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Synergistic induction and temporal enhanement of bone formation by osteogenic protein-1 (OP-1) and transforming growth factor beta-1 (TGF-B1) combinations in rats

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SYNERGISTIC INDUCTION AND TEMPORAL ENHANCEMENT OF BONE FORMATION BY OSTEOGENIC PROTEIN-1 (OP-1) AND TRANSFORMING GROWTH FACTOR BETA-1 (TGF-ß1) COMBINATIONS IN RATS.

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 1999.

DECLARATION

I, Thato Nelly Matsaba declare that this thesis is my own work and has not been submitted before for any degree or examination at this or any other university.

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05th day of NOVEMBER, 1999.

ABSTRACT

Several members of the bone morphogenetic protein/osteogenic protein (BMP/OP) and transforming growth factor beta (TGF-ß) families are molecular regulators of cartilage and bone regeneration. However, their precise mode of signal transduction and combined interactions are poorly understood. The presence of several molecular forms of these growth factors suggests multiple functions *in vivo* as well as synergistic interactions during both embryonic bone development and regeneration of cartilage and bone in postnatal life. A functional bioassay for identification of osteogenic proteins within the bone matrix has been established. The heterotopic bioassay in rats, together with the improved purification methods, has led to the purification of native BMPs. In rats, heterotopic and orthotopic implantation of TGF-ß singly fails to initiate new bone formation whereas implantation of BMPs/OPs elicit the local differentiation of new bone, at both sites.

This study presents data which shows that co-administration of TGF-ß1 with OP-1 delivered by a collagenous carrier, and implanted in the subcutaneous site of rats results in synergistic induction of bone formation. Changes in histological, biochemical and molecular response of the effects of the morphogens were studied over 7, 12 and 21 days post implantation. Induced cartilage and bone were analyzed by alkaline phosphatase activity, calcium content, Northern blots and histological examination of subcutaneous implants in the rats.

Single applications of TGF-ß1 (0.01µg, 0.03µg and 0.1µg) on days 7, 12 and 21 gave rise to negligible alkaline phosphatase activity (0.03-0.09 U/ mg protein) and calcium content (0.05-0.6 µg/mg tissue). Histological examination showed that all TGF-ß1 implants did not exhibit any sign of bone formation.

On day 7, OP-1 implants (0.1µg and 0.3 µg) elicited negligible alkaline phosphatase activity and calcium content. However, higher doses of OP-1 (1 µg and 3 µg) elicited alkaline phosphatase activity of 0.1 U/mg protein and 0.2 U/mg protein. Combination TGF-ß1 (0.01µg, 0.03µg and 0.1µg) with OP-1 (0.1µg, 0.3µg, 1µg and 3µg) slightly increased the activity of alkaline phosphatase activity (0.2 U/mg protein-0.4 U/mg protein). OP-1 singly gave rise to very low calcium levels of 0.4 µg/mg tissue to 0.5 µg/mg tissue. Addition of TGF-ß1 to OP-1 resulted in calcium content rising from 0.2 to 1 µg/mg tissue. Histologically, the specimens of single OP-1 applications did not show any sign of bone formation. On addition of TGF-ß1 to OP-1 the specimens showed signs of the beginning of chondroblastic differentiation.

On day 12 alkaline phosphatase activity elicited by single applications of OP-1 ranged from 0.1 U/mg protein to 1 U/mg protein. Addition of TGF- ß1 increased the alkaline phosphatase from 0.8 U/mg protein to 7 U/mg protein. Calcium levels resulting from single applications of OP-1 ranged from 0.1 to 15 µg/mg tissue. After addition of TGF-ß1 to OP-1 calcium levels rose from 5 to 20 µg/mg tissue.

Histological analysis showed formation of cartilage in specimens both of OP-1 solo and OP-1 in combination with TGF-ß1.

On day 21 alkaline phosphatase activity was reduced to a range of 0.1-0.5 U/mg protein upon single applications of OP-1. Addition TGF-ß1 resulted in a further decrease in alkaline phosphatase activity. Calcium levels were 10-68 µg/mg tissue on single applications of OP-1. Addition of TGF-ß1 to OP-1 increased the calcium levels in the range of 2-70 µg/mg tissue. Histological examination of the 3 µg OP-1 solo specimens showed complete chondrolysis whereas the OP-1 (3µg)/ TGF-ß1 (0.03 and 0.1 µg) specimens showed the differentiation of bone marrow.

Tissues generated in the rat subcutaneous space at 7, 12 and 21 days post implantation elicited mRNA expression of OP-1, BMP-3 and TGF-ß1. These results indicate that at least in part, the matrix-induced endochondral bone formation involves the expression of some members of the TGF-ß superfamily. Type II collagen (chondrogenesis marker) and type IV collagen (angiogenesis marker) mRNAs were also detected on days 12 and 21, respectively.

The present data suggests that TGF-ß up regulates the temporal activity of OP-1 to induce bone formation. Co-administration of TGF-ß1 to OP-1 caused an increase in the alkaline phosphatase activity and calcium content, markers of bone formation, which implies that when TGF-ß is mixed with OP-1, the cascade of bone formation

is accelerated. These results may have important therapeutic implications. The rapidity of tissue morphogenesis with bone marrow formation is important for regeneration of bone in older patients where repair phenomena are temporally delayed and the healing process is slower than in younger patients. The expression of multiple members of the TGF-ß superfamily indicates that the cascade of bone formation incorporates some members of the TGF-ß superfamily and this may form the basis for synergistic molecular therapeutics for cartilage and bone regeneration in clinical contexts.

DEDICATION

To my parents, for their love and support over many years.

