OSTEO-INDUCTIVE POTENTIAL OF DIFFERENT DOSES OF RECOMBINANT HUMAN OSTEOGENIC PROTEIN-1

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DECLARATION

I, Petrus Johannes Odendaal declare that this dissertation is my own work. It is being submitted for the degree of Master of Dentistry at the University of Pretoria, Pretoria. It has not been submitted before for any degree or examination at this or any other University.

.....(Signed)

...15...day of December..., 1999

This work has been performed at the Bone Research Laboratory, Department of Orthopaedics, University of the Witwatersrand, Johannesburg, and the Medical Research Council.

DEDICATION

I dedicate this work to

God, our creator, who blessed me with good health and a sound mind to enable me to complete this dissertation,

my parents who instilled in me the drive to always complete whatever I set out to do,

my wife, Almarie, who supported me with love and understanding.

ABSTRACT

Members of the bone morphogenetic protein (BMP) family induce endochondral bone formation *in vivo*. The ability of various doses of recombinant human osteogenic protein-1 (rhOP-1), to induce bone in the non-human primate, has been demonstrated at 15 and 30 days. This study evaluates the efficacy of different doses of rhOP-1 after a 90-day observation period.

We examined the efficacy of a single application of rhOP-1 to induce bone in the rectus abdominis of the baboon (*Papio ursinus)*. Doses of 0.1, 0.5 and 2.5 mg of rhOP-1 in conjunction with 100 mg of bovine guanidinium-extracted insoluble collagenous bone matrix (ICBM) as carrier were implanted in the rectus abdominis of two adult female baboons. Specimens were harvested on day 90 and subjected to alkaline phosphatase determination and histological and histomorphometric determination, on serial undecalcified sections cut at 7 µm.

Doses of 0.5 and 2.5 mg of rhOP1 induced heterotopic bone formation. Histological analysis showed ossicles composed of mineralized bony trabeculae, interspersed with bone marrow and remnants of ICBM. A nearly continuous osteoid rim supporting osteoblasts lined trabeculae and was interrupted by areas of mineralized bone resorption. Multinucleated giant cells characterized areas of bone resorption. The parameters of bone induction, as evaluated by histomorphometry and biochemical analysis, demonstrated comparable activity of 0.5 and 2.5 mg rhOP-1.

SAMEVATTING

Lede van die been morfogenetiese proteïen (BMP) familie induseer endochondrale beenformasie *in vivo*. Die vermoë van verskillende dosisse rekombinante menslike osteogene protein-1 (rmOP-1) om been in die nie-menslike primaat te induseer, is alreeds na 15 en 30 dae aangetoon. Hierdie studie evalueer die effektiwiteit van verskillende dosisse rmOP-1 na 'n 90-dae observasie periode.

Ons het die effektiwiteit van 'n enkele aanwending van rmOP-1 om been in die rectus abdominis van die bobejaan (*Papio ursinus*) te induseer, ondersoek. Dosisse van 0.1, 0.5 en 2.5 mg rmOP-1, met 100 mg runderagtige guanidinium-geëkstraheerde onoplosbare kollageneuse beenmatrys (OKBM) as draer, is in die rectus abdominis van twee volwasse vroulike bobejane ingeplant. Monsters is na 90 dae geoes en onderwerp aan alkaliese fosfatase bepaling. Ongedekalsifiseerde reekssnitte van 7 µm-dikte is histologies en histomorfometries ondersoek.

Beide 0.5 en 2.5 mg dosisse rmOP-1 het heterotopiese beenformasie geïnduseer. Histologiese analise het ossikels getoon wat bestaan uit gemineraliseerde benige trabekels, vermeng met beenmurg en oorblyfsels van OKBM. Trabekels was uitgevoer met byna aaneenlopende osteoïede krae, belyn deur osteoblaste. Hierdie osteoïede krae was onderbreek deur areas van gemineraliseerde beenresorpsie. Veelkernige reuseselle het areas van beenresorpsie gekarakteriseer. Die parameters van beeninduksie, soos deur histomorfometrie en biochemiese analise geëvalueer, het vergelykbare aktiwiteit met 1.5 en 2.5 mg rmOP-1 getoon.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

ALP:	alkaline phosphatase
BMP:	bone morphogenetic protein
cAMP:	cyclic adenosine monophosphate
cDNA:	complementary deoxyribonucleic acid
CDMP:	cartilage-derived morphogenetic protein
DBM:	demineralized bone matrix
DDP:	decapentaplegic protein
DNA:	deoxyribonucleic acid
FRCC:	fetal rat calvarial cells
Gdn-HCl:	guanidinium-HCl
HBC:	human bone cell
h:	human
ICBM:	insoluble collagenous bone matrix
IGF:	insulin-like growth factor
IGFBP:	insulin-like growth factor binding protein
MNCs:	multinucleated cells
mRNA:	messenger ribonucleic acid
OP:	osteogenic protein
rh:	recombinant human
TGF:	transforming growth factor
TRAP:	tartrate-resistant acid phosphatase
Vg-1:	vegetal protein

CHAPTER ONE: INTRODUCTION

Lacroix (1945), a pioneer of bone induction, hypothesized that endochondral ossification is dependent on a group of substances he called osteogenins.

Since then, the osteogenic potential of putative bone matrix proteins has been demonstrated by intramuscular and subcutaneous implantation of demineralized bone matrix (DBM), which locally initiates cartilage and bone differentiation (**Urist, 1965; Reddi & Huggins, 1972; Reddi, 1981**).

Subsequently, the observation that the osteogenic activity could be dissociatively extracted from the DBM as a soluble component and reconstituted with the inactive insoluble collagenous bone matrix (ICBM), provided a functional bioassay for the identification of bone morphogenetic proteins (BMPs) within the bone matrix (**Sampath & Reddi, 1981; Sampath & Reddi, 1983; Wang *et al.*, 1988; Luyten *et al.*, 1989; Sampath *et al.*, 1990**).

The amino-acid sequencing of naturally-derived BMPs helped to identify (by molecular cloning techniques) a family of highly conserved secreted proteins that initiate cartilage and bone differentiation *in vivo* (**Wozney *et al.*, 1988; Reddi, 1992; Wozney, 1992**), collectively called the bone morphogenetic protein family.

Furthermore, BMPs are members of a superfamily of differentiating molecules, the transforming growth factor-ß supergene family, that include the transforming growth factor-ßs (TGF-ßs) themselves, activins and inhibins, the growth and differentiating factors (GDFs) (**Celeste *et al.*, 1990; Özkaynak *et al.*, 1990; Özkaynak *et al.*, 1992; McPherron**

& Lee, 1993; Cunningham *et al.*, 1995) and the cartilage-derived morphogenetic proteins (CDMPs) (**Chang *et al.*, 1994**).

To date, over 20 members of the BMP/osteogenic protein (OP) family have been isolated (**Reddi, 1998**).

Several studies have demonstrated the ability of individual recombinant human (rh)BMPs/OPs, in conjunction with an ICBM, to induce local endochondral bone formation in extraskeletal sites (**Wang *et al.*, 1990; Hammonds *et al.*, 1990; Sampath *et al.*, 1992**).

These studies have shown that the induction of cartilage and bone in postnatal models recapitulate the events that occur in the normal course of embryonic development and therefore constitute the foundation for the therapeutic use of BMPs/OPs (**Reddi, 1992**).

In 1992 Sampath and co-workers utilized recombinant deoxyribonucleic acid (DNA) technology to produce human osteogenic protein-1 (hOP-1, also known as BMP-7) in mammalian cells for the first time (**Sampath *et al.*, 1992**). Examination of the effects of this recombinant osteogenic protein, in the rat subcutaneous bone induction model, demonstrated that recombinant human osteogenic protein-1 (rhOP-1) can induce new bone formation in a manner that is highly reproducible and dose-dependant. Histological evaluation demonstrated that as little as 5-10 ng of rhOP-1/25 mg of rat collagen matrix carrier is sufficient to induce endochondral bone formation. The quantification of bone formation by calcium content demonstrated that the quantity and rate of bone formation is dependent upon the dose of rhOP-1. The bone-forming activity, however, reached a

plateau at a concentration of 1 µg rhOP-1 /25 mg of matrix carrier. The availability of recombinant hOP-1 in large quantities thus permitted, for the first time, the evaluation of its therapeutic potential in pre-clinical studies.

In a series of animal studies Cook *et al.* (**Cook *et al.*, 1994a; Cook *et al.*, 1994b; Cook *et al.*, 1995**) evaluated the effects of rhOP-1 in the treatment of large, non-union, long-bone, segmental defects. Different concentrations of rhOP-1 were evaluated with various bone matrices as a carrier. Results demonstrated that the biological effect of rhOP-1 was dose-dependent. It also appeared that there was a threshold of osteogenic protein, above which no additional benefits were observed.

Large primates share a remarkable similarity with respect to bone remodeling with man (**Schnitzler *et al.*, 1993**). Pre-clinical studies in non-human primates are critical to test the efficacy of recombinant molecules under evaluation for regenerative procedures.
In 1996, Ripamonti *et al.* demonstrated the therapeutic utility of a single application of rhOP-1 by inducing complete regeneration of large critical-size cranial defects in adult baboons (*Papio ursinus*) (**Ripamonti *et al.*, 1996a**). It is possible that the concentration of 2.5 mg of rhOP-1 per gram of bovine or baboon bone matrix used for these experiments, was substantially more than that required for this specific defect and animal model, occasionally resulting in heterotopic osteogenesis along the pericranium and temporalis muscle. The results obtained with the bovine collagenous carrier, however, suggest that the human (allogeneic) matrix may not be a requirement for the therapeutic application of hOP-1.

The dose of osteogenic material implanted is clinically and commercially important, since it will be most cost-effective to use the least concentrated implant yielding the desired effect.

While the optimal concentrations of osteogenic protein remained uncertain, it was clear from the previous studies that larger animals, with longer life expectancies and slower metabolic rates, required more osteogenic protein for adequate bone induction. In general, the regenerative capacity of mammalian bone is believed to be inversely proportional to position on the phylogenetic scale (**Urist, 1965; Urist, 1989**). In summary, after implantation of a given dose of osteoinductive protein, smaller animals are expected to form more new bone, to form it faster, and to reach maximum mechanical strength earlier, as compared with larger animals.

