TGF-ß triggers a cascade of events leading to new bone formation in the course of 49 days (Beck et al. 1991). They used the critical size defect (CSD) model (a defect large enough not to be able to heal) (Schmitz and Hollinger 1986), drilling a 12mm large hole into the parietal bone and filling it with TGF-ß mixed with 3% methylcellulose. Bone formation was minimal in the control groups.

Since then very different materials have been investigated for the delivery of TGF-ß to bone, such as calcium sulphate implants and polylactic-coglycolic acid devices (Gombotz et al. 1994), gelatin sponge (Bosch et al. 1996), wax-like biodegradable polymer ceramics (Schmitt et al. 1998), tricalcium phosphate coated titanium implants (Lind et al. 1996) and demineralized bone matrix (McKinney and Hollinger 1996; Moxham et al. 1996; Ripamonti et al. 1996). The results have been encouraging, but limited bone formation has also been reported (Bosch et al. 1996; Ripamonti et al. 1996; Wikesjö et al. 1998).

Centrella and coworkers noted that the effect of TGF-ß on bone cell replication is biphasic and depends on both the TGF-ß concentration and the cell density in monolayer culture (Centrella et al. 1987). Dose dependence becomes more complicated when TGF-ß is released from different vehicles and when different animal species are used. Doses of between 0.4µg and 40µg have most commonly been used with conflicting results (Lind et al. 1996; McKinney and Hollinger 1996). Ripamonti and coworkers suggested that the limited bone formation is the result of TGF-ß's capacity to stimulate the proliferation of only periosteal and endosteal cells rather than to initiate bone cell differentiation, as does bone morphogenetic protein (Ripamonti et al. 1996). They also noticed a synergism in the function of TGF-ß and BMP in a heterotopic baboon experiment and postulated that TGF-ß might act as a chemotactic and mitogenic factor for responding precursor cells for subsequent induction by BMP (Ripamonti et al. 1997).

Coral has also been evaluated as a growth factor carrier, mainly in granular form. Damien and coworkers applied bone morphogenetic like protein and basic fibroblast growth factor (bFGF) to CC and noticed good ossicle formation only when BMP was present, whether with or without bFGF. The implants were placed into the subcutis of rat and they postulated that coral-collagen was a good carrier vehicle for BMP and should be tested in a

bony site (Damien et al. 1993). BMP and coral granules were later evaluated in spinal fusions in rabbit but solid fusions were obtained only in BMP groups, not in controls (Boden et al. 1997).

There are very few reports of TGF-ß in association with CC. Arnaud and coworkers tested 1µg of TGF-ß1 with coral granules and fibrin glue in a CSD model in rabbits and obtained significantly better bone formation with this combination at 2 months than with TGF-ß1 in methylcellulose or in fibrin glue alone (Arnaud et al. 1994). Another investigation showed only marginal bone growth stimulation at 4 weeks in a canine periodontal defect model with granular coral, hydroxyethyl starch and 20µg of TGF-ß1. The authors postulated that the healing interval of 4 weeks was too short or that the experimental model itself diminished the effect of TGF-ß1 (Wikesjö et al. 1998).

AIMS OF THE PRESENT STUDY

The experiments described here were designed to investigate the properties of natural coral and hydroxyapatite and their capacity to serve as bone substitutes. The specific aims were:

1. to examine the capacity of bone marrow (I) or TGF-ß1 (III) to induce bone ingrowth into porous HA (I) and CC (I, III) implants;

2. to study the effect of tissue ingrowth on the mechanical properties of HA and CC implant (II); and

3. to examine the resorption rate and pattern of coral implants when placed in extraosseal (I) and in orthotopic (III, IV) sites and to establish the extent to which the implants were replaced by new bone.

MATERIALS AND METHODS

The study comprises three animal experiments on rats (I-III) and one prospective study on humans (IV).

The study followed the principles of the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientic Purposes* and was approved by The Research Animal Commission of Helsinki University Central Hospital and by the Provincial Administrative Board (I-III).

All patients enrolled in the study responded to the Informed Consent protocol approved by our Institutional Committee on Human Research and stated that they found it acceptable (IV).

Animals and patients

Experimental animals, anaesthesia and post-operative follow-up (I-III)

The experiment was conducted on 113 Wistar rats ranging in weight from 250g to 650g. The numbers of implanted blocks at different follow-up periods are given in Table 2. The animals were anaesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg, Mebumat®, ORION, Finland) and were given procaine penicillin (300 000 IU/kg, Procapen®, ORION, Finland) intramuscularly before the operation. Postoperatively no immobilization was used. The rats were allowed an *ad libitum* diet of rat chow and water and inspected daily for general health.

Patients (IV)

The study group comprised 10 patients, one female and nine male. Five of the patients were unable to walk because of tetra- or paraparesis. The median age was 37 (range 25-79) years.

Another 10 patients, likewise one female and nine male, acted as controls. Five patients were para- or tetraplegic. The median age was 55 (range 17-78) years.

Implants

Natural coral (I-IV)

The coral implant material in all studies was calcium carbonate, $CaCO_3$ (Biocoral®, INOTEB, St. Gonnery, France), a porous natural coral of the genus *Porites*. Its mean porosity is 50% and pore size around 150µm, all the pores interconnecting with each other. 99% of the original material consists of calcium carbonate, the remaining 1% comprising

Table 2. Experimental design

HA=hydroxyapatite, CC=coral , m=with marrow, c=control

	weeks	HAm	HAc	CCm	CCc		total
	3	6	5(6)	6	6		
I	6	5(6)	5(6)	6	6		
	12	7		6			
	24	6					
		25	12	18	12		67

Values in parenthesis indicate original number of implanted blocks.
Three blocks were discarded because of technical failures in processing.

		HAm	HAc	CCm	CCc	femur	
II	dry	7		6(7)			
	wet	6		6		5	
	3 weeks	6	6	6	6		
	6 weeks	6	6	6	0(6)		
	12 weeks	6	6				
		31	18	24	6	5	84

One block was discarded because of technical failure in testing.
Six blocks could not be tested due to resorption and deformation of the implant.

	weeks	empty	CCc	CC/1μg	CC/5μg	CC/25μg	
III	3	4	4	4	4	4	
	8	4	4	4	4	4	
							40

Each animal received one implant (or served as a control without any implant)
μg indicates the amount of TGF-β1 used

organic materials, which are eliminated in the purification process.

Hydroxyapatite (I-II)

The hydroxyapatite (Interpore 200®, INTERPORE INTERNATIONAL, Irvine, USA) was manufactured from natural coral skeleton by a hydrothermal exchange reaction in which the calcium carbonate skeleton is converted to calcium phosphate. In this process the trabecular structure of the coral remains unchanged. The implants derive from the same coral species, *Porites,* as the natural coral implants used, and are structurally alike.

Transforming growth factor beta (III)

Lyophilized recombinant human transforming growth factor beta1 was obtained from Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA, USA). The specific bioactivity of the TGF-ß1 released from the CC carrier was determined by a cell growth inhibition assay (GIA). The assay measured the capacity of active TGF-ß1, diluted from the lyophilized implants, to inhibit the growth of mink lung epithelial cells in a dose-dependent manner when added in different concentrations (Ikeda et al. 1987).

Preparation of implants

Studies I-II

In studies I and II similarly shaped CC and HA blocks were inserted. The HA blocks were cut with a diamond saw longitudinally in the main pore direction, taking anisotropy into account. The implant size was 3x3x6mm for both materials. The CC blocks were prefabricated by the manufacturer.

The bone marrow obtained from the femur was mixed with 1ml of saline and the implant was immersed in the solution for 5 minutes before insertion in to the muscle.

An implant of the same material without bone marrow served as a control.

Study III

Four kinds of CC implant were prepared: coral implants as such and implants with 1µg, 5µg and 25µg of TGF-ß1. Disks, 2mm thick and with a mean weight of 0.78g, were cut with a diamond saw from a 30mm long cylinder (Biocoral®, BIO-CTV 1-2). The TGF-ß1 was diluted to phosphate-buffered-saline (PBS) in three different concentrations. 0.03ml of one of the three solutions was added to an implant, resulting in disks containing 1µg, 5µg or 25 µg of TGF-ß1. The im-

plants were then freeze-dried and kept at +4°C until used.

Eight rats received no implant and served as negative controls. Another eight rats received coral implants alone; 24 animals received coral implants containing 1µg, 5µg or 25 µg of TGF-ß1 (Table 2).

Study IV

The coral block used (Figure 4) was wedge shaped and measured 30x30x12-4mm (Biocoral®, BIO-CGI 1-1).

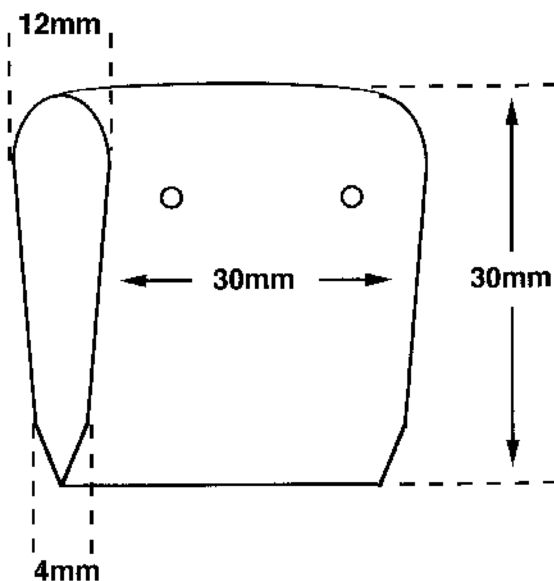

Figure 4. The natural coral implant used in study IV.