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CONTENTS

		page
Declaration		ii
Abstract		iii
Dedication		vii
Acknowledgements		viii
Contents		ix
List of figures		xii
List of Tables		xvi
1	**Introduction**	1
1.1	Purification of BMPs	3
1.2	Identification of BMPs	7
1.3	The TGF-ß superfamily	7
1.4	Bone morphogenetic proteins	8
1.5	Role of BMPs	9
1.6	BMP-7/OP-1	11
1.7	Role of BMP-7/OP-1	11
1.8	TGF-ß	13
1.9	Role of TGF-ß	14
1.10	Other growth factors	21
1.11	Co-operative action of growth factors	24
1.12	Synergy	24
1.13	Synergy in biological systems	25
1.14	Pre clinical and clinical applications for BMPs	27
1.15	Isobolographic analysis	28
1.16	Aims	31

2	**Materials and methods**	32
2.1	Experimental animals	32
2.2	Selection of experimental animals	32
2.3	Morphogens	33
2.3.1	rh OP-1	33
2.3.2	TGF-$ß_1$	33
2.4	Insoluble collagenous bone matrix	33
2.5	The rat heterotopic model	34
2.5.1	Experimental design	34
2.6	Preparation of implants	35
2.7	Analytical procedures	35
2.7.1	Tissue harvest, histology and biochemistry	35
2.7.2	Histology	35
2.7.3	Biochemical markers of bone formation	36
2.7.4	Reagents	36
2.8	Protein Lowry assay	37
2.8.1	Reagents	37
2.9	Calcium assay	38
3	**Northern blot analysis**	38
3.1	Preparation of total RNA	38
3.2	Formaldehyde agarose gel electrophoresis	40
3.2.1	Reagents	40
3.3	Sample preparation and gel electrophoresis	40
3.4	RNA transfer to membrane	41
3.5	Probes and hybridization	41
3.6	Amplification of constructs	42
3.7	Transformation of E. coli	43
3.8	Preparation of plasmid DNA	43

3.9	Preparation of probe labelled with alpha 32 PdCTP	44
3.10	Hybridization of probes and detection	45
3.11	Signal quantitation	46
3.12	Statistical analysis	46
4	**Results**	47
4.1	Seven days implantation	47
4.2	Twelve days implantation	62
4.3	Twenty one days implantation	73
5	**Discussion and concluding statements**	87
6	**References**	98

LIST OF FIGURES

Figure 1. Example of an isobologram.

Figure 2. Biochemical results of implantation of OP-1 and TGF-ß1both singly and in combination in the rat subcutis. Generated tissues were harvested at 7 days post implantation and alkaline phosphatase activity was determined as described in the methods.

Figure 3. Calcium content of tissues generated by implantation of OP-1 and TGF- ß1, both singly and in combination in the rat subcutis. Generated tissues were harvested at 7 days post implantation and calcium content was determined as described in the methods.

Figure 4. Toluidine blue stained sections of tissues generated in the subcutaneous space of the rat by ICBM solo and TGF-ß1 solo. The tissues were processed as described in the methods.

Figure 5. Toluidine blue stained sections of tissues generated in the subcutaneous space of the rat by OP-1 and TGF-ß1, both singly and in combination 7 days post implantation. The tissues were processed as described in the methods.

Figure 6. Northern blot analyses of gamma actin, OP-1, BMP-3 and TGF- ß1mRNA species generated in the rat subcutaneous space at day 7. Replicate generated tissues (n=4) were pooled and total RNA was extracted and hybridized as described in the methods.

Figure 7. mRNA signal quantitation of gamma actin, OP-1, BMP-3 and TGF-ß1 at day 7. Replicate generated tissues (n=4) were pooled and total RNA was extracted and hybridized as described in the methods.

Figure 8. Biochemical results of implantation of OP-1 and TGF-ß1both singly and in combination in the rat subcutis. Generated tissues were harvested at 12 days post implantation and alkaline phosphatase activity was determined as described in the methods.

Figure 9. Calcium content of tissues generated by implantation of OP-1 and TGF- ß1, both singly and in combination in the rat subcutis. Generated tissues were harvested at 12 days post implantation and calcium content was determined as described in the methods.

Figure 10. Toluidine blue stained sections of tissues generated in the subcutaneous space of the rat by OP-1 and TGF-ß1, both singly and in combination 12 days post implantation. The tissues were processed as described in the methods.

Figure 11. Northern blot analyses of gamma actin, OP-1, BMP-3 and TGF- ß1mRNA species generated in the rat subcutaneous space at day 12. Replicate generated tissues (n=4) were pooled and total RNA was extracted and hybridized as described in the methods.

Figure 12. mRNA signal quantitation of gamma actin, OP-1, BMP-3 and TGF-ß1 at day 12. Replicate generated tissues (n=4) were pooled and total RNA was extracted and hybridized as described in the methods.

Figure 13. Biochemical results of implantation of OP-1 and TGF-ß1both singly and in combination in the rat subcutis. Generated tissues were harvested at 21 days post implantation and alkaline phosphatase activity was determined as described in the methods.

Figure 14. Calcium content of tissues generated by implantation of OP-1 and TGF- ß1, both singly and in combination in the rat subcutis. Generated tissues were harvested at 21 days post implantation and calcium content was determined as described in the methods.

Figure 15. Toluidine blue stained sections of tissues generated in the subcutaneous space of the rat by OP-1 and TGF-ß1, both singly and in combination 21 days post implantation. The tissues were processed as described in the methods.

Figure 16. . Northern blot analyses of gamma actin, OP-1, BMP-3 and TGF-ß1mRNA species generated in the rat subcutaneous space at day 21. Replicate generated tissues (n=4) were pooled and total RNA was extracted and hybridized as described in the methods.

Figure 17. mRNA signal quantitation of gamma actin, OP-1, BMP-3 and TGF-ß1 at day 21. Replicate generated tissues (n=4) were pooled and total RNA was extracted and hybridized as described in the methods.

Figure 18. Northern blot analyses of collagen type II and type IV collagen species generated in the rat subcutaneous space at days 12 and 21 respectively. Replicate generated tissues (n=4) were pooled and total RNA was extracted and hybridized as described in the methods.

Figure 19. mRNA signal quantitation of collagen type II type IV at days 12 and 21 respectively. Replicate generated tissues (n=4) were pooled and total RNA was extracted and hybridized as described in the methods.

LIST OF TABLES

Table 1. Implantation protocol of OP-1 and TGF-ß1 in the rat subcutaneous space.

1 INTRODUCTION

For decades, the phenomenon of bone formation by induction has captured the minds of bone biologists. Bone has a remarkable potential for repair and regeneration. Lacroix (1945) observed that bone implants are capable of inducing new bone in the recipient. Based on this observation, the author hypothesised that in bone there may be substances that might initiate bone growth, which he named osteogenins. This was followed by the studies of Urist (1965) and Reddi and Huggins (1972) who first used *in vivo* assays to study bone-inductive properties of the bone matrix and then stimulated many other groups to contribute to the growing knowledge of the molecular and cellular signals involved in endochondral bone formation.