The homology of the OPs among various mammalian species, and the finding that rhOP-1 can induce brisk new bone formation or defect healing in a variety of animal species, indicates that the osteogenic proteins themselves are probably not species specific. The differential response of various animal models to the effects of rhOP-1 is an important consideration in determining the concentration of OP-1 composites for clinical use.

Determination of the optimal concentration of rhOP-1 to be implanted will require analysis of a considerable number of specimens and will probably vary depending on the experimental model that is used.

Important advantages of the use of highly purified recombinant proteins include the elimination of the risk of transmission of infection, which is associated with the transplantation of natural bone extracts, and the avoidance of possible immunogenic

problems arising from the implantation of impure products. The rhOP-1 implant should provide an alternative to the use of autogenous bone graft and allogeneic bone in the reconstruction of bone defects caused by trauma, neoplasia, or infection. OP-1 may also prove to be useful in prosthetic fixation, spinal cord arthrodesis, and the primary treatment of fractures.

The ultimate goal of osteogenic protein research is successful clinical application in man. It is with this goal in mind that the study was conducted.

CHAPTER TWO: REVIEW OF THE LITERATURE

2.1 The discovery and isolation of OP-1

Many bone-inducing proteins have been extracted from bovine bone. In 1990, Sampath and co-workers purified a bovine bone-inductive protein, with apparent molecular weight of 30 kDa. This protein, designated as OP, is composed of disulfide-linked dimers. Upon reduction, the dimers yield two subunits of 18 and 16 kDa. The 18-kDa subunit is the bovine equivalent to the protein product of the human OP-1 gene and the 16-kDa subunit is bovine equivalent of the protein product of the human BMP-2A gene. Both bovine proteins are capable to induce bone *in vivo* (**Sampath *et al.*, 1990**).

In their strategy to isolate the gene(s) that encode osteogenic proteins, Özkaynak *et al.* (**1990**) isolated two tryptic peptides from bovine osteogenic protein preparations. The amino acid sequences of these peptides revealed considerable homology to two members of the TGF-ß supergene family, namely decapentaplegic protein (DDP) of *Drosophila* and vegetal protein (Vg-1) of *Xenopus*. This information led the researchers to construct a synthetic consensus gene which they used as a probe to screen human genomic libraries. This resulted in the isolation of a novel human gene, OP-1, related to murine gene, Vgr-1 (**Özkaynak *et al.*, 1990**).

Extensive purification and amino acid sequence analysis of protein extracts of demineralized bone enabled Celeste *et al.* to clone BMP-1, BMP-2, BMP-3 and BMP-4. Further biochemical characterization of the proteins present in active BMP preparations, indicated that additional molecules related to BMP-2 were present. The complementary

deoxyribonucleic acid (cDNA) clones corresponding to human BMP-5, BMP-6 and BMP-7 were subsequently isolated. It is now clear that this BMP-7 is identical to OP-1 cloned previously by Özkaynak *et al.* in the same year (**Celeste *et al.*, 1990**).

2.2 The structure of OP-1

BMPs are dimeric molecules, a conformation that is critical for their biological activity *in vivo*. Indeed, reduction of the single intermolecular disulfide bond results in loss of activity (**Wozney *et al.*, 1988; Luyten *et al.*, 1989**).

The mature BMP monomer consists of about 120 amino acids with seven cysteine residues forming a cysteine knot in the core of the protein (**Wozney *et al.*, 1988; Reddi, 1994**).

To understand the mode of action of OP-1 and to provide a basis for the development of small molecule therapeutics, a sound knowledge of the three-dimensional structure of OP-1 is essential. The three-dimensional structure of mature OP-1, reported to 28 nm resolution, reveals striking differences with the structure of transforming growth factor ß-2. Although there is limited sequence identity between OP-1 and TGF-ß2, they share a common polypeptide fold. These structures constitute a basis for proposing the OP-1/TGF-ß2 fold as the primary structural motif for the TGF-ß superfamily as a whole, and give insight into how these growth factors interact with their receptors (**Griffith *et al.*, 1996**).

2.3 Observation of the effects of OP-1

2.3.1 Effects of OP-1 in cell cultures

In vitro studies of the biological actions of OP-1 in cell culture systems have been crucial to identify responding cell populations and to advance our knowledge of the actions of OP-1 at the cellular level.

2.3.1.1 Bone cell cultures

rhOP-1, produced in mammalian cells, demonstrates a capacity to induce new bone formation in the subcutaneous tissues of the rat. Evaluation of the effects of rhOP-1 on rat bone cell cultures enriched by osteoblasts, reveals a dose dependent cell proliferation and collagen synthesis. rhOP-1 is effective in specifically stimulating the synthesis of markers of the osteoblast phenotype, e.g. alkaline phosphatase, osteonectin, parathyroid hormone-mediated cyclic adenosine monophosphate (cAMP), and deposition of mineralized nodules **(Sampath *et al.*, 1992).**

Does OP-1 act on human bone cells *in vitro* as well? To answer this question, the effects of rhOP-1 on cellular proliferation and alkaline phosphatase (ALP) activity were studied in human bone cell (HBC) and human TE85 osteosarcoma cell cultures. Both HBCs and TE85 osteosarcoma cells showed increased proliferation when treated with OP-1, but ALP activity was only increased in TE85 osteosarcoma cells. $1,25(OH)_2D_3$ modulated OP-1 induced ALP activity in HBCs in a biphasic manner. These results indicate that OP-1 acts directly on human osteoblast-like cells to modulate cell proliferation and differentiation **(Knutsen *et al.*, 1993).**

rhOP-1 induces undifferentiated mesenchymal cells into bone forming cell lineage *in vivo* and stimulates the expression of osteogenic and chondrogenic phenotypes *in vitro*. However, its effects on osteoclastogenesis or bone resorption were still not known. An *in vitro* study to analyse the effects of rhOP-1 on rat bone marrow cells in culture provided the evidence that OP-1 could play an important role in bone remodeling. rhOP-1 stimulated the formation of tartrate-resistant acid phosphatase-positive (TRAP) multinucleated cells (MNCs) and markedly increased the capacity of 1,25-$(OH)_2D_3$ (a strong activator of osteoclastogenesis) to induce osteoclast-like cell formation and bone resorption **(Hentunen *et al.*, 1995)**.

Long-term cultures of fetal rat calvarial cells (FRCC) are widely used as *in vitro* models in the study of bone formation. The three distinctive phases in the developmental sequence of bone formation is defined by distinct patterns of gene expression, including those of matrix proteins. The expression of different bone matrix proteins, e.g. fibronectin, collagen type I, bone sialoprotein, osteopontin, alkaline phosphatase and osteocalcin, are indicators of the differentiated state of the cultures, therefore it is possible to analyse how various factors might affect osteogenesis at successive stages of bone development. Independent and combined effects of OP-1 and TGF-ß1 on primary cultures of FRCC, at different stages of cellular differentiation, show that OP-1 has selective effects on bone matrix protein expression that are dependent on the differentiated state of the cells **(Cheifetz *et al.*, 1996)**.

2.3.1.2 Organ and chondrocyte cell cultures

In recent studies, it has been shown that rhOP-1 stimulates cartilage growth in organ cultures of metatarsal long bones of the mouse embryo **(Dieudonné *et al.*, 1994)** and promotes the maturation and hypertrophy of chick chondrocytes in monolayer or

suspension cultures. The terminal differentiation of chondrocytes, characterized by cell hypertrophy, is a prerequisite for the onset of endochondral bone formation *in vivo* **(Chen *et al.*, 1995)**. In contrast, it has been suggested that when added to primary cultures of bovine articular chondrocytes, which are not destined for further differentiation, rhOP-1 stimulates matrix synthesis without promoting chondrocyte hypertrophy. This reflects a response specific to articular chondrocytes, as opposed to other cartilaginous cells **(Chen *et al.*, 1993)**.

The first study on the effects of rhOP-1 on fully differentiated human articular chondrocytes in culture, demonstrates that rhOP-1 is a more potent stimulator of the synthesis of cartilage-specific matrix macromolecules (aggrecan and type II collagen) than other stimulating factors tested for comparison, including TGF-ß1 and activin A **(Flechmacher *et al.*, 1996)**.

The correlated expression of both OP-1 ligand and OP-1 binding receptors in developing mouse long bones suggests a regulatory role for OP-1. OP-1 affects the expression of marker genes of chondrocyte differentiation, in organ cultures of embryonic mouse metatarsals, by acting on two steps in endochondral ossification. First, cell proliferation is enhanced, particularly so in the perichondrium where cells start to express the chondrocyte phenotype. Second, the terminal differentiation of mature chondrocytes into hypertrophic chondrocytes is inhibited. These effects, combined with the known pattern of OP-1 ligand and BMP receptor expression in the embryo, suggest that OP-1 plays a local role in the cascade of events during endochondral ossification **(Haaijman *et al.*, 1997)**.

2.4 Pre-clinical studies and possible clinical applications

2.4.1 Cartilage repair

Repair of articular cartilage defects is still a major challenge in modern medicine, because the factors that initiate cartilage formation, maturation and repair are poorly understood. The study of the healing process of both partial-thickness and full-thickness articular cartilage lesions led to the conclusion that it is essential to expand the existing cell population, to increase the total pool of healthy cells that contribute to the matrix repair. This could be obtained by increased proliferation, chemotaxis, or both, of cells from the neighbouring tissues such as the underlying bone and synovium. When collagen type I gel, containing OP-1, was placed in surgically created full-thickness articular defects in rat knee joints, complete healing of the defects resulted. The regenerated tissue contained cells resembling mature joint chondrocytes. These results may lead to a new strategy for biological repair of damaged joint surfaces in humans **(Grgic *et al.*, 1997)**

2.4.2 Bone repair

The use of BMPs/OPs promise to become a clinically important alternative to autogenous bone grafts and allogeneic implants in the treatment of large bone defects. A mammal's capacity for bone repair is roughly inversely proportional to its position on the phylogenetic scale. Thus, pre-clinical studies in non-human primates are critical to test the efficacy and safety of recombinant molecules under evaluation for future regenerative procedures in humans.

hOP-1 has been shown to induce regeneration in large segmental defects of the appendicular skeleton, in a dose-dependant manner, in rabbits, dogs and monkeys **(Cook *et al.*, 1994a; Cook *et al.*, 1994b; Cook *et al.*, 1995)**.

rhOP-1, at concentrations of 0.1, 0.5 and 2.5 mg per g of matrix, induces complete regeneration of full thickness calvarial bone defects in the adult non-human primate. This demonstrates the therapeutic utility of a single application of rhOP-1 **(Ripamonti *et al.*, 1996a)**.