Surgical procedures

Bone marrow induced osteogenesis (I) and compressive strength (II)

Autogenous bone marrow was harvested from the left femur through the knee joint. A parapatellar incision was cut and the patella shifted laterally. A hole was drilled into the intercondylar space and the marrow was sucked out with a 20ml syringe and 18-G needle.

The left latissimus dorsi muscle was obtained through a dorsolateral incision. It was freed from its distal insertions and the implant was rolled inside the muscle. The muscle was sutured to prevent dislocation of the implant. Right latissimus dorsi muscle with an implant of the same material without bone marrow served as a control.

TGF-ß1 and coral implant (III)

A paramedian incision was made down to the periosteum of the right parietal bone. A CSD defect, 6mm in diameter, was then prepared in the parietal bone with a specially fabricated 6mm trephine. The craniotomy bone disk with the attached periosteum was removed, leaving the dura intact. After copious irrigation with saline, the defect was either left empty or filled with coral implant, depending on the experimental protocol. The coral implant made contact with the bone edges of the defect. Two 4-0 nylon sutures were fixed across the defect to keep the implant in place,

and the wound was closed with running nylon sutures in one layer.

Natural coral as filling material at iliac crest (IV)

In the study group a 30x30mm bicortical bone graft was harvested from the iliac crest. In one patient the block was removed from the posterior and in the others from the anterior part of the crest. The bone graft was used for spinal fusion in eight patients, for humeral fracture correction in one and for recurrent humeral dislocation surgery in one. The coral implant was fitted to the defect with a soft impaction mallet without any fixation. The overlying soft tissues were sutured in layers.

A similar bone graft was harvested in the control group but the defect was left empty. A cervical fusion due to trauma was performed in all patients.

Methods of analysis

Sampling and fixation techniques

The animals were killed with an overdose of pentobarbital (I-III).

To prepare the samples for further processing in study I, the muscle around the implant was dissected.

In study III the parietal bone was cut around the defect area with a diamond saw and the whole block, with or without the implant, was harvested.

In study IV a cylinder-shaped biopsy was taken perpendicular to the implant at the iliac crest, without any imaging assistance, while the patient was under local anaesthetic. The cylinder measured 3 or 6 x 10-20mm.

All the samples, except those in study II, were dehydrated in increasing concentrations of ethyl alcohol-water solution and embedded in methylmethacrylate.

In study II the implants were immersed in saline after harvesting and a compression test was performed within 24 hours.

Histology (I, III and IV)

5μm-thick sections were cut in the centre of the implant or defect area, with microtome (Polycut S, Reichert-Jung, Nussloch, Germany) and stained with the Masson-Goldner method (Goldner 1938) for histological and histomorphometric analysis (Figure 5a-c).

The specimens were studied by ordinary light microscopy. The quality of bone was assessed and the number of osteoblasts, osteocytes and giant cells and the amount of fat and bone marrow were evaluated on each sample. Special interest was focused on the

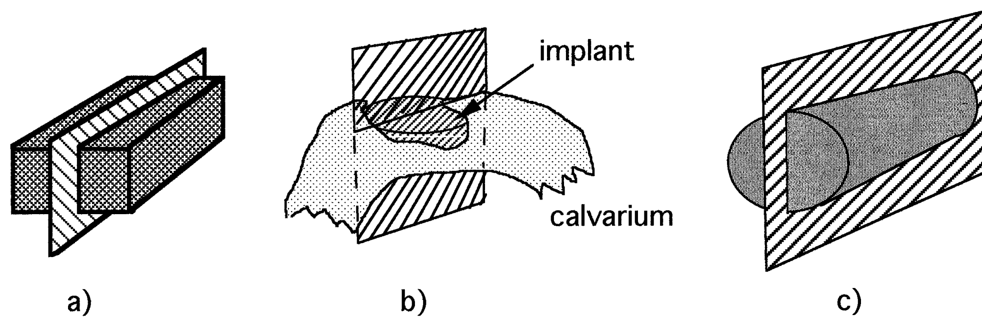

Figure 5. Longitudinal 5μm-thick sections were cut in the centre of the block for histological and histomorphometric analysis; a) study I, b) study III, c) study IV.

amount of macrophages and lymphocytes/neutrophils as signs of inflammation.

In study IV the amount of fibrous tissue, bone and marrow was evaluated on a four-step scale (-, +, ++, +++). The structure of the coral matrix was evaluated, (-) implying completely lost and (+++) wholly preserved original structure.

Histomorphometry (I and III)

For the quantitative histomorphometric analysis (I), a Leitz Diaplan light microscope was linked via video camera to a semiautomatic computerized analysis system (MOP Videoplan®, KONTRON, Munich, Germany).

In study I the entire section area was measured at x150 magnification. The variables to be measured were total implant cross-sectional area, bone area, fibrotic tissue area and the void area in pores. Three samples were excluded because processing failed.

Bone formation was compared using the total original cross-sectional area of the implant (before implantation) as a reference, not the true, resorbed area alone.

In study III a newer camera (SensiCam, PCO, Kelheim, Germany) and analysis system (analySIS, Soft-Imaging Software, GmbH, Münster, Germany) were used.

Three fields were selected for the assessment of new bone formation (Figure 6A). The sums of the individual measurements in these three fields were then used in the statistical analysis. Two further fields were measured at the edges of the implant to obtain information on the implant-calvaria interface (Figure 6B). The variables to be measured were new bone, fibrotic tissue and implant matrix areas, and also the shortest distance between the

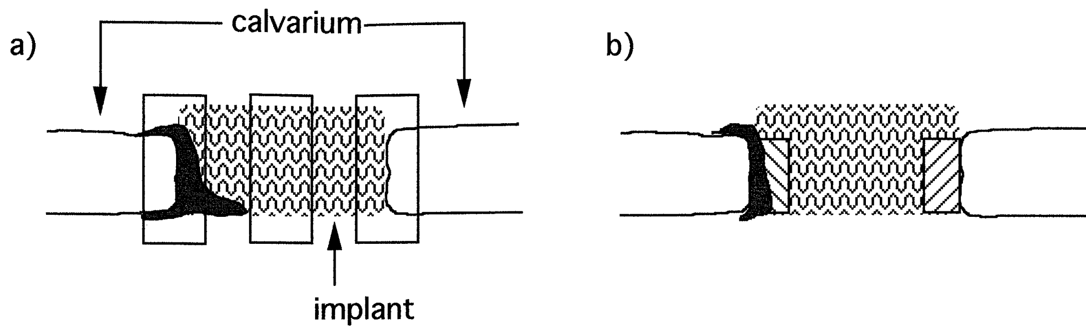

Figure 6. A) Schematic calvarial section showing the three fields used for histomorphometry (III). The middle point of the marginal field is placed at the original cut edge of the defect in order to measure the newly formed bone around the margin. Each area measures 1.87mm x 2.73mm = 5.11mm². B) Another two fields were selected for histomorphometry of the implant-calvaria interphase. They measure 0.96mm x 0.65mm=0.63mm².

opposite bone margins of the defects. The total implant area was determined to establish whether or not resorption had taken place.

Radiographic techniques (IV)

In the coral group, the donor site was imaged with quantitative CT scans every 6 months. Density was measured at the central point of the remaining implant. The scans were made with a Philips Tomoscan TX 60 and a GE HiSpeed Advantage CT scanner. Implant mineral density was quantified in equivalent mineral density (mg/ml) using lucite calibration phantoms from Image Inc. Contiguous 3mm-thick scans were performed. Plain x-rays were taken from the iliac crest perpendicular to the implant while the patient lay obliquely on the table. The remaining implant size was assessed semiquantitatively as 76-100%, 51-75%, 26-50% or 0-25% of the original size.

In the control group, only one plain x-ray was taken and quantitative tomography was performed after the follow-up period to examine the fate of the donor site defect without the influence of the coral substitute.

Compressive test (II)

After the implants had been harvested, they were immersed in saline. A compression test was performed within 24h using a JJ 5003 tensile/compression testing device (JJ LLOYD INSTRUMENTS, Southampton, UK). The compression rate was 10mm/min.

Testing was performed vertically towards the smallest area of the specimen. Compressive strength was calculated from the load-strain curve by the ratio of the ultimate compressive load to the cross-sectional area of the specimen. The elastic compressive modulus was taken to be the slope of the load strain-curve in the most linear portion (Figure 1). Altogether 54 implanted blocks were tested. In addition, implants of both materials in natural form (dry) and after being immersed in saline (wet) for 24h were measured. Five diaphyses of rat femurs cut from the cortical part of the bone were also tested (Table 2).

Statistics

The Kruskal-Wallis nonparametric test was used for statistical analysis in study I and $p<0.05$ was considered significant. Values were given as means (±SEM).

As well as the Kruskal-Wallis test a multiple comparison with the nonparametric version of Tukey's test was performed when more than two experiments were analysed (II).