The fundamental work of Urist (1965), Reddi and Huggins (1972) has provided evidence for the existence of a bone morphogenetic protein (BMP) complex in the bone matrix (Urist *et al.*, 1984; Sampath *et al.*, 1987). At the time, it was still unclear whether the bone-forming activity in the demineralised bone matrix was due to the combined action of several factors present in bone, a separate protein, or a family of proteins as yet to be characterised and sequenced. Native BMPs were isolated from the demineralised bone matrix and purified to provide information for amino acid sequences (Wang *et al.*, 1988; Luyten *et al.*, 1989). This work led to expression cloning and characterisation of recombinant human BMPs (Wozney *et al.*, 1988).

The purification and molecular cloning of the BMPs has set the stage for novel therapeutic approaches to correct congenital and acquired craniofacial and orthopaedic conditions (Ripamonti and Vukicevic, 1995 for review). Furthermore, the purification of BMPs has laid the foundation for the cellular and molecular dissection of bone development and regeneration.

Bone formation occurs in two different ways; first, there is intramembraneous ossification, which is the direct development of bone from mesenchyme, for example, in the skull. Second, there is an intervening stage of cartilage that precedes bone formation from mesenchymal cells as is the case in long bones. This process is called endochondral bone development (Reddi, 1981). Endochondral bone formation can take place in the embryo, during fracture healing or in post foetal life after induction by implanted demineralised bone matrix (Reddi, 1981; 1992).

Embryonic events give rise to five types of cells which are responsible for the production of the five skeletal tissues in the body namely: osteoblasts producing bone, chondroblasts producing cartilage, odontoblasts producing dentine, cementoblasts producing cementum, and ameloblasts producing enamel (Urist, 1980).

The first sign that indicates imminent skeletogenesis, is the condensation of mesenchymal and mesodermal cells to form a primordium of bone. These

condensing cells either arise locally, in the position the bone will occupy eventually, as in the formation of the embryonic vertebrae and the formation of a fracture callus, or they migrate from elsewhere to the site at which skeletogenesis will occur as in the migration of ectomesenchymal cells into the embryonic mandible. In post foetal life, the repair and maintenance of tissues deploys embryonic-like mechanisms. The matrix-induced bone formation mimics the embryonic sequence of bone formation.

1.1 PURIFICATION OF BMPS

Identification of osteogenic proteins in the bone matrix was not easy because of the small quantity of proteins that are tightly bound to the extracellular matrix of bone. This problem was solved by the solubilization of the bone-inductive fractions from the bone matrix (Sampath and Reddi, 1981). The osteoinductive nature of the demineralised bone matrix (DBM) is nullified by dissociative extraction into two components: a soluble protein extract and an insoluble residue which is mainly insoluble collagen (insoluble collagenous bone matrix or ICBM). Reconstituting the two components (Sampath and Reddi, 1981) can restore the osteoinductive capability. It has also been shown that the factors responsible for osteoinductivity reside in the solubilized component and when reconstituted with an appropriate carrier, the osteoinductivity can be restored (Sampath and Reddi, 1981). This operational reconstitution was a hallmark in the field of bone induction, since it allowed the identification of the factors responsible for osteoinductive activity (Sampath and Reddi, 1981).

Sampath and Reddi (1981) performed classic experiments to show that the osteoinductive activity of demineralised bone matrix was soluble. It was shown that guanidinium chloride extraction of demineralised bone matrix inactivates it, but reconstitution with the soluble component restored the osteoinductivity in the rat heterotopic bioassay. The subcutaneous implantation of the osteogenic activity in rats led to new bone formation typically by day 12 (Sampath and Reddi, 1981).

Bone formation by induction can be achieved by the intramuscular or subcutaneous implantation of DBM. DBM is prepared by crushing and sieving dehydrated diaphyseal shafts of rat femurs and tibias to particle sizes of 74-420 μm, then demineralising the powder with 0.5M hydrochloric acid (HCl).The matrix is then washed and dehydrated with ethanol and ether (Reddi and Huggins, 1972). These were the classical experiments of Urist and co-workers (Urist, 1965) (intramuscular implantation) followed by the subcutaneous implantation by Reddi and co-workers (Reddi and Huggins, 1972). A dissociative extraction was eventually performed to try to identify the bone inductive substance or substances in a very important experiment (Sampath and Reddi 1981). This was achieved with 4M guanidinium hydrochloride (Gnd.HCl) resulting in the insoluble collagenous bone matrix (ICBM) and protein extracts. The dissociative extraction yielded a complete loss of the ability of bone matrix to induce bone formation. Sampath and Reddi (1983) showed that the bone-inductive potential was not species-specific by using solubilized osteogenic fractions from several different species, which meant that a bone-inductive fraction

from bovine bone matrix could induce bone in the rat when reconstituted with rat insoluble collagenous bone matrix. This observation provided a functional bioassay for identification of osteogenic proteins within the bone matrix (Sampath and Reddi, 1981; Sampath and Reddi, 1983; Wang *et al.*, 1988; Luyten *et al.*, 1989; Sampath *et al.*, 1990).

The solubilized protein components of the bone matrix were reconstituted with their residues and assayed for their potential to induce bone formation. The total loss of the biological activity of bone matrix after dissociative extraction was restored when the extracted soluble components were reconstituted with their respective inactive insoluble residue. The components extracted from DBM were then characterised by polyacrilamide slab gel electrophoresis and gel filtration.

The heterotopic bioassay in rats, together with the improved purification methods led to the purification of native BMPs (Sampath *et al.*, 1987; Luyten *et al.*, 1989; Wang *et al.*, 1989; Sampath *et al.*, 1990). Ripamonti *et al.* (1992) purified BMPs from baboon bone matrix and showed that the implantation of bovine derived osteogenic fractions intramuscularly in baboons and subcutaneously in rats induced bone formation, provided that the factors were reconstituted with baboon ICBM.