Ripamonti *et al.* **(1997)** demonstrated that recombinant human (rh)TGF-ß1 induces endochondral bone formation in extraskeletal sites of adult baboons. Even more interestingly, rhTGF-ß1 and rhOP-1, delivered by the same device, synergize to induce large ossicles in extraskeletal sites of the baboon as early as 15 days after implantation. These results provide evidence for a novel function of TGF-ß1 in the primate and the scientific basis for synergistic molecular therapeutics for the rapid regeneration of cartilage and bone **(Ripamonti *et al.*, 1997)**.

The posterior region of the maxilla is one of the most difficult areas to successfully place dental implants due to poor bone density and the close proximity of the maxillary sinus. Sinus augmentation procedures have become a successful means to gain adequate bone volume and density to place dental implants in this area. However, the current augmentation techniques result in a wide variation of the type and volume of the bone generated. In the maxillary sinus of non-human primates, comparable radiographic and histological findings of bone formation have been demonstrated by implanting either natural bone minerals or a device containing 2.5 mg rhOP-1/g of collagenous matrix (Bio-

Oss, Geistlich Biomaterials, Switzerland). The rhOP-1 device may offer significant advantages over current grafting techniques for the sinus augmentation procedure, as well as for other oral and maxillofacial bone regeneration procedures **(Margolin *et al.*, 1998)**.

2.4.3 Periodontal repair

The regulation of periodontal tissue regeneration is still poorly understood. The expression patterns of BMP-2, BMP-3, BMP-4 and OP-1 during tooth morphogenesis suggest that specific BMPs/OPs may also be used for repair and regeneration of dentine and periodontal tissues in postnatal life.

A single application of rhOP-1 in conjunction with a collagenous matrix on surgically denuded root surfaces of adult baboons resulted in substantial induction of cementogenesis. This clearly reveals that rhOP-1 has a specific function to repair and regenerate periodontal tissues **(Ripamonti *et al.*, 1996b)**.

An ascending dose range of OP-1 promotes periodontal wound healing in surgically created supra-alveolar class III furcation defects in the canine model. All 3 concentrations of OP-1 (0.75, 2.5 and 7.5 mg OP-1/g collagen matrix) showed pronounced stimulation of osteogenesis, cementogenesis and formation of new attachment **(Giannobile *et al.*, 1998)**.

2.4.4 Renal repair

Data obtained from clinical studies in rodents suggest that OP-1 prevents the loss of kidney function due to ischaemia and may provide a basis for the treatment of acute renal failure **(Vukicevic *et al.*, 1998)**.

2.5 Mechanism of action of OP-1

With regard to the effects of BMPs on bone formation, the obvious question is how the application of a single BMP, such as OP-1, can initiate and perpetuate the entire developmental cascade of cellular events, ultimately leading to bone formation. Although it is possible that a single protein, acting through a single receptor, can stimulate more than one second messenger signaling pathway, the complexity of osteogenesis makes it unlikely that OP-1 is directly responsible for all the cellular activities involved in bone induction. It is likely that some of the effects of OP-1 may be mediated by regulating the action of known peptide growth factors, e.g. insulin-like growth factors (IGFs) that mediate growth and differentiation in bone cells.

The IGFs are the most abundant growth factors produced by human bone cells and stored in bone. The IGF regulatory system has been shown to regulate local bone formation *in vitro* (**Mohan & Baylink, 1991a; Mohan & Baylink, 1991b; Canalis *et al.*, 1988**) and *in vivo* (**Zapf & Froesch, 1986; Johansson *et al.*, 1992**).

An *in vitro* study of the mechanism, by which a BMP produces a multitude of effects on bone cells, demonstrates that OP-1 can act locally by modulating the IGF regulatory system. OP-1 enhances the secretion of IGF-II and regulates the balance between stimulatory (IGFBP-5) and inhibitory (IGFBP-4) classes of IGF binding proteins (IGFBPs). In addition to its effects on IGFBPs production, OP-1 also decreases the proteolysis of IGFBP-3 and IGFBP-5, which may in part be modulated by the increase in IGF-II secretion. Thus, OP-1 and other BMPs may recruit peptide growth factors to mediate some of their effects on bone formation (**Knutsen *et al.*, 1995**).

The effects of exogenous IGF-I on bone cell differentiation and on mineralized bone nodule formation, induced by OP-1, strongly suggest that the OP-1 down-regulation of IGFBPs (especially IGFBP-5) is an important mechanism by which OP-1 and IGF-I synergize to stimulate FRCC (**Yeh *et al.*, 1997**).

Overall, the positive modulation of these IGF components by OP-1, may enhance IGF availability, and subsequently promote IGF bioactivity in human bone cells.

To delineate the molecular mechanisms by which OP-1 exerts its effects on the IGF system, the time-course effects of OP-1 on the expression of IGFBP-3 messenger ribonucleic acid (mRNA) in human SaOP-2 osteosarcoma cells has been evaluated. OP-1 stimulates IGFBP-3 expression in human osteoblast cells by a mechanism that largely promotes the production of IGFBP-3 nuclear transcripts. This process requires *de novo* protein synthesis, and overrides an OP-1 induced targeted degradation of IGFBP-3 steady state mRNA (**Hayden *et al.*, 1997**).

To further our understanding of the effects of OP-1 on the regulation of gene expression of components of the IGF system, Yeh and co-workers examined whether changes in mRNA levels in FRCCs were the consequence of a change in transcription, in mRNA stability, or both. They found that OP-1 regulates IGF-I gene expression at the post-transcriptional level, but regulates IGF-II and IGFBP-5 gene expression at the transcriptional level (**Yeh *et al.*, 1998**).

2.6 Cell membrane receptors

The temporal and spatial expression of BMP-2/-4, OP-1 and BMP type II receptors, during fracture healing in rats, shed some light on the possible roles of these osteogenic proteins in intramembranous and endochondral ossification. OP-1 may act predominantly in the initial phase of endochondral ossification of fracture healing in rats, while BMP-2/-4 act throughout this process. These findings suggest that BMPs, acting through their BMP receptors, may play major roles in the modulation of the sequential events leading to bone formation **(Onishi *et al.*, 1998)**.

OP-1 induces proliferation and differentiation of HBCs in culture **(Knutsen *et al.*, 1993)** and modulates the production of IGF system components. However, the demonstration of high-affinity receptors for OP-1 on HBCs confirms that the effects of OP-1 are mediated, at least in part, by specific receptors **(Malpe *et al.*, 1994)**.

TGF-ßs and BMPs transmit their signals through a heteromeric complex of two transmembrane serine/threonine kinase receptors, known as type I and type II receptors. Type II receptors for TGF-ßs bind ligand by themselves, whereas type I receptors need type II receptors for ligand binding. In contrast to TGF-ßs, BMPs show specific binding to the type I and type II receptors individually. However, the co-operation between types I and type II receptors is essential for both optimal binding and signal transmission by BMPs. One type II BMP receptor can interact with multiple type I receptors and *vice versa*, thereby generating multiple type I and type II receptor combinations. Thus, BMPs and TGF-ßs may induce different cellular responses, depending on which type of receptors

are expressed by the cell **(Weiser *et al.*, 1995; ten Dijke *et al.*, 1996: Massagué, 1996; Weis-Garcia & Massagué, 1996)**.

2.7 Temporal and spatial expression of OP-1 in other tissues than bone

Although BMPs initiate, promote, and maintain chondrogenesis and osteogenesis, they are also involved in the morphogenesis of organs other than bone. The recent findings that BMPs/OPs are widely expressed, and not solely confined to skeletal systems, strongly suggest that they have multiple functions, and may play critical roles as soluble mediators of vertebrate development, morphogenesis and organogenesis. These findings may lead to novel strategies for therapy beyond bone.

The distribution of OP-1 in a variety of tissues during human embryonic development suggests that, in addition to bone formation, OP-1 could have other important regulatory roles in human embryogenesis, with high binding affinity to basement membrane components (**Vukicevic *et al.*, 1994**).

The spatial and temporal expression of OP-1 patterns in extraskeletal sites of the developing human and mouse has been analyzed in detail by *in situ* hybridization. OP-1 transcripts are present in a variety of tissues, indicating local or systemic release or both, particularly in the development of the kidney, limb bud, bone, tooth, heart and intestine. The widespread distribution of OP-1 expression correlates with development as a result of epithelial and mesenchymal interactions (**Helder *et al.*, 1995**).

The induction of the lens and the development of the eye involve a complex set of signals and tissue interactions. OP-1 is initially expressed in the optic vesicle and subsequently in the neural retina, the placodal epithelium, lens and cornea. The use of OP-1 monoclonal anti-bodies to block the action of OP-1, leads to anophthalmia in whole rat embryo cultures. These results reveal that OP-1 mediates the inductive signals involved in vertebrate eye development (**Solursh *et al.*, 1996**).

The definitive mammalian kidney forms as the result of reciprocal interactions between the ureteric bud epithelium and metanephric mesenchyme. OP-1 mRNA is expressed in the ureteric bud epithelium before mesenchymal condensation and is subsequently seen in the condensing mesenchyme and during glomerulogenesis. Thus, OP-1 is required for metanephric mesenchyme differentiation and plays a functional role during kidney development (**Vukicevic *et al.*, 1996**).

Analysis of the effects of the administration of BMP-2 and OP-1 locally, at different stages and locations during the development of the chick limb bud, indicates that in addition to the proposed role for BMP-2 and OP-1 in the establishment of the antero-posterior axis of the limb, they may also play direct roles in limb morphogenesis (**Macia *et al.*, 1997**).