In study III the differences in the means of the measured variables were compared with one-way analysis of variance and Tukey's Studentized Range Test. The values are given as means (±SD), and P values smaller than 0.05 were considered statistically significant. In study IV the density measurements were tested by Friedman's two way analysis of variance (ANOVA) by ranks and the Wilcoxon signed-rank test.

RESULTS

Study I

Histology

None of the control implants showed bone formation. A giant cell reaction and invasion of macrophages were observed in both materials. Small foreign-body type granulomas could be detected in some of the CC implants. No acute inflammation was seen in any of the specimens. The CC implant was successively deformed, whereas the HA implants were unchanged in size and form. Practically all the pores in both control materials were filled with fibrovascular tissue at 3 weeks.

Bone was formed in all implants with bone marrow. At 3 weeks abundant osteoblastic activity was seen directly on the surface of the pores. Bone formation had proceeded so far that scattered osteocytes and woven bone could be detected (Figure 7a and b). In both implants types numerous giant cells and macrophages were seen but only in direct contact with the foreign material, never adjacent to new bone formation. Only scattered lymphocytes were present, and no acute inflammation could be detected. The pores were almost completely filled with fibrovascular and bone tissue.

At 6 weeks bone marrow, too, was present, and both materials showed less osteoblastic activity than at 3 weeks. In some of the CC specimens mature, lamellar bone were also noted. In the CC specimens there were fewer giant cells and macrophages than at 3-weeks but in the HA specimens the number was unchanged. Some fibrovascular stroma, but no fat, could be seen in the pores in the periphery of the blocks of both groups.

At 12 weeks bone marrow was still present in both types of material. The amount of mature bone had increased and there was less osteoblastic activity. In the CC specimens only a few giant cells or macrophages could be seen. In contrast, giant cells and macrophages were evident in the HA specimens in all areas where bone did not line the implant material. The CC implants were deformed and nearly all the coral material not lined by bone had been phagocytized. In CC blocks the innermost part of the implant was occupied by bone marrow surrounded by mature bone, and the implant resembled an ossicle (Figure 7c and d).

In the HA implants bone marrow was still present at 24 weeks, although fat cells had

gradually invaded the pores and replaced the marrow. There was some osteoblastic activity, but most of the bone tissue was already quite mature and contained abundant osteocytes. Some giant cells could still be detected. The size and form of the implant were unchanged (Figure 7e and f).

No cartilage was detected in any implant. Bone formation did not show a centripetal pattern; instead bone was scattered all over the sample. New bone always appeared adjacent to the implant matrix.

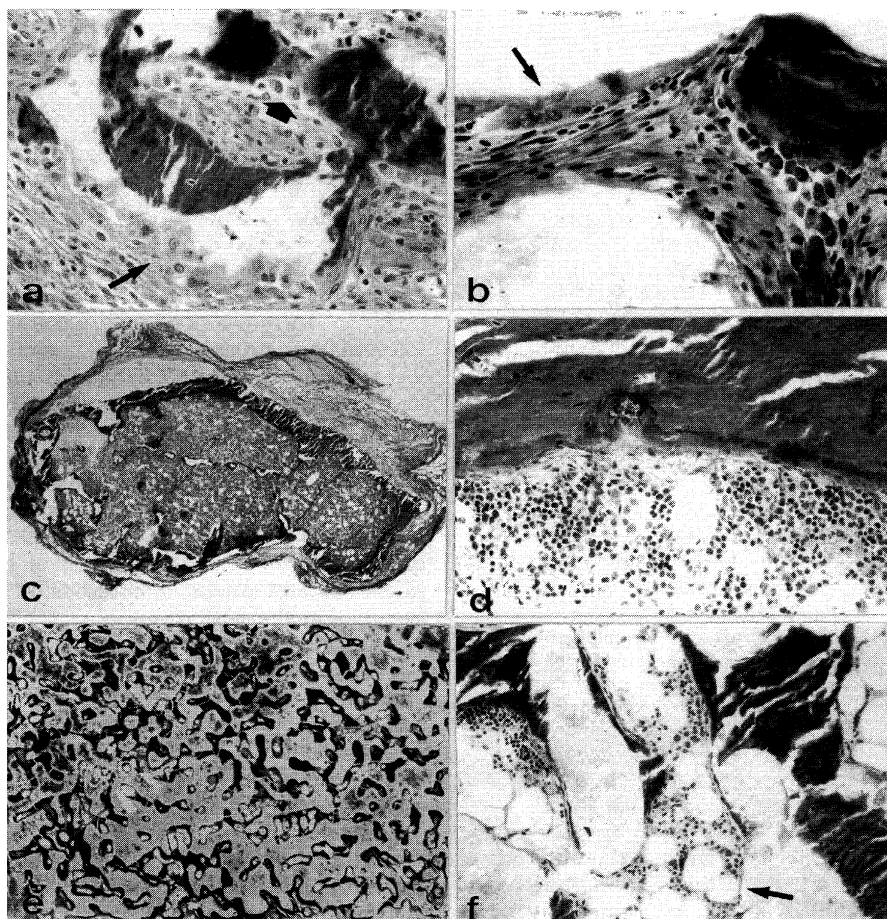

Figure 7. Bone formation in CC and HA implants with marrow (I)
a) CC at 3 weeks. Abundant osteoblastic activity (big arrow). Matrix (white) is partly phagocytized (arrow), x200.
b) HA at 3 weeks. Giant cells and macrophages lying directly on matrix (arrow), x260.
c) CC at 12 weeks. Implant has lost shape and form, bone marrow occupies central part and is surrounded by lamellar bone (dark) and matrix, x80.
d) Same specimen as in c: Osteoblastic activity and osteoid seam are still seen, x400.
e) HA at 24 weeks: Form and shape of matrix (grey) is preserved, bone (dark) invades entire implant, x80.
f) Same specimen as in e: Fat gradually replaces bone marrow (arrow), x400.

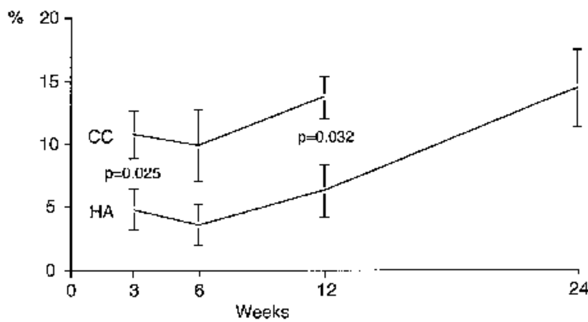

Figure 8. Bone ingrowth into CC and HA implants with bone marrow (I).
Bone ingrowth in the implant is given as percentages of mean (±SEM) bone area related to original total cross-sectional area of implant.
At 3 and 12 weeks the difference between materials is significant.

Table 3. Resorption of coral implants (I)

	mean (range)	
weeks	CC (%)	control (%)
3	87 (92-84)	86 (93-77)
6	62 (84-19)	61 (88-0)
12	40 (60-20)	

Values are mean total cross sectional areas in percentages related to original area before implantation. Maximum and minimum values are shown in parenthesis.

Histomorphometry

At 3 weeks there was significantly more bone in CC than in HA blocks, 10.8% and 4.8%, respectively, of the original cross sectional area (Figure 8). The CC implants were reduced to 87% of the original area (Table 3).

At 6 weeks the extent of bone formation had not changed significantly, the mean size of the CC blocks having decreased in the marrow group to 62% and in the control group to 61% of the original. The variation ranged from 88% to nil. In the control group one block was completely resorbed

At 12 weeks bone formation in the HA group was 6.3% and in the CC group 13.7%. The difference between the two materials was significant. The increase was not statistically significant within either group when compared with 3 or 6 week samples.

The size of the CC blocks was reduced to 40% and all the blocks remained.

At 24 weeks 14.4% of bone was found in the HA implants. Bone occupied 38% of the porous area (Figure 9).

Study II

This experiment was a continuation of the first study, and the protocol was similar up to the harvesting of the implants. There were no 12 week testing for CC, because the first study showed that the implant has been resorbed by that time point; neither was the 24-week HA group included.

Resorption

The coral implants maintained their shape after 3 weeks even though some resorption had already occurred. At 6 weeks the blocks treated with marrow could still be tested but the control blocks had deformed to such an extent that they could no longer be measured. All the HA blocks had preserved their shape, even at 12 weeks.