Subcutaneous or intramuscular implantation of demineralised bone matrix (DBM) gives rise to a sequence of developmental, morphogenetic and biochemical events

resulting in the differentiation of endochondral bone (Urist, 1965; Reddi and Huggins, 1972; Reddi, 1981). The developmental cascade of matrix-induced endochondral bone formation consists of chemotaxis and attachment of mesenchymal stem cells to the matrix, multiplication of progenitor cells followed by cartilage differentiation, bone differentiation and finally hematopoiesis (Reddi, 1981). On day-1 of the cascade, there is fibronectin deposition on the matrix, facilitating cell attachment. Multiplication of mesenchymal cells follows between days 2 and 3, resulting in chondroblast differentiation at day 5; appearance of cartilagenous matrix follows at day 7 as shown by sulphate incorporation into proteoglycans and appearance of chondrocytes. The beginning of angiogenesis and cartilage resorption occurs around day 9. New bone formation in association with calcium incorporation occurs at day 11, then extensive bone remodelling with hematopoietic marrow formation as evidenced by iron incorporation at day 21. The endochondral development programme summarises the embryonic development and the induction of bone repair and regeneration in post foetal life, and it also enables molecular studies at the different stages of endochondral bone formation as well as a better understanding of the role of endogenous and exogenous growth and differentiation factors involved.

1.2 IDENTIFICATION OF BMPS

The purification of BMPs was followed by molecular cloning and expression of recombinant human proteins (Wozney *et al.*, 1988; Hammonds *et al.*, 1991; Sampath *et al.*, 1992). Amino acid sequence of bone-inductive factors was identified leading to the elucidation of the genetic code and expression of a new family of proteins called the bone morphogenetic proteins (BMPs).

1.3 THE TGF-ß SUPERFAMILY

The transforming growth factor beta (TGF-ß) superfamily is a group of molecules which comprises a large group of structurally related signalling proteins that are secreted as dimers and then cleaved after an Arg-X-X-X-Arg site to release biologically active carboxy-terminal domains containing seven highly conserved cysteine residues. The group consists of BMPs (Wozney *et al.*, 1988; Ozkaynak *et al.*, 1990; Celeste *et al.*, 1990); activins and inhibins (Schwall *et al.*, 1988); the 60 A (Wharton *et al.*, 1991); the *dpp* gene products of *Drosophila melanogaster* (Padget *et al.*, 1987; Ferguson and Anderson, 1992); the vegetal Vg-1 gene products of *Xenopus Laevis* (Weeks and Melton, 1987); the Vg-1 related analogue of murine Vgr-1 (Lyons *et al*, 1989); the more distantly related growth and differentiating factors (Lee, 1991; McPherron and Lee, 1993) and the cartilage derived morphogenetic proteins (CDMPs) (Chang *et al.*, 1994) and finally, the TGF-ßs themselves.

1.4 BONE MORPHOGENETIC PROTEINS

The BMPs comprise a growing family of more than 14 proteins. Of these, 9 have been shown to singly induce heterotopic bone formation *in vivo* when expressed recombinantly. The first BMPs to be identified were BMP-1, BMP-2, BMP-2b (now called BMP-4) and BMP -3 (Wozney *et al.*, 1988). Bmp-1 was later found not to be a member of the TGF-ß family, since it is a misnamed protease (Reddi 1996; Kessler, 1996). Other novel members were later isolated and cloned such as BMP-5, BMP-6, BMP-7 (OP-1), and BMP-8 (OP-2) (Celeste *et al.*, 1990; Ozkaynak *et al.*, 1990). There are now about 30 members in the BMP family. Other members of the TGF-ß' superfamily related to BMPs include activin and inhibin, the Mullerian inhibitory substance (MIS), the growth and differentiation factors (Lee, 1990; Alexandra *et al.*, 1993; Cunningham *et al.*, 1995) and the cartilage derived morphogenetic proteins (Chang *et al.*, 1994). According to their primary amino acid sequences, BMP-2 to BMP-8 are grouped into 3 main subfamilies.

BMP-3 is the only member of its group (BMP-3 group) with a 43-49% identity with each of the other groups (Luyten *et al.*, 1989). The second group, the BMP-7 subfamily constitutes BMPs -5, -6, -7 and -8 which share an average of 89% amino acid identity with each other.

The third group consists of BMP-2 and -4 that share a 92% amino acid identity. Other minor subfamilies include BMP-9, -10, and -15 which fall under one group

and the growth and differentiation factors which also make another subfamily made up of GDF-1, GDF-3, GDF-8, GDF-9, GDF-11/BMP-11, GDF-12 and GDF-14.

1.5 ROLE OF BMPS

BMPS have wide-ranging extraskeletal roles in development, as suggested by the following observations:

(I) Localisation studies in both human and mouse tissues have demonstrated high levels of mRNA expression and protein synthesis for various BMPS in lung (BMP-3, -4, -5 and -6) (Vukicevic *et al.*, 1994; Bellusci *et al.*, 1996; Urase, 1996), kidney (BMP-3, -4 and -7) (Ozkaynak *et al.*, 1991; Luo *et al.*, 1995; Vukicevic *et al.*, 1994; Helder *et al.*, 1995; Dudley and Robertson, 1997; Godin *et al.*, 1998), small intestine (BMP-3 and -7) (Ozkaynak *et al.*, 1991), heart (BMP-2, -4, -6 and -7) (Lyons *et al.*, 1995; Helder *et al.*, 1995;) teeth (BMP-3, -4 and -7) (Vainio *et al.*,1993; Francis *et al.*, 1994; Aberg *et al.*, 1997; Barlow and Francis, 1997; Jernvall *et al.*, 1998; Peterkova *et al.*, 1998; Helder *et al.*, 1998) and limb bud (BMP-2, -4, -5 and -7) (Lyons *et al* ., 1990; Francis *et al.*, 1994; Lyons *et al.*, 1994; Helder *et al.*,1995). Vukicevic *et al.*, (1998), reported that BMP-7 reduces severity of injury after ischemic acute renal failure in rats. They showed that BMP-7 preserves kidney function, minimises infarcation and cell necrosis, and decreases the number of plugged tubules; suppresses inflammation by down regulating the expression of intercellular adhesive molecule, and prevents the accumulation and activity of

neutrophils; maintains the expression of the vascular smooth muscle cell phenotype in pericellular capillaries ; and reduces programmed cell death during the recovery.
(ii) BMP-4 and -7 are key molecules in epithelial-mesenchymal interactions, (Vainio *et al.*, 1993; Francis *et al.*, 1994; Helder *et al.*, 1995; Barlow and Francis, 1997; Aberg *et al.*, 1997; Jernvall *et al.*,1998; Peterkova *et al.*, 1998; Helder *et al.*, 1998).