In developing dental tissues of rodents, OP-1 has distribution and expression patterns similar to those of other BMP members. OP-1 is present throughout all stages of embryonic and neonatal tooth development. However, analysis of tooth development in OP-1 deficient mice shows no alteration in tooth patterning, tooth morphogenesis, or cytodifferentiation of odontoblasts and ameloblasts. This suggests that OP-1 is not an

essential growth factor for tooth development, possibly because of functional redundancy with other BMP members or related growth factors (**Helder *et al.*, 1998**).

CHAPTER THREE: AIM OF THE STUDY

The aim of this study was to evaluate the efficacy of a single application of different doses of recombinant human osteogenic protein-1 in conjunction with bovine insoluble collagenous bone matrix for bone induction in the rectus abdominis of adult female baboons (*Papio ursinus*).

CHAPTER FOUR: MATERIALS AND METHODS

4.1 Selection of the animals

Two clinically healthy adult female Chacma baboons (*Papio ursinus*), with a mean weight of 20.65 kg ±2.25 kg, were selected from the primate colony of the University of the Witwatersrand, Johannesburg. Both animals had normal haematologic and biochemical profiles **(Melton & Melton, 1982)**. Skeletal maturity was confirmed by radiographic evidence of closure of the distal epiphyseal plate of the radius and ulna.

4.2 Ethics approval

The research protocol was approved by the Animal Ethics Screening Committee of the University of the Witwatersrand, and the Research Committee of the Faculty of Dentistry, University of Pretoria. This research was conducted according to the *Guidelines for the Care and Use of Experimental Animals* prepared by the University of the Witwatersrand, Johannesburg and in compliance with the *National Code for Animal use in Research, Education and Diagnosis in South Africa.*

4.3 Housing conditions

Following standard quarantine procedures, the animals were housed individually in suspended wire-mesh squeeze-back cages in the non-human primate unit of the university, 1800 m above sea level. Animals were caged in rooms kept under slight negative pressure

(-25 kPa) with controlled ventilation (18 filtered air changes per hour), temperature (22° C ± 2°), humidity (40% ± 10%), and photoperiod (lights on from 0600 to 1800 hours).

4.4 Diet

The diet of the animals consisted of a balanced protein-fat-carbohydrate diet with vitamins (thiamine, riboflavin and nicotinic acid) and mineral supplements (Ca:P=1.2:1), and a soft dietary intake of sweet potatoes, pumpkins and oranges mixed in a ratio of 3:1 with a protein-vitamin-mineral dietary supplement (**Dreyer & De Bruyn, 1968**). The baboons had access to tap water *ad libitum*.

4.5 Recombinant morphogen and carrier matrix preparation

Recombinant human OP-1, a kind gift of Creative BioMolecules (Hopkinton, MA, USA) was prepared as previously described (**Sampath *et al.*, 1992**). Mature OP-1 is a glycosylated 36-kD homodimer of 139-amino acid residue chains. Stock solutions of hOP-1 were prepared in 50% ethanol, 0.01% trifluoroacetic acid, and the protein concentration was determined using an extinction coefficient of 2 for a 1 mg/ml solution at 280 nm.

From a therapeutic perspective, a carrier matrix is required for local delivery of BMPs/OPs to obtain a morphogenetic response. The extracellular matrix of bone is an optimal substratum for cell recruitment, attachment, proliferation and differentiation (**Sampath & Reddi, 1981; Reddi, 1994**), as well as a delivery system for BMPs/OPs, both naturally-sourced or in recombinant form (**Sampath & Reddi, 1981; Sampath *et al.*, 1992; Ripamonti *et al.*, 1996a**). The carrier used in this study to deliver rhOP-1 was ICBM, the

inactive residue obtained after dissociative extraction of bovine bone matrix with 4 M guanidinium-HCl (Gdn-HCl) **(Sampath & Reddi, 1981; Ripamonti *et al.*, 1991b)**. Collagenous bone matrix (74-420 µm particle size) was prepared from diaphyseal segments of bovine cortical bones. After demineralization in 0.5 M HCl at room temperature, the bone matrix was dissociatively extracted in 4 M Gdn-HCl containing a mixture of protease inhibitors (5 mM benzamidine / 0.1 M 6-aminohexanoic acid / 0.5 mM phenylmethylsulfonyl fluoride / 5mM *N*-ethylmaleimide) **(Sampath & Reddi, 1981)**. The resulting ICBM, inactive after extraction of osteogenic proteins, was washed three times with distilled water, dehydrated in ethanol and ether, and used as carrier for rhOP-1. For the preparation of samples suitable for heterotopic implantation in the baboon, OP-1, in 50% ethanol and 0.01% trifluoroacetic acid, was combined with ICBM and lyophilized. Implants were prepared in sterile 5 ml capacity polypropylene tubes by adding 0.1, 0.5 and 2.5 µg of rhOP-1 to 100 mg of ICBM. Aliquots of carrier matrix were prepared with respective liquid vehicles without rhOP-1 as control.

4.6 The primate heterotopic model: OP-1 implantation designs

The primate heterotopic model has been described in detail **(Ripamonti *et al.*, 1991b; Ripamonti *et al.*, 1992b)**. Briefly, food was withdrawn from the animals on the evening before surgery with access to water *ad libitum*. On the day of surgery, the animals were immobilized by intramuscular ketamine-HCl (Ketalar®, Parke-Davis, 8 mg/kg body weight) and anesthetized with intravenous thiopentone sodium (Intraval Sodium®, RPR, 15 mg/kg body weight). Using sterile operative techniques, lyophilized pellets of collagenous matrix alone or containing different doses of rhOP-1 were implanted bilaterally in ventral intramuscular pouches created by sharp and blunt dissection of the rectus abdominis

muscle. A total of eight pouches were prepared per animal, four on each side of the midline. The longitudinal pouches were 2 cm long, 6 to 7 cm lateral to the midline, and were vertically separated by at least 4 cm of intervening muscle.

Animal 1

Right side: Upper two pouches received control pellets.

Lower two pouches received pellets each containing 0.5 mg of OP-1.

Left side: All four pouched received pellets containing 0.1 mg of OP-1

Animal 2

Right side: All four pouches received pellets containing 0.5 mg of OP-1.

Left side: All four pouches received pellets containing 2.5 mg of OP-1.

Each pouch was further enlarged lateral to the incision line to accommodate 2 pellets, one on each side or the incision line. Each animal thus received a total of 16 pellets. Whilst superiorly and laterally surrounded by muscular tissue, implants were resting directly over the peritoneal fascia. Incisions were closed in layers by suturing the fasciae and the superficial tissues with atraumatic resorbable sutures (Vicryl®, Ethicon, USA). Post-operative pain was controlled by intramuscular buprenorphine-HCl (Temgesic®, R & C, 0.3 mg). Individually housed animals were monitored and fed as described above.

4.7 Tissue harvesting, processing, histology, histomorphometry and biochemical analysis

Anaesthetized animals were killed with an intravenous overdose of intravenous thiopentone sodium (Intraval Sodium®, RPR) on day 90.

Generated specimens were dissected free of soft tissue, divided in half and weighed.

One half of each specimen was processed for undecalcified histology. These specimens were dehydrated in ascending grades of ethanol and embedded in a polymethymethacrylate resin (K-Plast, Medim, Germany). Serial sections were cut at 7 µm using tungsten-carbide knives and a motor-driven microtome (Polycut-S, Reichert-Jung, Germany). Sections were stained using the free-floating method with Goldner's trichrome stain for undecalcified bone.

Sections were analyzed at 100x with a Provis AX70 research microscope (Olympus Optical Co., Japan) superimposing the Zeiss graticule over the sources selected for histomorphometry **(Parfitt *et al.*, 1987)**.

A calibrated Zeiss Integration Platte II with 100 lattice points was used to calculate, with the point-counting technique **(Parfitt, 1983)** the fractional volumes (in %) of each histological component, namely newly formed bone and soft tissues (fibrovascular and marrow tissues) **(Ripamonti *et al.*, 1993)**. Bone values were determined by calculating its mineralized and osteoid components **(Ripamonti *et al.*, 1993)**. Morphometry (volumes) was performed on two sections per implant, representing two levels, approximately 400

µm apart from each other. Morphometry was performed on three sources per section. Each source represented a field of 3.136 mm^2.

The other half of each specimen was homogenised in 2 ml of ice-cold 3 mM sodium bicarbonate containing 0.15 M NaCl, pH 7.8. After centrifugation, the alkaline phosphatase activity of the supernatant was used as index of bone formation by induction as described in appendix A **(Reddi & Huggins, 1972; Ripamonti *et al.*, 1991b)**.

CHAPTER FIVE: RESULTS

5.1 Clinical examination and harvesting

Palpation of the abdomen of the first baboon revealed a single discrete nodule in an area implanted with a control pellet. In the second baboon, three well distinct nodules of varying size could be identified by palpation. The section of the specimens revealed a hard, whitish structure that contained reddish, haemorrhagic areas of vascular tissue.

5.2 Histological examination

The only specimen harvested from the first baboon consisted entirely of collagenous bone matrix within fibrovascular tissue (***Figure 1***). However, in a single section, a small island of mineralised bone was seen at the periphery of the implanted ICBM (***Figure 2***).
In the second animal, both concentrations of rhOP-1 (0.5 and 2.5 mg) induced bone formation. Newly generated tissue had grown to form large ossicles, with a planar-convex morphology, extending for several centimeters along the fascial plane (***Figure 3***). The ossicles were covered by bundles of muscle fibers on the outer aspect and by peritoneal tissue on the inner aspect (***Figure 4***). The mineralised tissue was composed of bony trabeculae interspersed with bone marrow and remnants of ICBM (***Figure 5***). Trabeculae were lined by a nearly continuous osteoid rim supporting osteoblasts and interrupted by areas of mineralised bone resorption (***Figure 6***). The irregular and corrugated foci of resorption were characterised by the presence of multinucleated osteoclasts (***Figure 7***). Resorption was more frequent at the outer than the inner surface of the ossicles (***Figure 8***).

The bone marrow consisted of roughly equal amounts of fibrovascular and adipose tissue (***Figure 9***). Small amounts of residual matrix were still present but completely embedded in mineralized bone (***Figure 10***). The ossicles generated by implants with 2.5 mg of rhOP-1 contained more residual matrix as seen in a comparison between ***Figures 4 and 5***. No cartilage was observed in any of the specimens.