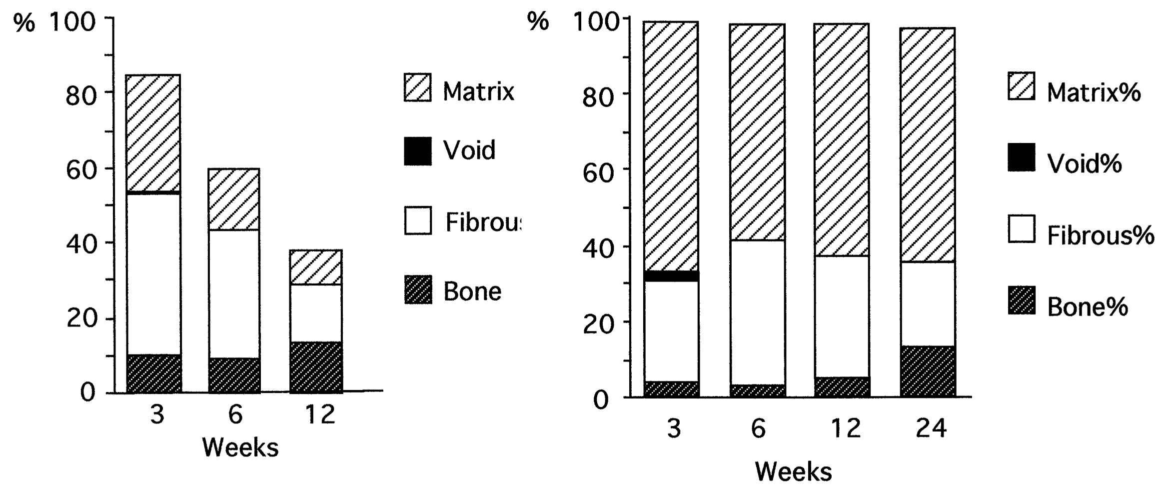

Figure 9. Tissue ingrowth into implants with bone marrow (I). Relative amounts of matrix and different tissue components and relative sizes of implants are presented for different follow-up periods. 100% is original total cross sectional area of implant.
Left side: coral implants, right side: hydroxyapatite implants.

Table 4. Compressive test results for hydroxyapatite

	% bone	compressive strength (MPa)		compressive modulus (MPa)	
	HAm	HAm	HAc	HAm	HAc
dry		5.7±1.6		151.3±55.3	
wet		2.6±0.7		75.5±37.0	
3weeks	13.5±11.4	6.9±2.3	4.7±2.3	96.4±66.9	137.0±78.0
6weeks	9.1±9.6	7.6±3.9	5.9±2.6	215.9±146.8	213.7±93.3
12 weeks	15.4±14.2	11±3.8	6.0±1.9	177.7±98.1	119+89.7

HAm = hydroxyapatite with bone marrow
HAc = control
% bone = Bone ingrowth is given as percentages of mean (± SD) bone area relative to porous cross-sectional area of implant

Table 5. Compressive test results for coral

	% bone	compressive strength (MPa)		compressive modulus (MPa)	
	CCm	CCm	CCc	CCm	CCc
dry		14.1±1.7		346.0±176.5	
wet		9.7±2.8		234.2±107.6	
3 weeks	20.1±8.8	2.3±1.4	0.9±0.3	33.6±26.3	14.1±11.8
6 weeks	25.0±16.4	4.0±3.2		80.7±89.8	

CCm = coral with bone marrow
CCc = control
% bone = Bone ingrowth is given as percentages of mean (± SD) bone area relative to porous cross-sectional area of implant

Compressive test

The test data are presented in Figure 10 and Tables 4 and 5. In the HA group with bone marrow, compressive strength was significantly higher at 3, 6 and 12 weeks than in the wet blocks without any tissue ingrowth (Figure 11a). The highest mean strength, 11.0MPa, was achieved at 12 weeks. The compressive strength did not, however increase significantly over time after 3 weeks in either the marrow or the control groups. The increase in strength in the control implants was significant at 6 and 12 weeks when compared with the wet blocks. The HA marrow group showed higher strength values than did the controls at 12 weeks but not at 3 or 6 weeks. There was no difference in the elastic compressive modulus over time within the HA with marrow or within control groups, nor when the groups were compared with each other.

The highest compressive strength in coral blocks was measured in the dry group (14.1MPa) after which the strength decreased, in the marrow group to 2.3MPa at 3 weeks. A slight, but non-significant, increase in compressive strength was observed in the marrow group at 6 weeks (4.0MPa).

The decrease in strength was highly significant when the dry CC blocks were compared with the 3-and-6 week specimens in the marrow group and also with the control implants. The 3-week coral control blocks were the weakest of all implants (0.9MPa). At 3 weeks strength values were significantly
higher in the coral marrow group than in the controls.

The elastic compressive modulus was lower in both implanted coral groups at 3 weeks than the dry blocks

The compressive strength and modulus values of dry coral were higher than those of the dry HA blocks. The values of wet coral were also higher than those of wet HA. The five diaphyseos samples of the cortical rat femur tested gave a mean compressive strength of 116MPa.

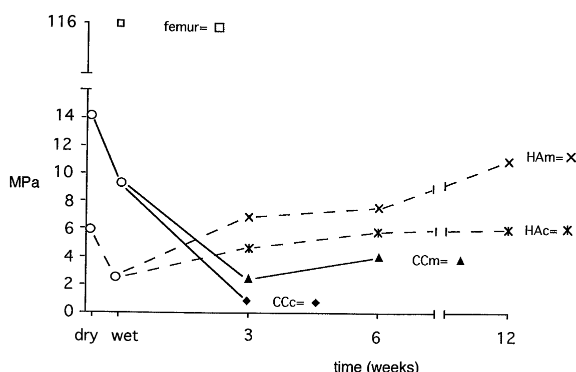

Figure 10. Compressive strength of implants (II).
Dry and wet implants are marked with O and are the same for blocks with marrow and for controls.

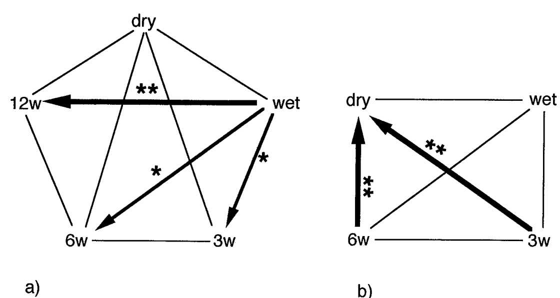

Figure 11. Statistical significance of compression strength testing of HA and CC implants with marrow (II).
a) hydroxyapatite, b) coral. The arrow shows the direction of the increase in strength.
*=p < 0.05, **=p < 0.01

Study III

Macroscopic observations

All the control coral blocks were clinically mobile at removal in contrast to the treatment groups, in which all implants, exept one, were firmly fixed to the calvarium. The one implant in the 8w/25µg group was partially unattached.

Growth inhibition assay

The mink lung epithelial cell test showed that the specific bioactivity of the TGF-ß1 released from the CC carrier was in active form.

Histology

In the coral control samples, the structure of the implants had already partly collapsed at 3 weeks (Figure 12A). In the TGF-ß1-treated implants, however, the shape and structure were still well preserved at 8 weeks (Figure 12B). In the non-treated samples, abundant reactive cells consisting of both giant cells and macrophages were noted in the pores of the coral implant. Marked resorption
of the biomaterial, leading to break-down of the structure, was also visible (Figure 12C). In the implants treated with TGF-ß1 the number of reactive cells was very low (Figure 12D). The control implants were completely filled with fibrous tissue ingrowth at 3 weeks whereas in the samples treated with 5 or 25µg of TGF-ß1, pores void of tissue could be observed (Figure 12E).

Only scattered lymphocytes, and no neutrophilic leucocytes, were detected, as none of the implants were infected.

The defect edge in the empty controls was roundish, with only marginal new bone formation. In the treatment groups, new bone had been produced mostly between te implant and the calvarial bone and above and underneath the defect edge and along the dura. Inside the implant, bone was found only to a lesser extent (Figure. 12F). In some cases, abundant osteoblasts and an osteoid rim could be seen after 8 weeks. In more mature areas, the new bone contained osteocytes and bone marrow cells (Figure 12F). Cartilage was not found.

Histomorphometry

In none of the groups did the newly formed bone bridge the defect, nor was there a statistical difference in the diameter of the hole. On the other hand, bone formation was statistically more abundant in all the 8-week treatment groups than it was in the empty or coral controls. There was more bone in the 5µg and 25µg groups at 8 weeks than at 3 weeks (Figure 13). The implant-calvaria interface showed more bone formation in the 8w/5µg group than in the coral controls and the amount of fibrous tissue in all the TGF-ß1 implants at 8 weeks was also lower than in the controls. The implant was statistically smaller in the 8 week coral control group than in the 8w/25µg group (Figure 14).