(iii) BMP-2 and-4 are involved in the signalling pathway that controls patterning in the developing chick limb (Francis *et al.*, 1994) and BMP-4 is a ventralising factor in early *Xenopus* development (Dale *et al.*, 1992; Jones *et al.*, 1992).

(iv) The homologues of the BMPs in *Drosophila,* the decapentaplegic (*dpp*) and 60 A gene products have the capacity to induce bone formation in mammals (Sampath *et al.*, 1993) and the human BMP-4 is able to confer the normal embryonic dorso-ventral patterning in *Drosophila* transformants defective in expressing *dpp* (Padget *et al.*, 1993).

At least some members of the BMP family are required for proper patterning in the vertebrate limb and in other skeletal structures as shown by the defects in skeletal structures caused by mutations and deletions of *BMP-5* gene in short ear mice (Kingsley *et al.*, 1992).

1.6 BMP-7/OP-1

Bone morphogenetic protein -7, also known as osteogenic protein-1 (OP-1), was originally isolated from bone based on its ability to induce bone formation *in vivo* (Sampath *et al.* 1990). It has been demonstrated that OP-1 has the ability to induce bone formation in non-human primates (Ripamonti *et al.*, 1996). OP-1 is synthesized in the kidney (Ozkaynak *et al.* 1991) and it is also present in the circulation ,which suggests that OP-1 may have a therapeutic potential for the regulation and treatment of metabolic bone diseases such as osteoporosis. OP-1 is a member of the TGF-ß superfamily and shares a common polypeptide fold with TGF-ß2 although there is limited sequence identity between them (Griffith *et al.* 1996). The authors elucidated the three dimensional structure of OP-1 and, based on their results, suggested that the structure of OP-1 confirms the proposal that the TGF-ß2 fold is prototypical for the conserved C-terminal region of the TGF-ß superfamily (Daopin *et al.* 1992; Schlunegger and Grøtter, 1992). OP-1, like other members of the TGF-ß superfamily, has diverse biological activities and plays an important role in the migration, proliferation, and differentiation of mesenchymal cells during embryogenesis and in the repair and regeneration in postnatal life.

1.7 ROLE OF BMP-7/OP-1

There is accumulating evidence that BMP-7 plays an important role in the regulation of many morphogenetic events during embryogenesis. The embryonic vertebrate kidney development takes place along the interaction of the epithelial ureteric bud

and the metanephric mesenchyme (Saxen,1987). Nephrogenesis follows this interaction. Luo *et al.*, 1995 and Dudley *et al.*, 1995, reported that BMP-7 (OP-1) deficient mice die shortly after birth due to poor kidney development. In situ hybridization analysis showed that the absence of OP-1 in mutant mice resulted in the absence of main molecular markers of nephrogenesis which suggests that OP-1 is a nephrogenic inducer hence very important for kidney development (Luo *et al.*, 1995; Vukicevic *et al.*, 1996; Godin *et al.*, 1998). Loss of OP-1 signalling in kidney results in apoptosis in the metanephric mesenchyme (Dudley and Robertson, 1997). Other abnormalities in OP-1 mutant mice include skeletal patterning defects of the rib cage, skull and polydactily in the hind limbs, kidney as well as eye defects. When rat embryos were cultured in the presence of OP-1 blocking antibodies, the rats all exhibited a size reduction or absence of eyes. There was a greater reduction in neural retinal development that led to the conclusion that OP-1 is essential for eye development (Solursh *et al.*, 1996). High levels of OP-1 mRNA expression were observed in murine kidney (Ozkaynak *et al.*, 1991) as well as in basement membranes (Vukicevic *et al.*, 1994). Helder *et al.* (1995) showed that in developing human embryos and foetuses, OP-1 was found to be highest in the foetal kidney and heart between 12 to 14 weeks of gestation, followed by other tissues including brain and lung. The above findings also suggested that Bmp-7 plays important roles in the initiation and progression of limb formation as well as being involved in the terminal differentiation of chondrocytes and subsequent bone formation. Bellusci *et al.* (1996) also showed that OP-1 plays an important role in mouse embryonic lung

development. OP-1 was reported to be a key molecule in epithelial-mesenchymal interactions (Vainio *et al.*, 1993). Hofmann *et al.* (1996) reported that OP-1 is involved in regulating proliferation and epithelial-mesenchymal interactions in the developing limb. OP-1 was also reported to mediate neural inhibition and epidermal induction in Xenopus embryos (Suzuki *et al.*, 1997). OP-1 was reported to play roles in the development of the heart, teeth, and small intestines.

1.8 TGF-ß

Assoian and the co-workers (1983) first identified TGF-ß isoforms as 25 kDa homodimers that induce normal rat fibroblast growth in soft agar culture. Active TGF-ß must be dissociated from a latent complex to become biologically active. The latent complex is a 100 kDa precursor that is produced by transfected Chinese Hamster Ovary (CHO) cells (Gentry *et al.*, 1987). In other types, such as fibroblasts and platelets, the latent TGF-ß complex also contains a protein called the TGF-ß binding protein. Bone cells are unique in making two latent forms of TGF-ß, one containing TGF-ß binding protein and one that does not.

The TGF-ß family is known to comprise 5 closely related proteins called TGF-ß-1 to- 5 (Roberts and Sporn, 1990). TGF-ßs -1 to -3 have been identified in mammalian cells, TGF-ß -4 in chicken and TGF-ß -5 in *Xenopus laevis.* The amino acid structure of the TGF-ßs is highly conserved between species (Roberts *et al.*, 1991). TGF-ß-1 is identical in man, monkey, pig, cow and chicken. TGF-ß-2 shares 71% homology

with TGF-ß 1 and the other TGF-ßs share homology in the range of 64% to 83% with TGF-ß-1 (Roberts *et al.*, 1991). The first 3 isoforms of TGF-ß have been found in human tissues. Using immunohistochemistry, Wataya-Kaneda *et al.* (1994) showed the distribution of TGF-ßs 1-3 in human skin: TGF-ß2 was localised mainly in the intercellular space of all the layers of the epidermis as well as weakly in the cytoplasm; TGF-ß3 was present in the subepidermal area of the dermis; and TGF-ß1 was not observed in either epidermis or dermis. These results suggested that TGF-ß2 and 3 may regulate the human skin function in an epithelial autocrine or mesenchymal-epithelial interaction manner. TGF-ßs upregulate the expression of collagen α II gene in periosteum-derived cells. Collagen type II is a marker for cartilage formation (Ballock *et al.*, 1997).