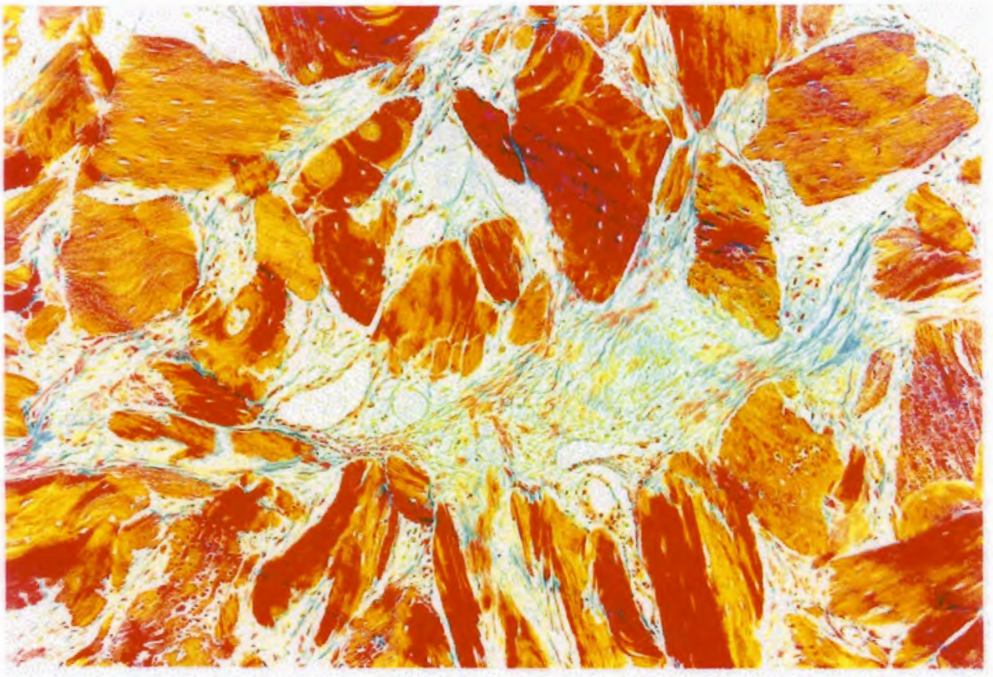

FIG. 1. Bovine collagenous matrix without rhOP-1 (control): particles of collagenous bone matrix within fibrovascular tissue (magnification 200 x)

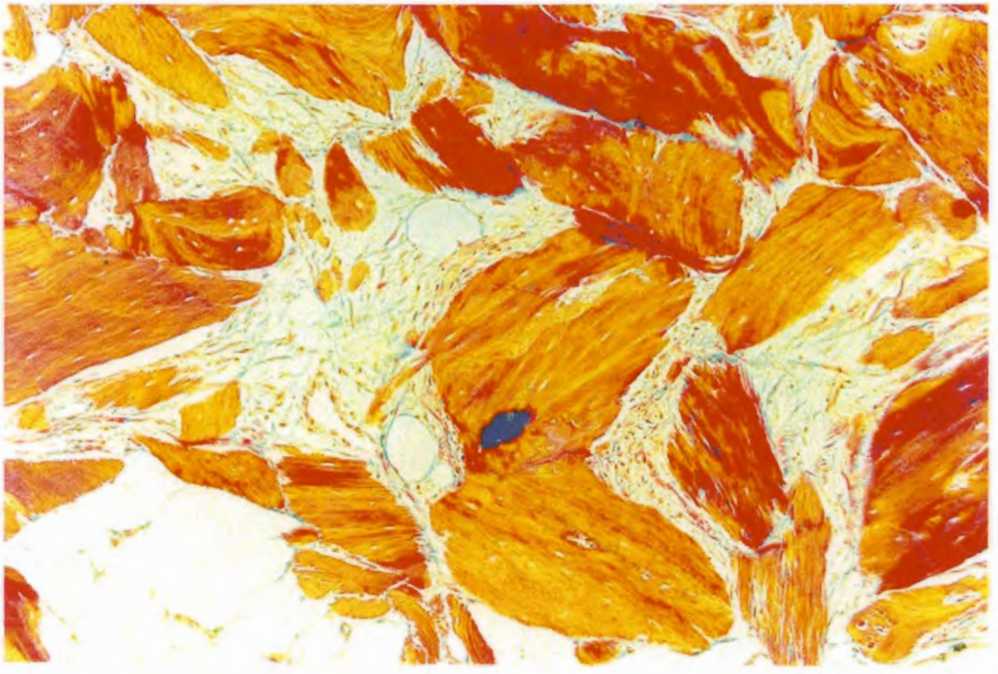

FIG. 2. Bovine collagenous matrix without rhOP-1 (control): particles of collagenous bone matrix within fibrovascular tissue (magnification 200 x)

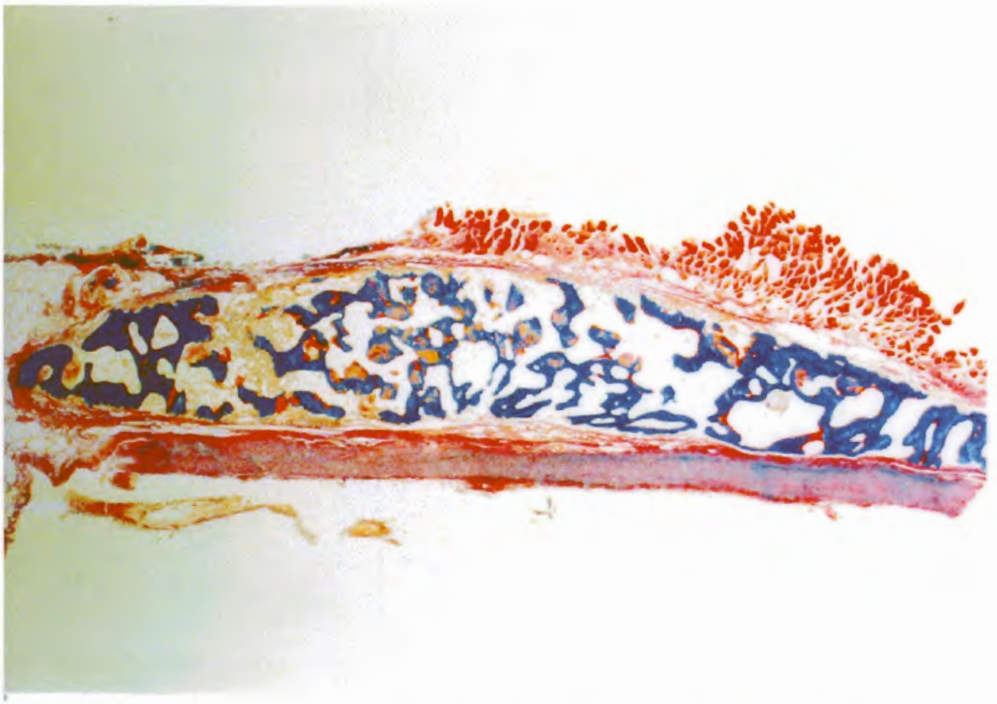

FIG.3. Bovine collagenous matrix and 2.5 mg of rhOP-1: a large ossicle formed between the abdominal muscle and the peritoneal tissue; trabeculae of mineralized bone across the entire specimen (magnification 12.5 x)

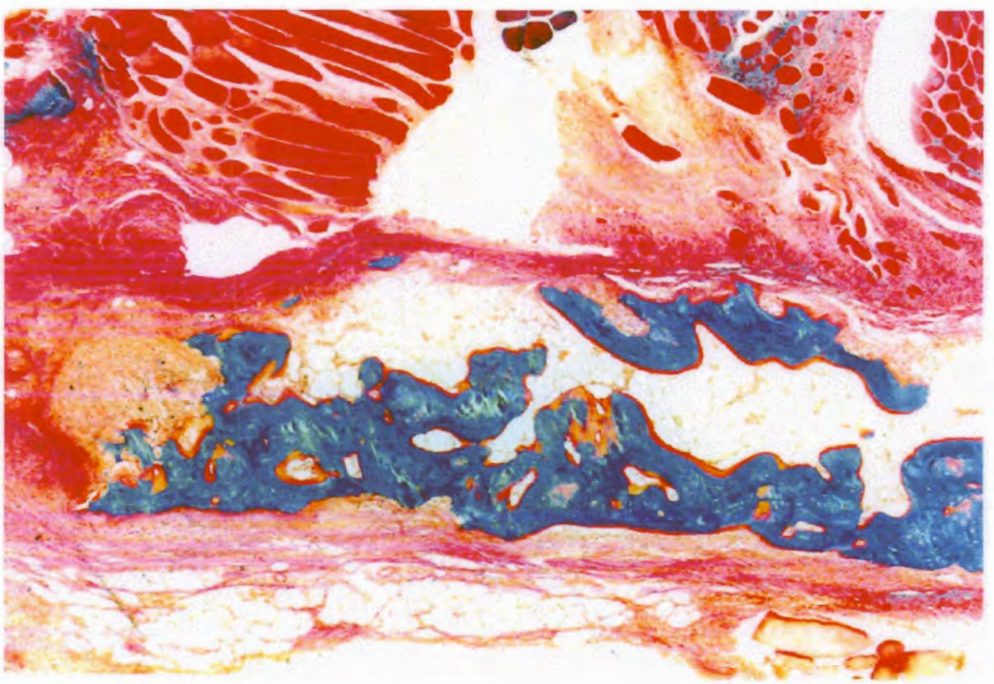

FIG. 4. Bovine collagenous matrix and 0.5 mg of rhOP-1: ossicle covered by muscle fibers on the outer aspect and by peritoneal tissue on the inner aspect (magnification 4 x)