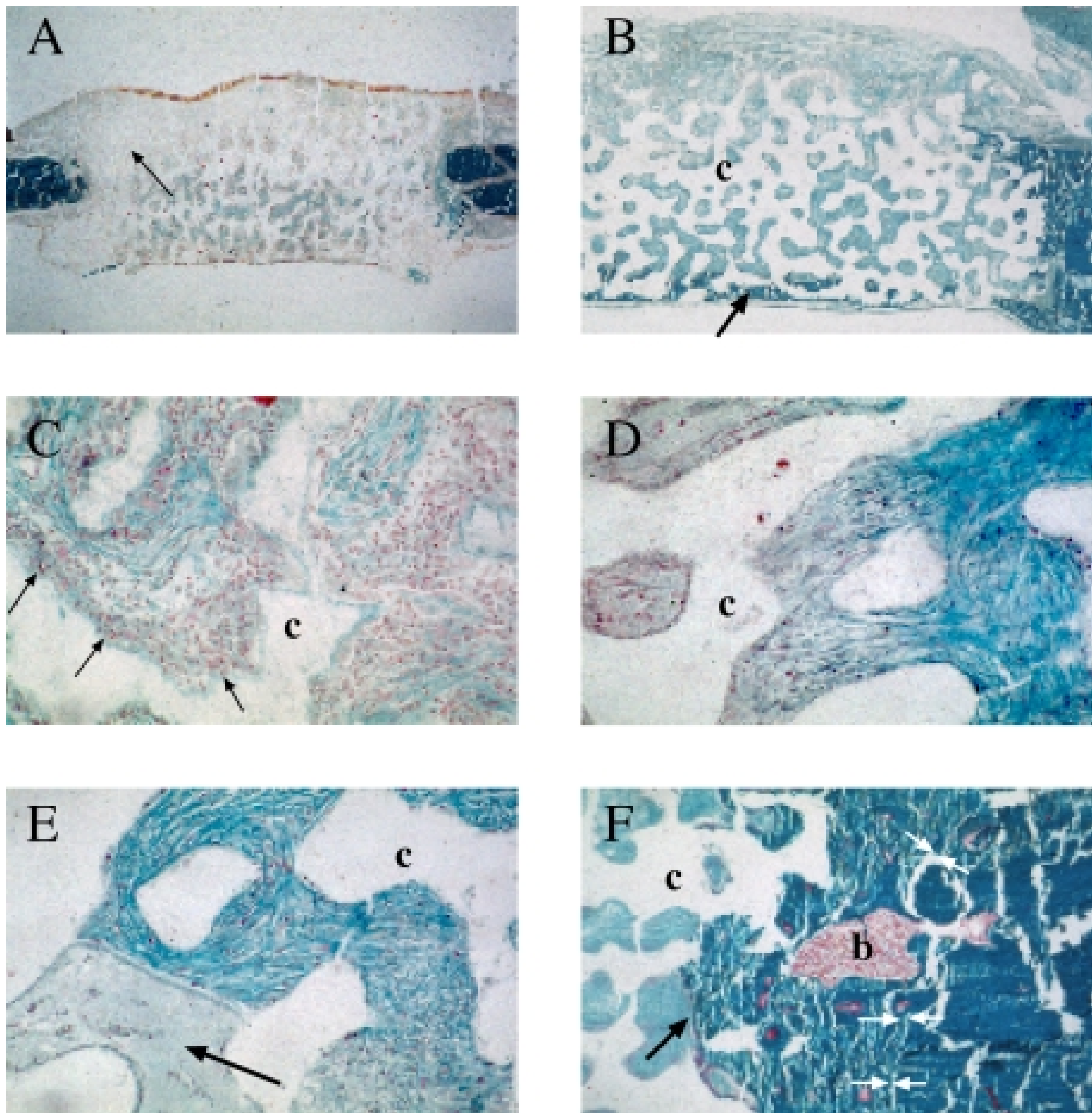

Figure 12. Histological samples from calvarial implants (III).
A) Coral control at 3 weeks (III). The coral structure has already partly collapsed (arrow). The calvarial margins have become roundish and only minimal bone formation can be seen on the left bone margin, x20.
B) 1µg TGF-ß1 implant at 8 weeks. Minor resorption has occurred in the upper part of the implant. The matrix structure (**c**) is well preserved. Bone has formed above and underneath the defect margin and is growing along the dura and inside the pores (arrow), x40.
C) Same specimen as in A. Abundant giant cells and macrophages lie directly on the matrix (arrow), x200.
D) Same specimen as in B. Only a few reactive cells can be observed. The pores are filled with fibrous tissue, x200.
E) 25µg TGF-ß1 at 3 weeks. Some pores are still void of any tissue ingrowth (arrow), x200.
F) 5µg TGF-ß1 at 8 weeks. White arrows point to the border of calvaria and new bone. Fibrous tissue fills the pores (light green) and an osteoid seam is present between the woven bone and fibrous tissue in the pore (black arrow). Bone marrow is visible in the new bone (**b**).

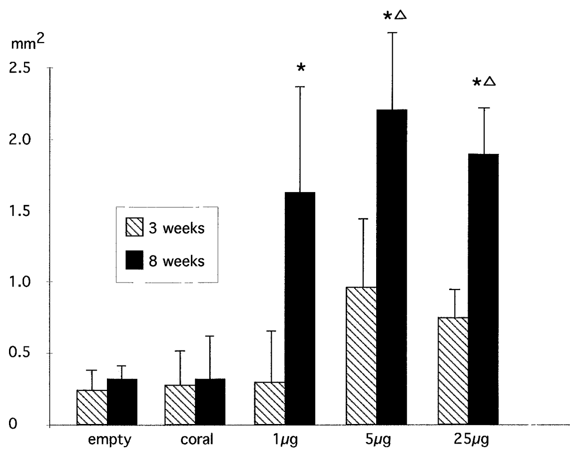

Figure 13. Bone formation in the calvarial defects (III)
Bone formation is given as the sum (±SD) of the bone area of the three fields measured in study III (Fig. 6A). The * symbol above the columns shows the statistical difference ($p<0.05$) from empty and coral controls. △ stands for enhanced bone formation ($p<0.05$) from 3 to 8 weeks inside the same TGF-ß1 dose group.

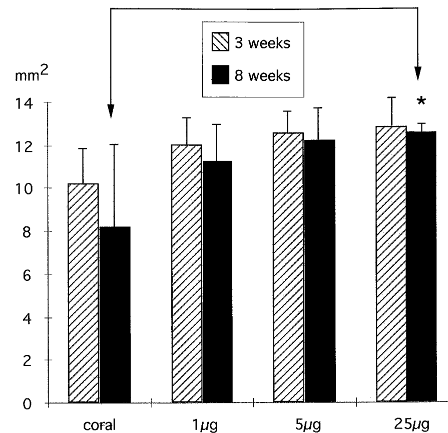

Figure 14. Implant resorption in the calvarial defects (III).
The * represents the statistically significantly lower ($p<0.05$) resorption of 25µg TGF-ß1 implant than in the 8-week coral control.

Study IV

Macroscopic observations

The immediate postoperative recovery of the donor sites was uneventful. All implants were clinically tightly fixed at 1 year. In one patient, pieces of the coral implant started to extrude after 1.2 years and the remaining implant was removed 1.7 years after the operation.

Radiographic findings

The implants showed up clearly in plain x-rays in the early postoperative phase, but after 6 months the contours had become indistinct and the exact boundaries of the blocks were difficult to detect. The central part remained more radio opaque, indicating that resorption was proceeding centripetally (Figure 15A and B).

CT scans showed the implants more clearly (Figure 15C-E). Although they had become smaller over time, there was no significant change in density, neither in the block remaining nor when the immediate postoperative density was compared with the final measurements. Fragmentation was noted in five patients; this most likely occurred through the existing two burrholes (designed for fixation) or through the hole caused by the biopsy .

In the control patients, the donor site was deformed, the remodelling of the bone making

the defect edges rounder and flatter but never filling the defect to its original shape.

Resorption

All implants resorbed to some extent, but none completely (Table 6). Three blocks did not change their resorption category from 1 year to the end of the follow-up period. The iliac crest did not regain its original form in any patient during the follow-up.

Table 6. Resorption of coral implants (IV)

Material remaining	1year	final
76-100%	2	1
51-75%	7	2
26-50%	0	5
0-25%	1	2

The values are the number of coral blocks, that fall into a certain resorption category at one year and in the end of the study.
The final follow-up time was mean 2.1 (range 1.6-2.3) years, median 2.1

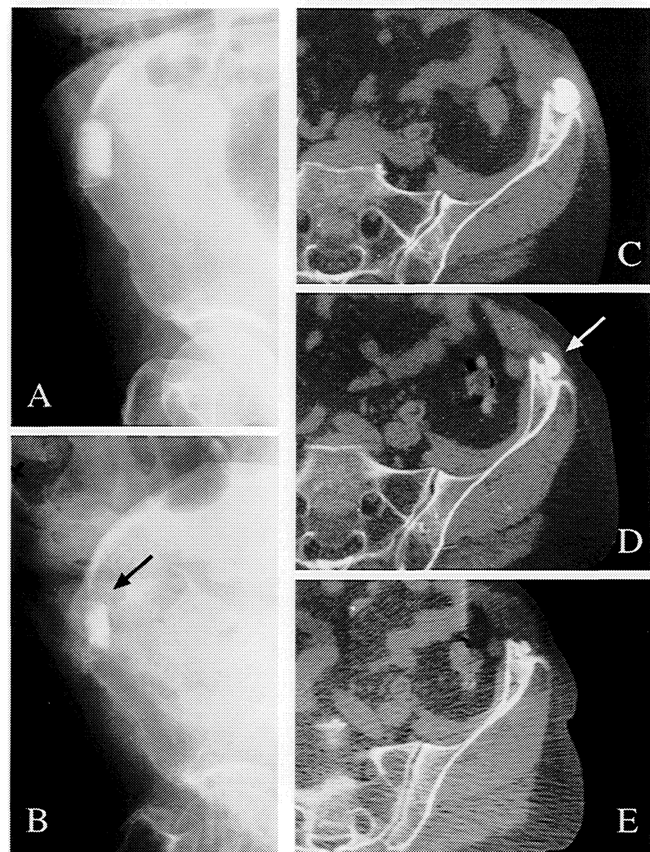

Figure 15. Plain x-rays and CT scans of two patients (IV). A and B are from a different person than C-E. The implant in CT scans C-E resorbed the most rapidly of all blocks.
A) X-ray of coral block 3 months postoperatively
B) The same block after 2.3 years. The arrow points to the resorbed part of the implant.
C) CT scan 2 months postoperatively. The implant is integrated with bone.
D) At 1 year, the implant has fragmented; the arrow points to the larger piece.
E) Only small fragments of coral remain after 2.1 years. The shape of the iliac crest has not been restored.