1.9 ROLE OF TGF-β

The TGF-ß family of cytokines is multifunctional and essential for survival. They play an important role in epithelial-mesenchymal interactions. The differentiation of epithelial and mesenchymal cells is enhanced by TGF-ß-related factors (Vukicevic *et al.*, 1996) which are often detected in both these cell types (Heine *et al.*, 1987). The factors also perform important roles in growth and development, inflammation and repair and host immunity. The mammalian TGF-ß isoforms (TGF-ß-1-3) are secreted as latent precursors and have multiple cell surface receptors of which at least two mediate signal transduction (Clarke and Coker, 1998). Extracellular matrix, neighbouring cells and other cytokines can modify autocrine and paracrine effects of

TGF-ßs. The important role of TGF-ß family is illustrated by the fact that the TGF-ß knockout mice die in utero and the remainders succumb to uncontrolled inflamation after birth (Kulkarni *et al.*,1993). Mice harbouring null mutations for TGF-ß isoforms demonstrate that each exerts discreet nonoverlapping functions during development. TGF-ß1 knockout mice reveal an important role for this cytokine in regulation of the immune system, with evidence for altered development, activation and function of various immune populations (Letterio and Bottinger, 1998). After normal growth for the first 2 weeks, TGF-ß1 null mice develop a rapid wasting syndrome by 3 to 4 weeks of age. The cause of death was found to be an excessive inflammatory response with massive infiltration of lymphocytes and macrophages in many organs, primarily in the heart and lungs (Kulkarni *et al.*, 1993). TGF-ß is very crucial in the process of epithelial maturation in the developing foetal lung. It has been demonstrated that foetal rat lung fibroblasts express all the 3 TGF-ß isoforms known in mammals while epithelial cells synthesise only TGF-ß 1 and 3. Isolated fibroblasts were shown to secrete all 3 isoforms (de Bortolli *et al.*, 1995). The role of TGF-ß homeostatic and pathogenic processes suggests numerous applications in the diagnosis and treatment of various diseases characterised by inflammation and fibrosis (Clark and Coker, 1998).

TGF-ß is known to antagonise a number of pro-inflammatory molecules such as interleukin-1, -6, tumour necrosis factor and interferon gamma, hence, TGF-ß null mice can be expected to have increased activity of pro-inflammatory cytokines that

may result in an uncontrollable pro-inflammatory cytokine cascade. In an inflammatory site, secretion or local injection of TGF-ß results in induction of leukocyte margination, chemotaxis and accumulation (Wahl *et al.*, 1993). Depending on time and dose, TGF-ß promotes adhesion of monocytes to the collagen type IV, laminin and fibronectin. TGF-ß also facilitates the movement of monocytes through the extracellular matrix by triggering the expression of type IV collagenase and the 92 and 72 kDa gelatinases (Wahl *et al.*, 1993).

Platelets have a high content of TGF-ß-1, and, this is believed to have an important role in the events of injury (Border and Ruoslahti, 1992). Upon platelet degranulation at the injury site, TGF-ß is released into the surrounding tissue (Assoian and Sporn, 1986). A complex sequence of events that promotes healing is then initiated by TGF-ß. These include: chemoattraction of monocytes and leukocytes (Wahl *et al.*, 1987), induction of angiogenesis (Roberts *et al.*, 1986) and control of the production of cytokines and other inflammatory mediators (Kulkarni *et al.*, 1993). TGF-ß induces repair in many ways, some of which are: stimulation of the synthesis of individual matrix components in the fibroblastic cells (Border and Ruoslahti,1992). It simultaneously blocks matrix degradation by decreasing the synthesis of the protease inhibitors (Edwards *et al.*, 1987). TGF-ß also increases the expression of intergrins and changes their relative proportions on the surface of the cells such that it could enhance adhesion to the matrix (Ignotz and Massague, 1987).

TGF-ß has been reported to increase steady state levels of its own message in normal and transformed cell cultures. Bascom *et al.* (1989) reported that TGF-ß exhibits a positive autocrine feedback in murine fibroblasts, where exogenous TGF-ß-1 induced expression of its own mRNA. TGF-ß-1 was capable of increasing its own message 2 to 3 fold within 3 hours in human lung carcinoma, human lung fibroblasts, human fibrosarcoma and rat osteosarcoma cell lines (Purcho *et al.*, 1988). An upregulation of TGF-ß-1 transcription has also been reported during bone fracture healing (Danielpour *et al.*, 1989). During injury treatment, the increase in TGF-ß-1 mRNA levels is transient and can occur as early as 2-3 hours after treatment. However, in bone fracture healing, the increase in TGF-ß mRNA occurs on day -5 and again on day -15 coinciding with the onset of membranous and endochondral ossification, respectively (Danielpour *et al.*, 1989). In marked contrast to BMPs/OPs, TGF ß-1 and -2, purified either from platelets or produced recombinantly, have to date failed to induce heterotopic bone formation in the *in vivo* bioassay in rodents, eliciting instead a fibrovascular response (Roberts *et al.*, 1986; Sampath *et al.*, 1987; Hammonds *et al.*, 1991). In marked contrast to previous heterotopic studies in rodents, Ripamonti *et al.* (1997) showed for the first time that recombinant human transforming growth factor ß-1 induces endochondral bone formation in extraskeletal sites of adult baboons. They also showed that TGF ß-1 and recombinant hOP-1 synergize in inducing large ossicles in extraskeletal sites of non-human primates as early as day 15 post implantation. Duneas *et al.* (1997) also showed that platelet-derived TGF-ß-1 induces endochondral bone formation in

heterotopic sites of primates, and interacts synergistically with recombinant human osteogenic protein-1 (hOP-1) resulting in the production of large ossicles both heterotopically in the rectus abdominis and orthotopically in calvarial defects of the baboon.