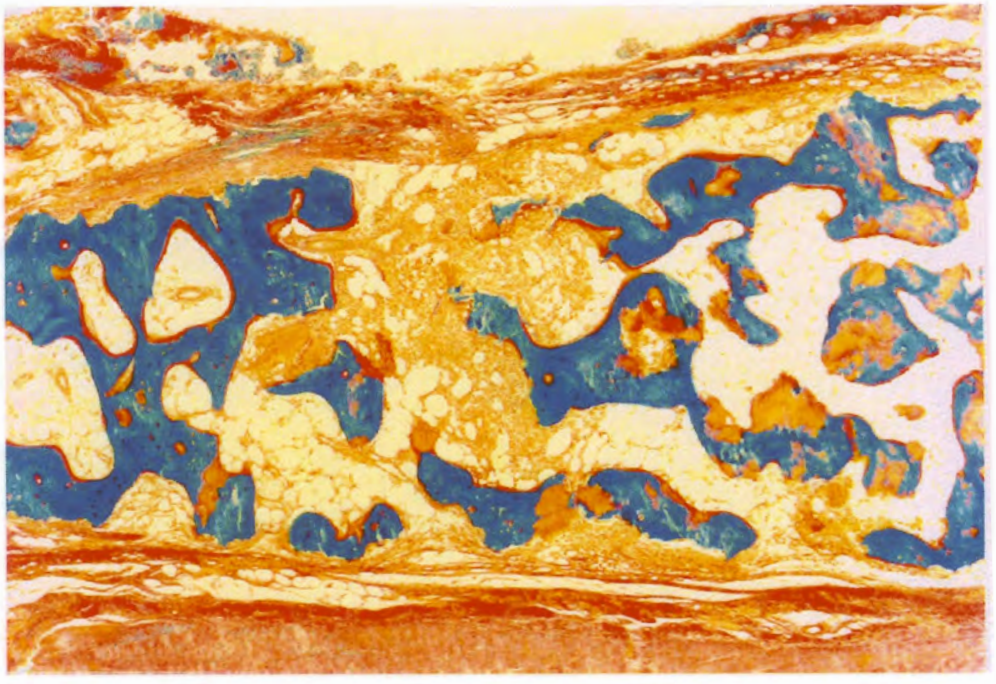

FIG. 5. Bovine collagenous matrix and 2.5 mg of rhOP-1: bony trabeculae interspersed with hematopoietic bone marrow; residual matrix in close association with mineralized bone (magnification 100 x)

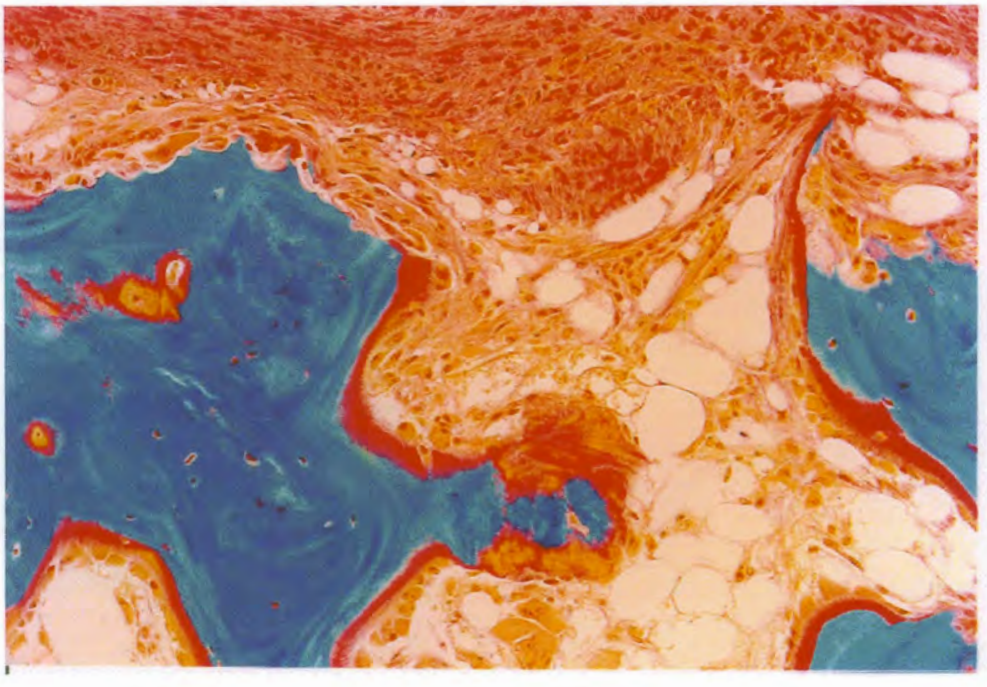

FIG. 6. Bovine collagenous matrix and 2.5 mg of rhOP-1: bony trabeculae lined by osteoid seams supporting osteoblasts and interrupted by areas of bone resorption (magnification 200 x)

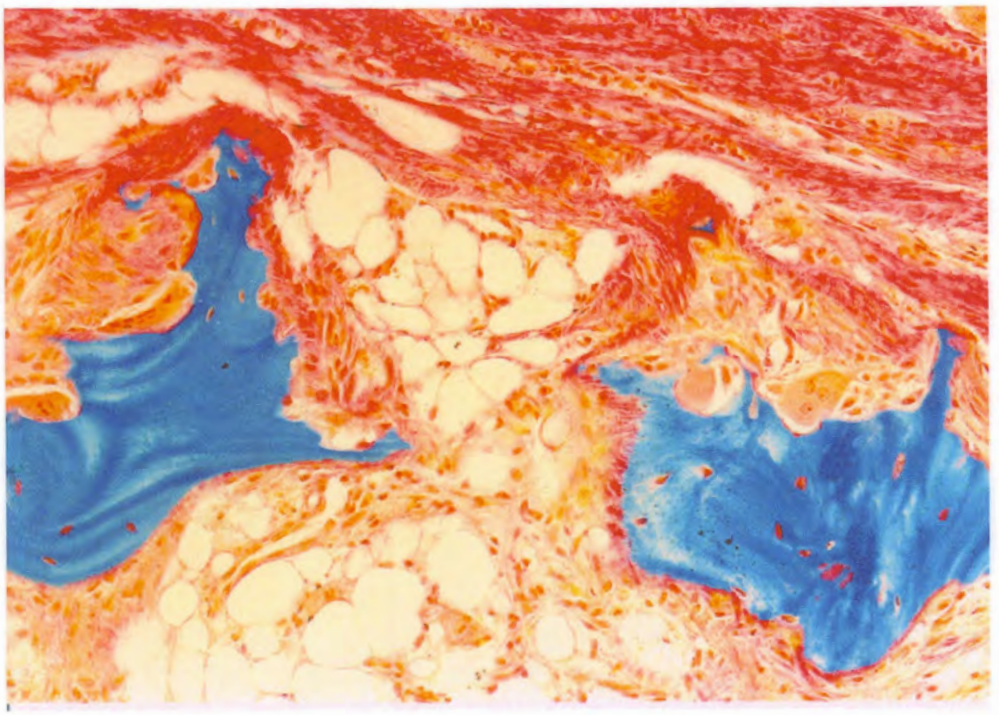

FIG. 7. Bovine collagenous matrix and 0.5 mg of rhOP-1: irregular and corrugated foci of bone resorption characterized by the presence of numerous multinucleated osteoclasts (magnification 200 x)

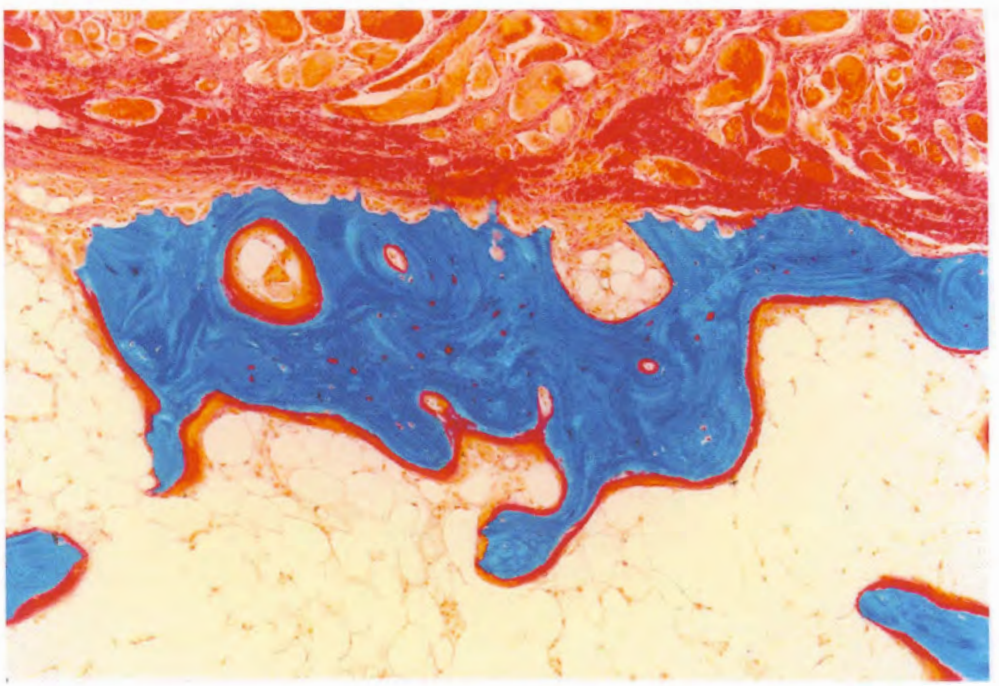

Fig. 8. Bovine collagenous matrix and 2.5 mg of rhOP-1: pronounced resorption of bony trabeculae on the outer aspect of the ossicle; continuous osteoid seam lining the trabeculae on the peritoneal aspect of the ossicle (magnification 200 x)