Histology

The histological findings are summarized in Table 7. Three of the ten samples were excluded from the histological study, as they contained mature bone and bone marrow without any signs of coral matrix. The x-rays and CT scans showed that at least 50% of the implant still remained in these samples, too, suggesting that they had been taken from adjacent normal bone and therefore did not represent the implant site.

Ingrowth of fibrous tissue was observed in six of seven samples and in two of them bone was also detected (Figure 16A and B). Only in one sample was the coral matrix totally devoid of tissue; this block resorbed the least of all implants (Figure 16C).Giant cells and macrophages were present in areas with tissue ingrowth. These reactive cells were in direct contact with the matrix, indicating their function in the resorption process (Figure 16D). Scattered lymphocytes were seen in a few samples, the majority not containing any lymphocytes or granulocytes at all.

One implant was infected at 1.2 years, 2 months after the biopsy (patient no. 7). The 1 year biopsy specimen was without leukocytes, suggesting that the implant became infected after the sample was taken. This implant had to be removed after 1.7 years. The matrix was well preserved in the particles removed, and some fibrous ingrowth and bone could be detected. Abundant granulocytes, a sign of infection, were also observed (Figure 16E and F).

Table 7. Histological findings at 1-year postimplantation

Patient	Matrix	Fibrous tissue	Bone	Marrow	Void	Inflammatory cells	Macrophages	Giant cells
1	+	+	++	-	-	+	+	-
2	++	+++	-	-	-	-	++	++
3	+++	-	-	-	+++	-	-	-
4	-	+	++	-	-	-	+	-
5	++	++	-	-	+	-	++	+
6	++	+	-	-	++	+	+	+
7	++	+	-	-	++	-	+	+

Seven biopsy samples out of 10 were included for histological examination and evaluated on a four-step quantitative scale from (-, nil) to (+++, pores fully occupied). As for the cells (three last colums), (+) stands for scattered cells, and (+++) for abundant amount of cells. Exeptionally, the matrix column presents morphology: (-) implies completely collapsed structure and (+++) wholly preserved structure. The patient numbers are as in Figure 16.

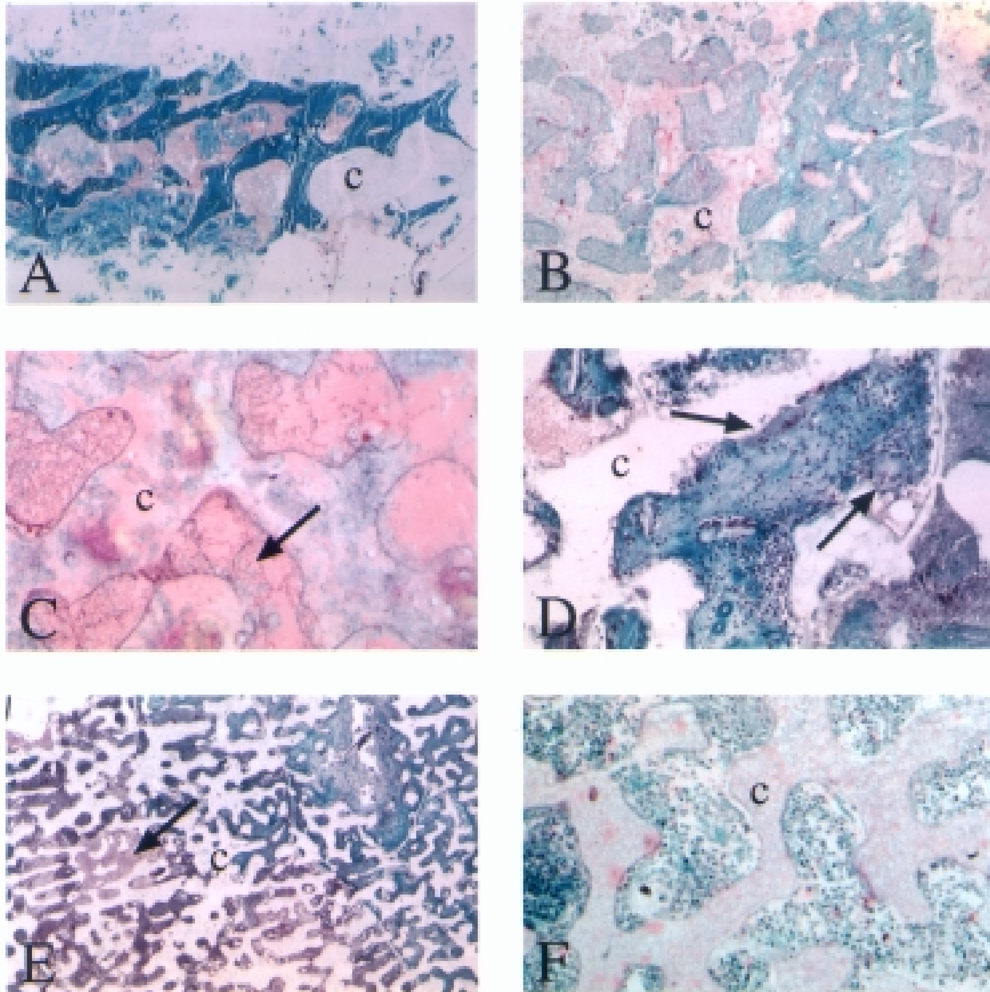

Figure 16. Histological samples from biopsies (IV). "C" indicates coral matrix. The patient numbers refer to the same patients as in Table 7.
A) Patient no. 1. Lamellar bone is seen around collapsed coral matrix, x15
B) Patient no. 2. Fibrous tissue fills all coral pores. No bone is seen and the coral matrix is partly absorbed, x40.
C) Patient no. 3. The matrix is completely preserved, no ingrowth is seen in the pores (arrow), x130.
D) Patient no. 6 Several multinucleated giant cells lie adjacent to the coral matrix (arrows). Fibrous tissue, but no bone, fills the pores, x150.
E) Patient no. 7.The pattern of coral matrix is well preserved. In some areas the pores are devoid of tissue ingrowth (arrow), whereas fibrous tissue and reactive, but no inflammatory, cells are present in most pores, x10.
F) Patient no. 7. The infected implant after removal at 1.7 years. The matrix is well preserved and the pores are filled with granulocytes, x120.

GENERAL DISCUSSION

Background

The porous structure of natural coral, genus *Porites*, was previously found to be similar to that of osteon evacuated cortical bone (Holmes 1979) and to allow tissue ingrowth (Hulbert et al. 1970; Chiroff et al. 1975). The hydrothermal exchange reaction enabled the calcium carbonate skeleton to be converted to a calcium phosphate replica. Hydroxyapatite (calcium phosphate) forms 85% of the inorganic part of bone, and it was only natural to develop implants that resemble bone tissue in constitution and structure. HA has many good qualities for a bone substitute but it resorbs very little. The use of HA as bone substitute has been widely documented since 1975 and new applications are still being developed (Costantino et al. 1992).

The coral skeleton is 99% calcium carbonate and can also serve as bone substitute. CC is resorbable but otherwise resembles HA.

Coral was chosen in the present investigation because of the promising reports published. Until the early 1990s there was no experience of calcium carbonate as a bone substitute in Finland, whether in clinical or in experimental works.

Even though experiments on natural coral as bone substitute started back in the 1970s (Hulbert et al. 1970; Guillemin et al. 1981), coral is still not a well known material. One reason is that most reports of the earlier experiments on coral were published in French.

Bone ingrowth

Bone marrow

When HA implants without bone marrow have been placed in bone defects, bone ingrowth into HA has been variable. In orthognatic patients, Holmes an coworkers found 18% bone invasion (compared to total measured area) in a follow up time of 4.7 to 16.4 (mean 9.3) months in HA implants (Holmes et al. 1988). Martin and coworkers placed HA blocks in cortical defects of dog humerus and radius. Bone ingrowth into pores increased from 52% at 16 weeks to 74% at 1 year (amount of bone relative to porous space). In the cancellous site, bone ingrowth was 38% at 4 weeks, then fell to 17% at 1 year (Martin et al. 1993). It would appear that the entire porous space of HA is seldom completely filled with bone (Rosen and McFarland 1990).

Bone ingrowth into CC is more difficult to measure because of resorption. CC loses its internal porous structure very quickly, in our study (I) after 6 weeks, and after that the bone does not actually invade the pores but replaces the matrix.

In studies III and IV we observed that when CC implants are placed into bone defects the amount of new bone without any inducing factor is scarce. The coral controls in calvarial defects (Figure 13) showed very little bone formation (III) and no bone could be detected in five of seven biopsies taken from the iliac crest implants at one year (IV).