TGF-ß isoforms are expressed temporally and spatially in various developing tissues undergoing epithelial-mesenchymal interactions such as the kidney, heart, skin, palate, hair, whisker follicles, lung, mammary gland and teeth (Lehnet and Akhust, 1988; Pelton *et al.*, 1989,1990; Millan *et al.*, 1991; Schmidt *et al.*, 1991). TGF-ß1 is predominantly found in mesenchymal tissues such as connective tissue, cartilage and bone, and in tissues derived from neural crest mesenchyme (Heine *et al* .,1987), while TGF-ß2 was reported to be present in early facial mesenchyme and in various epithelial, implying that this morphogen may regulate epithelial cell growth and differentiation of developing tissues (Pelton *et al.*, 1989). TGF-ß localisation is also seen when remodelling of mesenchyme and mesoderm occurs, as during formation of digits from limb buds, formation of the palate and formation of the heart valves (Heine *et al.*, 1987). The presence of TGF-ß is often coupled with angiogenesis (Roberts *et al.*, 1986). Upon TGF-ß treatment, mammary epithelial cells undergo a reversible transition to a mesenchymal phenotype (Miettinen *et al.*, 1994). This occurred in parallel with increased expression of the mesenchymal marker fibronectin, and a reorganisation of actin stress fibres from an epithelial to a fibroblastic pattern. This information suggests that TGF-ß can induce epithelial to

mesenchymal transdifferentiation (Mietten *et al.*, 1994) This transdifferentiation cannot be mimicked by activin and it appears to involve protein kinase-C. TGF-ß plays an important role in the process of epithelial maturation in the developing foetal lung. de Bortoli *et al.*, 1995 used an immunofluorescence approach to show that foetal rat lung fibroblasts are dominated by the 3 TGF-ß isoforms known in mammals (TGF-ßs -1, -2, -3), while epithelial cells synthesise TGF-ß -1 and -3. Isolated fibroblasts secrete the three isoforms (de Bortoli *et al.*, 1995).

In HIV infection, TGF-ß can contribute to noncytopathic mechanisms of immunodeficiency (Lotz and Seth, 1993). It can promote virus replication in infected monocytes and peripheral blood mononuclear cells under certain *in vitro* conditions (Lotz and Seth, 1993). Machuate *et al.* (1995) investigated the effect of recombinant human TGF-ß2 administration on trabecular bone loss induced by unloading in rats. Their results showed that systemic administration of TGF-ß2 enhances osteoblast precursor cell proliferation and type I collagen expression by osteoblasts, and prevents the impaired bone formation and osteopenia induced by unloading. In the development of a mouse embryo expression of TGF-ßs 1, 2, and 3 was observed in many tissues such as cartilage, bone, teeth, muscle, heart, blood vessels, lung, kidney, gut, liver, eye, ear, skin and nervous tissue. Furthermore, TGF-ß was observed in a wide variety of tissues and in all three primary embryonic germ layers. For example, TGF-ß1 was observed in the ectoderm, TGF-ß2 in the mesoderm, and TGF-ß3 in the endoderm (Pelton *et al.*, 1991). These results suggest that TGF-ßs1, 2,

3 act through both paracrine and autocrine mechanisms during mammalian embryogenesis.

TGF-ß is found in high concentrations in platelets (Assoian *et al.*, 1983), however, bone represents the most abundant source (Seyedin *et al.*, 1985). TGF-ß exists in two dimeric forms where TGF-ß1 predominates (Cheifetz *et al.*, 1987). Both type -1 and -2 have been identified in bone (Seyedin *et al.*, 1985). The presence of TGF-ß1 and -2 in bone suggests that they may play an important role in bone formation and repair (Centrella *et al.*, 1987). Carrington *et al.*, 1988, used the matrix induced bone forming system to examine the time course of appearance of TGF-ß and its localisation in developing endochondral bone. Their results revealed that both TGF-ß-1 and 2 were present during endochondral bone development when the critical transition from calcified cartilage to bone was taking place and in the presence of osteoblasts. These results suggest that TGF-ß may play an important role in regulating bone development *in vivo* as well as cell function *in vitro.* TGF-ß can act in a biphasic manner in that at high concentrations it inhibits mature osteoclastic bone resorption, and at low concentrations it inhibits osteoclast formation. Also, TGF-ß stimulates new bone *in vivo* but inhibits new bone formation *in vitro* (Kato *et al.*, 1980). A single application of rhTGF-ß-1 adjacent to cartilage was found to induce bone formation in rabbit ear full-thickness skin wounds (Beck *et al.*, 1991).

TGF-ß-2 functions as a local positive regulator of bone remodelling, and alterations in TGF-ß-2 of bone cells or in their responsiveness to TGF-ß-2 may contribute to the pathogenesis of metabolic bone disease (Erdebacher and Derynck, 1996).

1.10 OTHER GROWTH FACTORS

Extensive work has been undertaken to screen for additional members of the TGF-ß superfamily. It is during these screens that new genes were cloned and designated growth and differentiation factors (GDFs) (Lee, 1990; Mcpherron and Lee, 1993; Hotten *et al.*, 1994). These growth factors are a diverse group of regulatory agents that control cell survival, proliferation and differentiation.

It has been shown that the bone matrix acts as a store house for growth and regulatory factors (Hauchka *et al.*, 1986). GDF-5 may be required for the normal skeletal development of limbs as suggested by mutations of the GDF-5 gene in brachypodism mice (Storm *et al.*, 1994). Hotten *et al.* (1996) demonstrated that rhGDF-5 can induce chondrogenesis *in vivo* and *in vitro* in rat limb bud cells. Their results suggested that the action of GDF-5 may be relatively specific for chondrogenesis during the entire process of endochondral bone formation. It has been shown that rhGDF-5 stimulates mesenchyme aggregation and chondrogenesis in rat limb bud cells *in vitro*, and that partially purified rhGDF-5 induces cartilage and bone formation in muscular tissues of rodents *in vivo* (Hotten *et al.*, 1996).

The fibroblast growth factors (FGFs) have also been reported to induce formation of bone (Mundy, 1996). Mutations of the FGF receptor family cause abnormal skeletal defects (Reardon *et al.*, 1994; Rousseau *et al.*, 1994). For example, achondroplasia, a disorder of the growth cartilage characterised by short limbed dwarfism, is caused by a mutation in the transmembrane type 3 receptor for the FGF family (Rousseau, 1994). Crouzon's syndrome, which is an autosomal dominant condition associated with the premature fusion of the cranial sutures is associated with mutations in the FGF receptor gene.