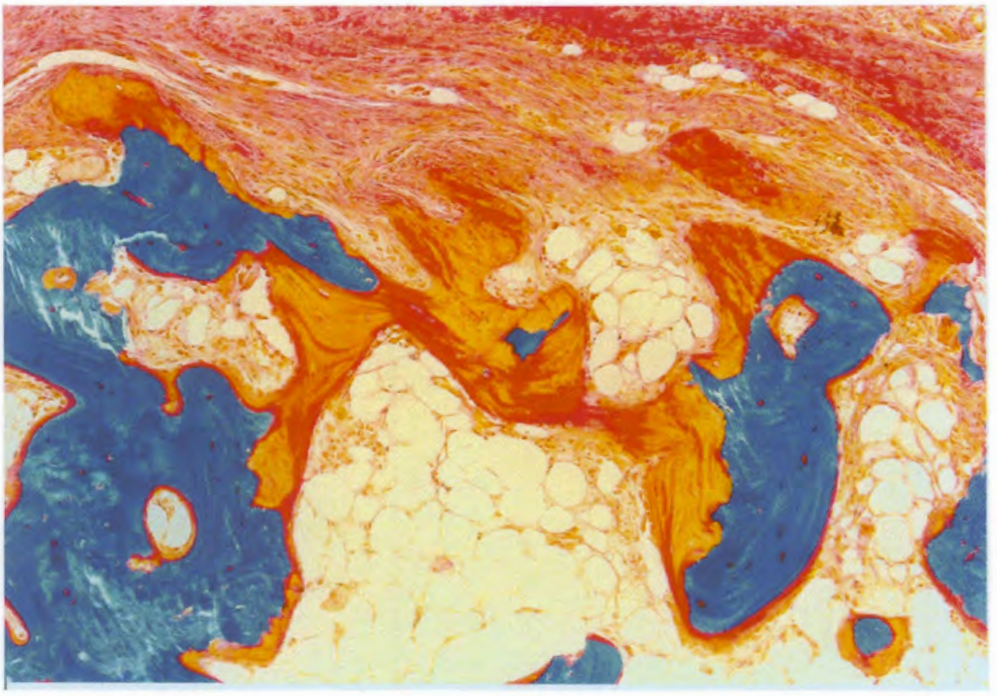

FIG. 9. Bovine collagenous matrix and 0.5 mg of rhOP-1: bony trabeculae with thick osteoid seams, within hematopoietic bone marrow (magnification 200 x)

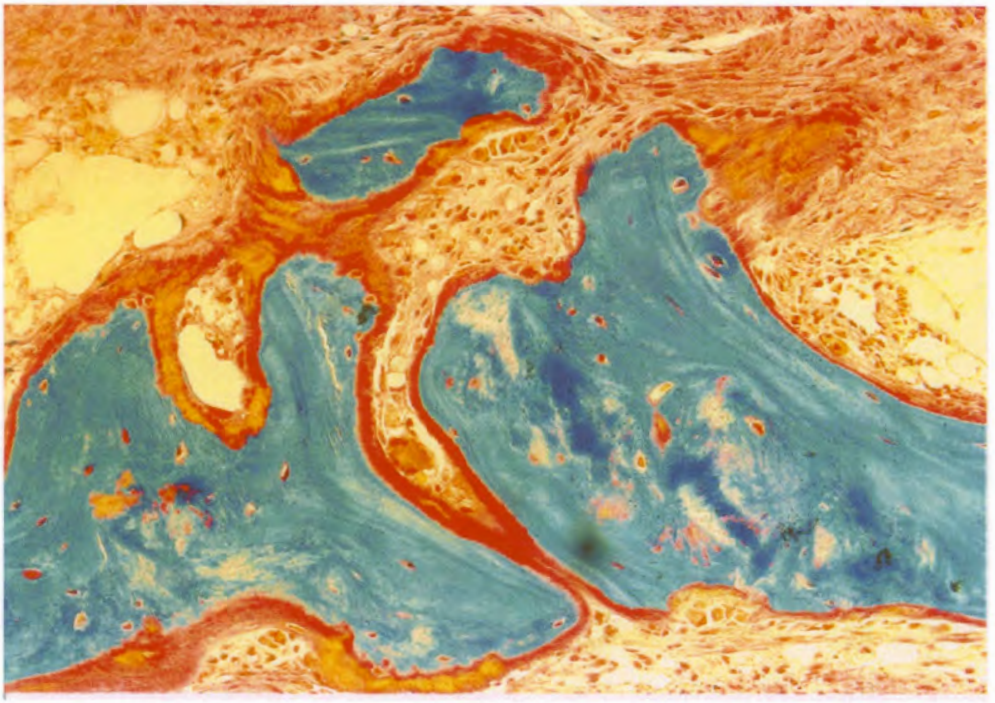

FIG. 10. Bovine collagenous matrix and 2.5 mg of rhOP-1: newly formed mineralized bone covered by osteoid seams (magnification 200 x)

5.3 Histomorphometry

Volume fractions (%) of mineralised bone and osteoid in ossicles generated by various morphogen concentrations on day 90 are shown in ***Table 1***. Implants containing 0.5 mg and 2.5 mg of rhOP-1/100mg ICBM produced comparable amounts of bone. The 2.5 mg concentration showed more variation in generated osseous tissue volume.

5.4 Biochemical indices of bone formation

5.4.1 Alkaline phosphatase (units/mg protein)

The alkaline phosphatase activity in rhOP-1-treated implants on day 90 is shown in ***Table 2***. The implants containing 0.5 mg and 2.5 mg of rhOP-1/100 mg of ICBM produced comparable amounts of alkaline phosphatase. The amount of alkaline phosphatase was more variable in specimens with a concentration of 2.5 mg.

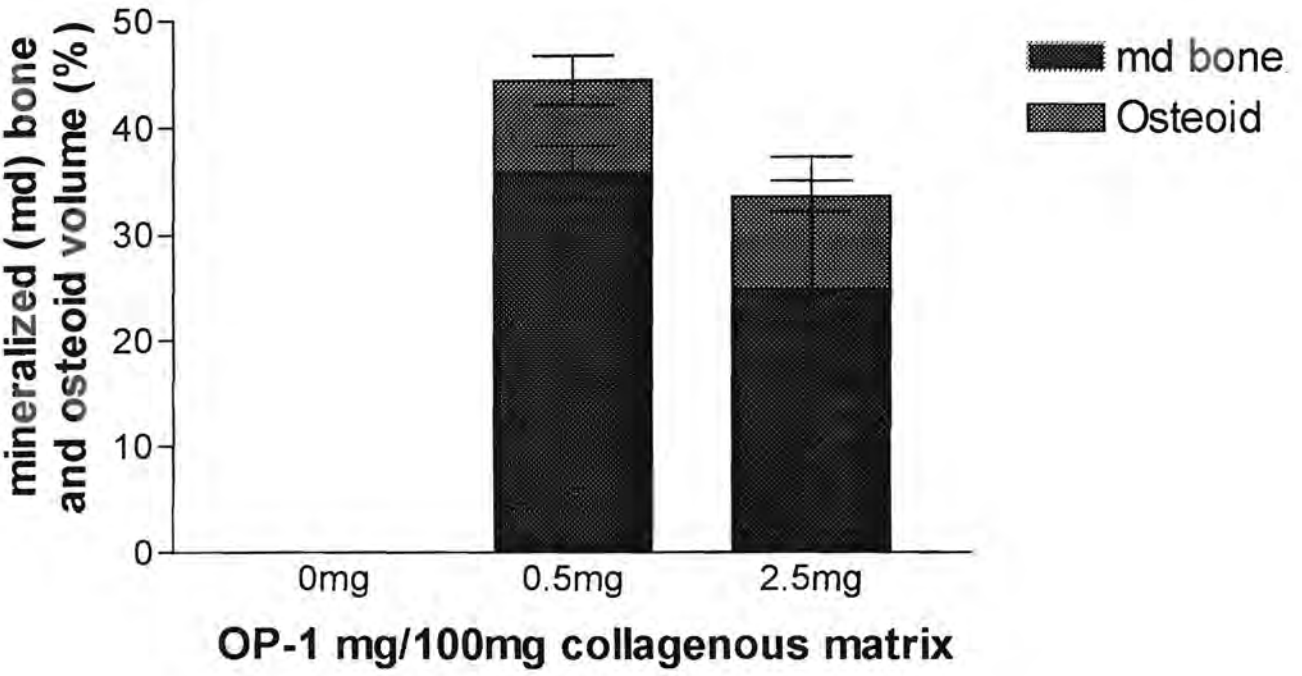

Table 1 Parameters of bone formation induced by rhOP-1 on day 90. Histomorphometric results of induced mineralized bone and osteoid volumes (in %) in the newly formed ossicles. OP-1 dose response.

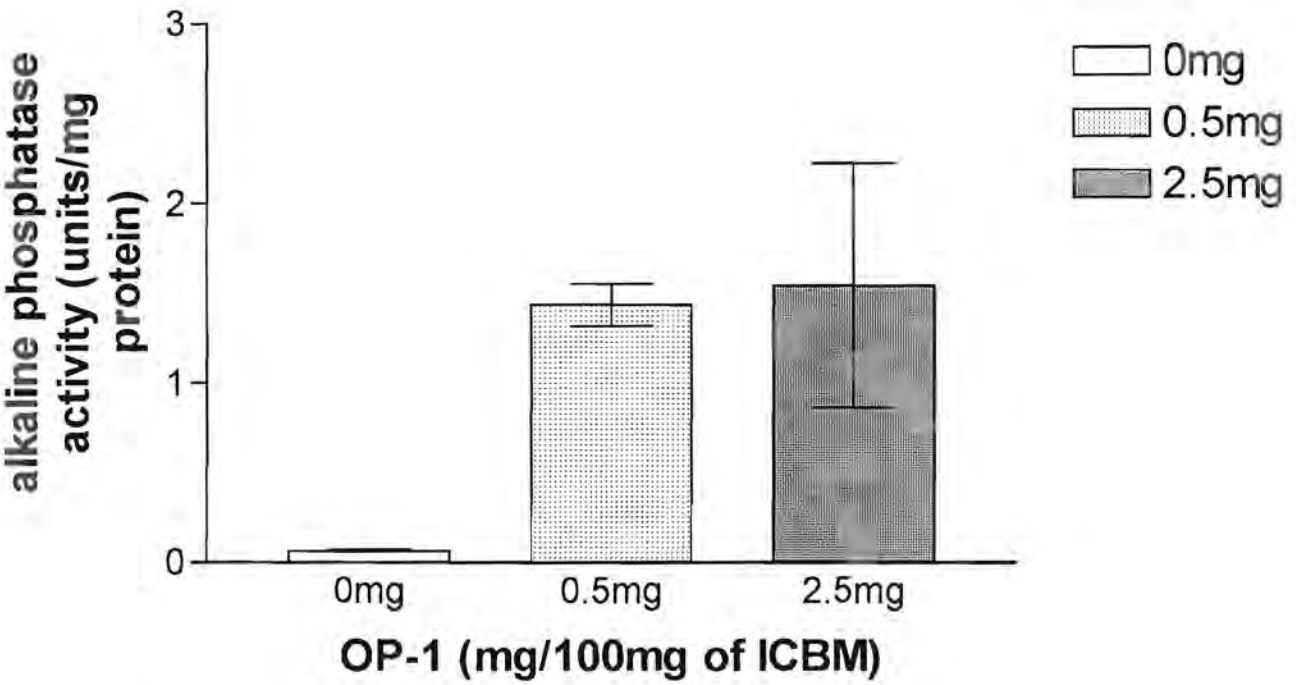

Table 2 Parameters of bone formation induced by rhOP-1 on day 90. Effect of rhOP-1 on alkaline phosphatase activity in the newly generated ossicles. The alkaline phosphatase activity of the supernatant after homogenization of implants was determined with *p*-nitrophenylphosphate as substrate at pH 9.3 and 37° C for 30 minutes. One unit of enzyme liberates 1 µmol of *p*-nitrophenol under the assay conditions. Alkaline phosphatase is expressed as units of activity per milligram of protein (**Reddi & Huggins, 1972; Ripamonti *et al.*, 1991a**). Protein concentration in the supernatant was measured by the method of **Lowry *et al.* (1951)**.