Adding bone marrow to porous calcium ceramics induces membranous bone formation in extraosseal sites. This has been shown with tricalcium phosphate blocks implanted subcutaneously in rats (McDavid et al. 1979), with HA blocks implanted intramuscularly in rabbits (Nade et al. 1983) and rats (Okumurai et al. 1991; Ohgushi et al. 1992) and also with CC implanted subcutaneously in rats (Ohgushi et al. 1992). In study I bone was formed in all implants placed intramuscularly with bone marrow but in none of the controls. More bone was produced in CC than in HA implants at 3 and 12 weeks, although at 12 weeks the CC blocks had been reduced to 40% of their original cross sectional size. The absolute amount of bone did not increase significantly within either group after 3 weeks, but there was a trend of increasing bone formation in HA implants at 24 weeks (p=0.051), when the amount of bone was about the same as in CC implants at 12 weeks (14.4%/13.7%, Figure 8). As the two implants are similar in structure, the difference in chemical constitution and in the resorption process of the CC matrix itself may affect bone formation.

Ohgushi and coworkers implanted CC and HA disks with bone marrow into subcutaneous pouches in rats. At 4 weeks bone occupied 19.1% (HA) and 22.0% (CC) of the porous area (Ohgushi et al. 1992). Our results (I) for intramuscular sites at 3 weeks were 13.5% (HA) and 20.1% (CC). Ohgushi found bone only in blocks immersed with bone marrow, not in those without it. This is in accordance with our findings here and also with previous reports where porous ceramics were used with bone marrow in extra osseal sites in rats (McDavid et al. 1979; Nade et al. 1983).

TGF-ß

The use of autogenous bone marrow requires a harvesting operation. The amount of marrow being limited, it would be ideal to

have a prefabricated, " off the shelf" bone substitute with bone induction capacity.

In recent years, transforming growth factor beta (TGF-ß) has been examined experimentally together with a variety of biomaterials to induce bone growth. Although promising, the results must be interpreted with great caution owing to the complex and multiple effects of TGF-ß (Beck et al. 1991; Gombotz et al. 1994; Ripamonti et al. 1997; Schmitt et al. 1998).

The data presented in study III demonstrate that TGF-ß1 applied to natural coral implant significantly enhanced new bone growth in the calvarial defect of nongrowing rats (Figure 13). The bone grew mainly at the margins of the defect but did not completely bridge the implant in any of the specimens during 8 weeks.

Bone formation was the result of "a single injection" of TGF-ß1 delivered from the NC implant. Beck and coworkers showed that a single injection of TGF-ß in 3% methylcellulose triggers a cascade of events leading to new bone formation in rabbit skull in the course of 49 days (Beck et al. 1991). Apparently TGF-ß can bind tightly to bone matrix in its active form, as shown when radioiodinated recombinant human TGF-ß1 in a 3% methylcellulose vehicle was applied to a skull defect in rabbit. Sixteen days later, radioactivity was still detected in the defect area where rhTGF-ß1 was incorporated into the matrix (Richardson et al. 1993). These results show that the effect of TGF-ß can be prolonged and that it probably takes place through various mechanisms.

Our findings are in accordance with the above data. The 5µg and 25µg implants showed a tendency towards enhanced bone formation at 3 weeks but the formation was not statistically significant until at 8 weeks when compared with the control implants (Figure 13). The influence of TGF-ß1 on bone formation may therefore last for several weeks.

Coral has also been used as a TGF-ß carrier but, to our knowledge, previous studies were performed with the granular form of the material and the results cannot as such be directly compared with ours (Damien et al. 1993; Arnaud et al. 1994; Boden et al. 1997; Wikesjö et al. 1998; Arnaud et al. 1999). Arnaud and coworkers tested 1µg of TGF-ß1 in association with coral granules and fibrin glue in a CSD model and obtained significantly better bone formation with this combination at 2 months than with TGF-ß1 in methylcellulose or in fibrin glue alone. Histological sections showed that bone growth proceeded around the gran-

ules, not inside them as with solid, porous coral material (Arnaud et al. 1994).

In our study, bone formation was enhanced by TGF-ß but was limited mainly to the defect margin area. The rats were 6 months old and can be considered mature, nongrowing animals. When porous hydroxyapatite implants were soaked in bone marrow cell suspension and implanted subcutaneously in young (8-week) and old (60-week) rats, the bone formation capacity was lower in the aged rats (Inoue et al. 1997). Similar results have been obtained in other experiments on old rats (Quarto et al. 1995). Thus age also seems to affect the capacity of TGF-ß to stimulate bone formation and may explain, at least to some extent, the limited bone ingrowth in the implants studied here.

We did not observe dose dependence on bone formation at different concentrations of TGF-ß1. The optimal dose has not previously been defined. A single injection of 1µg of TGF-ß1 into neonatal rat periosteum caused parietal bone thickening at 12 days but a 200ng dose resulted only in increased formation of osteoprogenitor cell layers and bone matrix at the early stage, not after 12 days (Tanaka et al. 1993). Recently, a much lower dose, 5 or 25ng, injected under the periosteum caused woven bone formation in 4-week-old rats (Fujimoto et al. 1999). However, the same study showed a decrease in bone formation when TGF-ß1 was injected into the periosteum repeatedly. Centrella and coworkers noted that the effect of TGF-ß on bone cell replication is biphasic and depends on both the TGF-ß concentration and the cell density in monolayer culture (Centrella et al. 1987). Dose dependence is even more complicated when TGF-ß is released from a vehicle. MacKinney and coworkers noted that a 40µg dose of TGF-ß1 in 3% methylcellulose promoted more bone formation than did 0.4 or 5µg in a rabbit CSD experiment (McKinney and Hollinger 1996). Earlier, Beck and coworkers had obtained similar results (Beck et al. 1993). In a recent rabbit CSD experiment using bone wax polymer as the carrier, a dose of 2µg of TGF-ß led to decreased bone formation at 12 weeks. It was speculated that this was due to the incomplete biodegradation of the carrier (Schmitt et al. 1998). Used in conjunction with TGF-ß1, gelatine sponge has stimulated very limited bone formation in rat calvaria (Bosch et al. 1996) at doses of 2, 5 and 10µg. The follow-up time was only 2 weeks and the rats were nongrowing, 6-month-old animals. In addition, the control defects were in the same animal, and thus the TGF-ß may have diffused to the control site.

These factors may explain the poor results. Ripamonti and coworkers obtained equally poor results in primates, and suggested that the limited bone formation was the result of TGF-ß's capacity to stimulate the proliferation of only periosteal and endosteal cells rather than to initiate bone cell differentiation, as does bone morphogenetic protein (Ripamonti et al. 1996).

These and other results show that the effect of TGF-ß on bone formation in animals depends, not only on the age and species of the animals, but also on the doses used, the location of the injection site and the type of delivery material employed.

Compressive strength

In the compressive strength experiment (II) we used HA and CC implants treated in the same manner as in study I. The results (II) should be related to those obtained in study I (Figures 8 and 10).

The implanted HA blocks were stronger than the wet, unimplanted specimens (Table 4 and Figure 11a). The compressive strength and modulus did not increase after 3 weeks. At 12 weeks a difference was noted in compressive strength between the HA with marrow and the control implants. The amount of bone in study I at 12 weeks constituted 15.4% of the porous area. It did not increase significantly after 3 weeks, although there was an increasing tendency at 24 weeks.

Piecuch and coworkers implanted HA blocks similar to ours on the edentulous ridge of dogs for 24 months. The compressive strength was greater than with dry (not implanted) specimens but was not related to the amount of bone ingrowth (Piecuch et al. 1984). Martin and coworkers implanted porous (porosity 65-75% and pore size 260-600 µm) HA blocks into cortical bone of the radius and cancellous bone of the humerus in dogs and noticed nonlinear correlation with bone ingrowth and compressve strength in the cortical site. The bone ingrowth (% bone of the porous space) increased from 22% at 4 weeks to 74% at 1 year and the increase in compressive strength and elastic compressive modulus was significant (Martin et al. 1993). Ohgushi and coworkers used an intramuscular rat model similar to that used here and induced bone formation with marrow into porous (400 µm) implant with 60% hydroxyapatite and 40% tricalcium phosphate. They found that the compressive strength and rigidity were already significantly higher at 4 weeks in blocks with bone formation than in controls

with only fibrous tissue ingrowth (Ohgushi et al. 1989).

Data from several sources show that the exact effect of bone ingrowth on the compressive strength and compressive elastic modulus of porous implants needs further study. The mode of tissue ingrowth differs, depending on whether the implants are placed in soft tissue or in direct contact with bone. The outcome is also affected by pore size and porosity (Le Huec et al. 1995).

The porous material with tissue ingrowth acts as a composite structure. The implanted block consists of the mineral matrix of the block, fibrovascular tissue and bony tissue. Each component affects on the compressive strength, but, with CC, resorption makes interpretation very difficult. The matrix resorbs and softens and the porous structure changes. The importance of new bone formation is thus underlined.

If bone ingrowth is insufficient, the effect of fibrous tissue on the strength becomes important. In study I, the pores of HA blocks without marrow were fully filled with fibrous tissue at 3 weeks. We observed a significant increase in strength in blocks without bone marrow when compared with wet blocks. The elastic compressive modulus did not change but fibrovascular ingrowth seemed to make the HA implant stronger, although not as strong as implants with bone ingrowth.