CHAPTER SIX: DISCUSSION AND CONCLUSIONS

As predicted form results obtained in rodents (**Sampath *et al.*, 1992**) and baboons (**Ripamonti *et al.*, 1997; Duneas *et al.*, 1998**), the present findings show that rhOP-1, at doses of 0.5 and 2.5 mg per 100 mg of bovine ICBM as carrier, initiate bone formation by induction in heterotopic sites of the adult baboon which is maintained up to 90 days after implantation. The present study differs from the study by Ripamonti *et al.* and Duneas *et al.*, in that the bone induction potential of rhOP-1 was evaluated on day 90, rather than on day 15 and 30.

The fact that no bone was induced in the first animal can possibly be explained by different healing potentials between individual animals.

Only four of the sixteen implants (containing 0.5 and 2.5 mg of rhOP-1) induced bone formation in the second animal. Whether this is the norm in bone induction assays could not be determined, as we could not find any references on the incidence of success when implanting BMPs/OPs in the abdomen. In this instance one can do no more than speculate on the possible factors that might have inhibited bone induction. Those factors could be excessive trauma during the surgical procedure, variability in the production of implants or repeated post-surgical trauma at the sites of implantation.

The size and the shape of the generated ossicles in the present study compares favorably with that observed in previous studies in the baboon (**Ripamonti *et al.*, 1997; Duneas *et al.*, 1998**). Histological analyses of the present specimens differ markedly from results obtained on day 15 and 30, in that no cartilage was observed. This could be explained by

the longer evaluation period. The tissue response elicited by subcutaneous implantation of BMPs/OPs is reminiscent of embryonic endochondral bone development (**Reddi, 1992; Wozney, 1992**). It stands to reason that the cartilage intermediate has been completely replaced by bone by day 90.

Comparison of histomorphometric measurements between the 0.5 and 2.5 mg of rhOP-1 specimens show comparable volumes of mineralized bone, osteoid and fibrovascular tissue. The 2.5 mg rhOP-1 specimens, however, show a larger volume of residual collagenous matrix. Due to the small number of animals and specimens, it is very difficult to draw any conclusions from this. It is likely that the rapid proliferative activity and subsequent induction of osteogenesis result in the presence of scattered remnants of the collagenous carrier within the center of the specimens.

The presence of bone marrow suggests that that the ossicles had reached maturity by day 90. This is consistent with the bone maturation expected in humans by day 90.

The presence of osteoid and areas of bone resorption show that the bone is undergoing active remodeling.

The expression of alkaline phosphatase by the two different concentrations of rhOP-1 in the present study is comparable. The small sample size, however, makes conclusions difficult.

Ripamonti *et al.* speculated on the use of optimal concentrations of rhOP-1 in the baboon orthotopic model (**Ripamonti *et al.*, 1996a**). He states that the 2.5 mg dose of hOP-1 was

possibly substantially more than required for the specific defect and animal model. The dose of osteogenic material implanted is clinically and commercially important. It would be premature to comment that the 0.5 mg dose of rhOP-1 used in the present study is the concentration of choice. More studies are needed to verify the results of this pilot study.

The biological functions of members of the BMP/OP family are not well understood. In postnatal models of osteogenesis, the BMPs/OPs recapitulate events that occur in the normal course of embryonic bone development. The most obvious proposal for the role of hOP-1 in vivo is as a molecular initiator for the regeneration of skeletal defects.

ADDENDUM

Alkaline phosphatase assay

Set-up

- Water bath, temperature 37° C.
- ***Substrate solution*** (modified): 0.005 M (5 mM) instead of 0.01 M (10 mM) solution of PNPP (para-nitrophenol phosphate disodium, Sigma 104, phosphatase substratum, crystalline, MW 371.1), pH 9.3. Add 371 mg of hydrated PNPP to 200 ml of double DW. Make fresh each time. Store PNPP in refrigerator, avoid moisture. Label as "Substrate solution for Alk Phosphatase".
- ***Barbitol buffer solution***: Sodium Barbitol 0.1 M (adjusted with HCl to pH 9.3. Add 20.6 g Barbitol to 900 ml DW and adjust to proper pH, then bring to one liter. Store in the cold labeled as "Barbitol buffer solution for Alk Phosphatase". For a limited number of implants prepare 500 ml (10.3 g) or 250 ml (5.15 g) of buffer solution.
- ***Base solution***: 0.1 N NaOH. 4 g of NaOH in one liter of DW. Label as "Base solution".
- Label 13 x 100 polypropylene tubes with appropriate sequential sample numbers, duplicates in one rack and singly in another rack to use as matching blanks. Identify and label first set of tubes with: "Alk". Identify and label first tube of matching blank with: "B Alk".
- Spectrophotometer (400 nm reading)

Method

1. Add 1ml of ***Substrate solution*** and 1 ml of ***Barbitol buffer solution*** to all tubes (3 tubes per sample).
2. Place single rack of blanks in water bath for 30 minutes exactly and stop reaction with 2 ml of 0.1 N NaOH (Base Solution). Remove rack of blanks from water bath.
3. Place double set of tubes in water bath and in sequence add 50 µl of sample supernatant to duplicates an to blanks in single rack.

Vortex. Generally one adds sample from 2-4 supernatant tubes per minute and stopwatch to complete a minute before continuing.

4. Stop reaction after 30 minutes with 2 ml of ***Base Solution***, stopping only 2-4 of the duplicates in the water bath per minute in the order that they were started.
5. Read on spectrophotometer at 400 nm in groups of 3, blank and duplicates for each supernatant sample. A hot sample will show a yellow color and generally read between 00080 and 00110. Optical density (OD) reading used is average of duplicates minus reading of blank.
6. Alkaline phosphatase activity is expressed as units activity/mg protein. One unit of alkaline phosphatase is defined as the enzyme activity that liberated 1 µmol PNPP/0.5 h at 37° C per mg of protein. Take the OD reading and multiply by 218.58 and divide by twice the amount of protein determined by the Lowry assay.

The above calculation is determined as follows: extinction coefficient 18.3. Total volume of read sample, 4 ml. The µmoles of PNPP "consumed" = OD x 4/18.3. To relate to mg protein, multiply this amount by 1000 since Lowry determines µg of proteins: 4/18.3 x 1000 = 218.58. Divide by twice the protein values since the volume used in the alkaline phosphatase assay is twice that used for the protein determination.

Lowry protein assay

Set-up

- Label with sequential sample numbers 13 x 100 polypropylene tubes, duplicates in single rack. Identify first set of tubes with: "Protein".
- Label at least 8 blank tubes (each labeled B).
- Label 4 tubes in duplicate (8 tubes) for protein standards (labeled S-20, S-40, S-60, and S-80).
- Spectrophotometer (750 nm reading).
- Dissolve 10 mg of Bovine Serum Albumin (BSA) in 10 ml of DW. Alternatively, defrost premeasured frozen aliquots of BSA (1 mg/ml in DW).
- ***Copper reagent*** (make fresh):
  a) ***Protein solution***: 100ml of 2 % Na_2CO_3 (w/v) in 0.1 NaOH (2 g of Na_2CO_3 + 400 mg of NaOH. Make 1 (one) l (20 g Na_2CO_3 + 4 g NaOH). Store at room temperature, labeled as "Protein stock solution".
  b) ***NaTartarate solution***: 2 % (w/v) of NaTartarate in DW. Add 200 mg to 10 ml DW. Add 1 ml of 2 % NaTartarate in DW. Add 1ml of 2 % NaTartarate for each 100 ml of Protein stock solution used.
  c) ***Copper sulfate solution***: 1 % (w/v) of $CuSO_{4.5}H_2O$. Add 100mg of $CuSO_{4.5}H_2O$ to 10ml DW. Add 1ml of 1 % (w/v) $CuSO_{4.5}H_2O$ for each 100 ml of Protein stock solution used.
  d) Stir and use solution within one hour.

Method

1. Place an appropriate volume of BSA in each standard (e,g. 20 µl of BSA in S-20 tubes).
2. Add 25 µl of sample supernatant in each sample tube (in duplicate).
3. Add DW to give final volume of 400 µl to duplicates of samples and protein standards.
4. Add 400 µl of DW to blanks.
5. Add 2 ml of ***Copper reagent*** to each tube and wait 30 minutes.
6. Add 200 µl of 1 N ***Phenol reagent*** to each tube (Fisher Diagnostics Phenol Reagent solution 2 N, Folin-Ciocalteau) diluted 1:1 with DW, **Vortex** immediately. For a limited number of implants, add 20 ml of ***phenol reagent*** to 20 ml of DW.
7. Wait a minimum of 30 minutes and read on spectrophotometer at 750 nm. Protein turns solution blue. The samples must be read between 30-90 minutes after the addition of the phenol reagent.
8. Combine the blanks tube solution together and place a volume in the reference cuvette and suck up a few runs of blank into the reading cuvette and adjust spectrophotometer to zero. Read standards and then the samples.
9. Protein values are determined by using standard values to obtain a standard curve and the protein in 25 µl of sample supernatant read from the curve, protein values generally range from 10-80 µg per 25 µl of supernatant.

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