The material strength and modulus of coral were high compared with those of HA but there was a very distinct decrease in strength at 3 weeks (Figure 10). After the implantation, the compressive strength and the compressive elastic modulus remained unchanged between 3 and 6 weeks in the marrow group. In study I bone occupied 20.1% of the porous area at 3 weeks in coral implants with marrow. In the mechanical test, the 3-week implants were stronger than the controls, which previously had shown only fibrous tissue ingrowth (Table 5). At 6 weeks, the coral blocks with marrow had partly resorbed, but the strength and modulus remained at the same level. This may be the result of two opposite reactions: the matrix resorbs slowly but at the same time the amount of bone related to the reduced implant size increases. The compressive strength and modulus for coral in our study were comparable to those of cancellous bone up to 6 weeks in the marrow group but were already very low at 3 weeks in controls.

Biodegradation

Well documented HA was chosen as a reference material for CC because of the similar-

ity in their structure. The limited resorption of HA (White and Shors 1986) was confirmed in our study (I-II). Whereas, the resorption of coral varied widely (Table 3 and 6). It has been suggested that the main factor in the resorption process is carbonic anhydrase, an enzyme abundant in osteoclasts (Guillemin et al. 1981). The enzyme lowers the pH at the osteoclast-implant interface, dissolving the calcium carbonate matrix (Chétail and Fournié 1969). Resorption is most active in the bone-matrix contact areas and proceeds centripetally (Braye et al. 1996). The same resorption pattern was noted here (I, III, IV), with or without the presence of bone tissue.

Resorption through carbonic anhydrase activity is not the only, possibly not even the most important, resorption mechanisms. There was no bone formation in control implants in study I but the resorption rate was similar to that in the bone marrow group (Table 3). In study II the control implants at 6 weeks could not been tested due to deformation. Giant cells were found in control implants (I) but we could not determine whether or not these cells were osteoclasts. In the orthotopic site (III), where abundant osteoclasts are present, the control implants showed resorption at 4 weeks but all the implants remained, albeit deformed, at 8 weeks. One would expect faster resorption if the carbonic anhydrase enzyme were the main factor in the resorption process.

Fricain and coworkers studied mouse macrophages and human fibroplasts *in vitro* and suggested that these cells are capable of phagosytizing coral. Direct contact between these cells and the coral matrix is a prerequisite for the process (Fricain et al. 1998, a). In fact, the control implants in study I were already filled with fibrous tissue at 3 weeks, which could explain the similarity in the resorption rate to that in the marrow group. The results of study IV are in accordance with this observation. One implant placed to the iliac crest (IV) had no tissue ingrowth at 1 year; this implant remained the largest block throughout the follow up period (Figure 16c). We also studied the histology of the one infected block 1.7 years after implantation, and found that the coral matrix was completely preserved in areas without tissue ingrowth (Figure 16 f). In contrast, the block that resorbed the fastest was fully occupied by bone and fibrous tissue at 1 year.

In study III the 25µg TGF-ß1 group showed less resorption at 8 weeks than did the controls (Figure 14). In general, the structure of the TGF-ß1-treated implants was much better preserved than that of the coral

controls, in which the matrix had partly collapsed (Figure 12A). Diminished resorption of CC has not previously been reported in conjunction with TGF-ß.

TGF-ß stimulates fibroblast growth (Leof et al. 1986; Franzen and Dahlquist 1994) but deactivates macrophages (Tsunawaki et al. 1988). The number of macrophages in our TGF-ß1 treated implants was lower than in our coral controls (Figure 12D). On the other hand, empty areas without any fibrotic tissue inside the CC implants could be detected in the 5 and 25µg 3-week groups, implying possible inhibition of fibrotic tissue growth (Figure 12E). We were unable to explain this apparent discrepancy in diminished fibrotic tissue growth and known fibrotic tissue stimulation by TGF-ß. It is nevertheless consistent with the diminished resorption of CC.

Resorption appears to proceed more rapidly in animals than in humans. When CC blocks were implanted in the cortex of the femur and tibia in pig and sheep, resorption at 1 month was 64% and 93%, respectively (Guillemin et al. 1989). Complete resorption of CC granules has been observed at 24 weeks in a connective tissue site in pigs (Naaman et al. 1994). In our experiment in the intramuscular site the coral implants were deformed at 6 weeks and one implant was completely resorbed in the control group (I). At 12 weeks the mean total cross-sectional area was 40% of the original (Table 3.)

In humans, degradation seems to be slower. 50% of the blocks used to fill the 10mm cranial burr holes resorbed completely at 1 year (Roux et al. 1988). In spinal fusions small fragments of natural coral blocks were found after 1 year (Pouliquen et al. 1989). When traumatic methaphyseal defects were filled with coral blocks, resorption times of over 4 years have been reported (de Peretti et al. 1996).

Our experiment showed that CC implants placed into the iliac crest were less than 50% of their original size after 2 years. None of the blocks resorbed completely and one implant was more than 75% of its original size (Table 6). The resorption is unpredictable and the mechanisms are not fully understood.

SUMMARY

The main purpose of this study was to examine bone ingrowth into hyroxyapatite and calcium carbonate bone substitutes when bone ingrowth was induced with bone marrow or TGF-ß. In addition, special interest focused on the resorption rate and pattern of CC implants.

A total of 113 rats received 167 HA or CC implants. CC implants were also placed into iliac crest defects in 10 humans.

In the first experiment CC and HA blocks were implanted in rat latissimus dorsi muscle with autogenous bone marrow to compare their bone-forming capability. A block without marrow placed in the opposite latissimus muscle served as a control.

The animals were killed at 3, 6 and 12 weeks and, in the hydroxyapatite group, also at 24 weeks. The sections were analysed histologically and histomorphometrically.

The second experiment examined the effect of tissue ingrowth on the compressive strength of the CC and HA implants. The implants were obtained in a process similar to that used in the first investigation.

In the third experiment 1μg, 5μg and 25μg of TGF-ß1 were added to CC blocks that were placed into a critical size defect in the parietal bone of rat. The implants were retrieved at 3 and 8 weeks and the capacity of TGF-ß1 to enhance bone formation was examined by histology and histomorphometry in undecalcified sections.

In the fourth investigation a bicortical bone graft was harvested from the iliac crest of 10 patients and the defect was filled with a CC block. The fate of the implant was monitored by X-rays and quantitative CT scans for a mean of 2.1 years. A biopsy was taken at 1 year. Another 10 patients with a similar defect but without any implant served as controls.

Study I showed that bone formation could be induced into calcium ceramics by bone marrow. More bone was found in CC than in HA implants at 3 weeks (10.8% versus 4.8%) and at 12 weeks (13.7% versus 6.3%, bone/total original block area). There was no bone in control implants.

TGF-ß1 induced significant, but limited, bone enhancement in the CSD experiment (III). None of the implants showed bone bridging over the defect.

In the orthotopic site in humans, at iliac crest site, only two of seven biopsies showed bone ingrowth into the implant (IV).

Bone ingrowth resulted in greater compressive strength in HA implants than in controls at 12 weeks (II). CC resorbed quickly and lost its compressive strength, which was originally higher than in HA. At 3 weeks the marrow group was stronger than the control CC specimens. Bone ingrowth seemed to maintain the strength of the coral implant even if it was dissolving. The mechanical strength of both materials was comparable to that of cancellous bone.

Resorption was not observed in HA implants. The resorption in CC implants was evident in the intramuscular site at 3 weeks and was similar in both control and bone marrow implants. At 12 weeks the cross-sectional area of the block had diminished to 40% of the original area.

Resorption was lower in the TGF-ß1 treated implants (III) than in the control implants. The number of macrophages and giant cells was reduced in the TGF-ß1 implants, which showed less resorption and more intact structure than did the coral controls. Void areas without any fibrous tissue ingrowth were found only in the TGF-ß1-treated implants, which may partly explain the reduced resorption.

Resorption proceeded centripetally and apparently more rapidly when accompanied by tissue ingrowth (I, II, III and IV). None of the implants placed into the iliac crest resorbed competely during follow-up (mean 2.1 year).

CONCLUSIONS

1) Bone marrow induced more bone ingrowth into CC than into HA implants in an intramuscular site, even though CC resorbed over time. Bone did not form without bone marrow. Fibrovascular and bone tissue already occupied the pores almost completely at 3 weeks.

   TGF-ß1 promoted significant, but only limited, bone ingrowth into CC implants in an orthotopic site. Bone formation did not depend on the TGF-ß1 dose. The age of the animals may have contributed to the limited response.

2) Bone ingrowth into HA enhances its compressive strength. CC dissolves and becomes weaker even if bone ingrowth seems to maintain the strength to some extent. The compressive strength of both materials is comparable to that of cancellous bone but is not sufficient for them to be used in weight bearing areas.

3) The resorption of CC varies widely in the intramuscular site. There was no difference between the control and marrow groups, which suggests that mechanisms other than resorption by the carbonic anhydrase enzyme may play a more important role in the resorption process.

   TGF-ß1 diminished the resorption of CC implants, possibly by reducing soft tissue ingrowth and by deactivating macrophages.

   In humans the resorption of large CC implants in an orthotopic site proceeds very slowly and the implant is only partially replaced by bone.

   If CC or HA implants are used as bone substitutes, bone formation should be induced either by bone marrow or, in the future, by a combination of different growth factors.

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