Iseki *et al.*(1997) showed that in the foetal mouse skull vault, fibroblast growth factor receptor type- II (FGFR-2) transcripts are most abundant at the periphery of the membrane bones and are mutually exclusive with those of osteopontin but coincide with sites of rapid cell proliferation. FGFR-2 protein was shown to be locally abundant, being present at low levels in FGFR-2 expression domains and at high levels in differentiated areas. The authors suggested that increased FGF/FGFR signalling in the developing skull, whether due to FGFR-2 mutation or to ectopic FGF-2, shifts the cell proliferation balance towards proliferation by enhancing the normal paracrine down-regulation of FGFR-2. Basic FGF (bFGF) has also been shown to induce complete differentiation of isolated metanephric mesenchyme in rats (Irina *et al.*, 1996). Wall and Hogan (1995) used in situ hybridization analysis to identify the expression of FGF-8 during brachial arch development in the chick. Their results showed that FGF-8 expression is initially broad and diffuse becoming

more tightly restricted, particularly in the epithelium of the posterior ectodermal margin of the 2nd branchial arch. These resu. suggested that this molecule was important in the branchial arch development in the chick.

Platelet derived growth factors (PDGF) are other powerful bone cell mitogens whose receptors are frequently expressed in bone cells (Graves *et al.*, 1984). When injected locally over mice calvaria, PDGFs induce new bone formation (Mundy, 1996). However, they were also found to increase bone resorption, which may be a limitation to their usefulness as potential therapeutic agents (Mundy, 1996).

Other important growth factors which have been studied for many years to identify their potential effects on bone formation are insulin-like growth factors (IGF). Both IGF I and II have powerful effects on bone formation *in vivo*. IGF II is the most abundant of the insulin-like growth factors that are present in the bone matrix. Both IGF I and II are being used in clinical trials for patients in an attempt to increase bone mass (Mundy, 1996). IGF II stimulates osteoblastic cell proliferation in several species. It has been demonstrated that IGF II and TGF-ß-1 increased total protein synthesis in normal nontransformed osteoblastic cells isolated from human bone (Strong *et al.*, 1990).

1.11 COOPERATIVE ACTION OF GROWTH FACTORS

In 1997, (Roth *et al)* used immunohistochemistry to demonstrate the possible roles of TGF-ß 1, 2, 3 and IGF I and II in premature cranial suture fusion in humans. Their results showed the presence of TGF-ß 1, 2, 3 and IGF I and II in prematurely fusing human cranial sutures which indicates that these growth factors may play a role in the biology underlying premature suture closure. The authors suggest that in future, manipulating the local expression of these growth factors at the suture site may enable plastic surgeons to modulate premature suture fusion. Most growth factors have a wider range of action than the biological activity for which they were originally named hence are better considered as multifunctional agents (Sporn and Roberts, 1988). The nature of growth factor action is dependant on the nature of other substances present. For example, TGF-ß quickens growth of certain fibroblasts *in vitro* in the presence of platelet-derived growth factors but hinders their growth if epidermal growth factor is present (Roberts *et al.*, 1985).

1.12 SYNERGY

The search for synergy has been going on since the last century, and has followed many tortuous paths especially during the last 30 years. Claims of synergism for the effects, both therapeutic and toxic, for chemical combinations are ubiquitous in the broad field of biomedicine, where over 20 000 articles from 1981 to 1987 included synergism as a key word (Greco and Laurence, 1998). Groups on the search for synergy have included scientists form the disciplines of pharmacology, toxicology,

statistics, mathematics, epidemiology, entomology, weed science and others (Greco *et al*, 1995). Their paths have independently found the same traits, crossed, bitter fights have ensured and alliances have been made, however the challenges of assessing the nature of intensity of agent interaction is universal and is especially critical in the chemotherapy of both infectious diseases and cancer (Greco *et al*, 1995).

Synergism is a word derived from Greek which means to work in unison. It is defined pharmacologically as an interaction between two agents which results in an effect greater than the sum of the effects attributable to each agent when applied singly. It is usually easy to classify the interaction of two agents where one is active and the other inactive when applied singly.

1.13 SYNERGY IN BIOLOGICAL SYSTEMS

Ripamonti *et al.* (1997) have revealed that TGF-ß was inactive in the rat subcutaneous site but active intramuscularly in the baboon and that co-administration with rh OP-1 led to a synergistic induction of bone formation 15 days post implantation in the adult primate. They also reported that TGF ß-1 and hOP-1 synergize in inducing massive ossicles in extraskeletal sites of the primate as early as 15 days post implantation. The addition of TGF-ß to rh OP-1 influences tissue induction, directing development toward a more chondrogenic phenotype. It was previously reported that TGF-ß does not induce osteogenesis in heterotopic sites in

rodents (Roberts *et al.*, 1986; Sampath *et al.*, 1987; Hammonds *et al.*, 1991) ,but Duneas *et al* (1997) showed that addition of comparatively low doses of pTGF-ß1 to rh OP-1 led to synergistically increased parameters of bone formation orthotopically 30 and 90 days post implantation in calvarial defects, and heretotopically in the rectus abdominis. Other cases of synergistic interaction of TGF-ß and other growth factors have been reported, for example, TGF-ß interacts synergistically with basic FGF in the induction of chondrogenesis in mice (Franz *et al*, 1994; Kimelman and Kirschner, 1987).

Another case of synergistic interaction was shown by Johnsons-Pais and Leach in 1996. They used a somatic cell genetic approach to study the reputation of liver/bone/kidney alkaline phosphatase gene expression in skeletal mineralisation and serves as a good index of bone formation. A series of hybrids constructed by fusion of the human osteosarcoma TE-85 with the mouse fibrosarcoma La-t- were reduced to extinction and then exposed to two factors known to influence alkaline phosphatase activity, namely 1,25-dihydroxy-vitamin D3 (1,25 D3) and transforming growth factor beta 1. For two hybrids, the combination induces reexpressing of alkaline phosphatase activity to the levels of TE-85 parent, neither factor alone could induce alkaline phosphatase reexpressing to the levels observed with the combination, and in one hybrid, the combination of factors synergistically increased alkaline phosphatase expression. 1,25D3 is involved in mobilising bone calcium stores and TGF-ß1 plays a critical role in bone remodelling.

Basic FGF has a limited capacity to induce muscle actin expression in animal hemisphere cells. Addition of TGF-ß (which by itself does not induce actin expression) raises this level of expression to levels normally induced in the embryo (Kimelman and Kirscher, 1987). The authors conclude that bFGF and TGF-ß increase cardiac actin expression in two different ways: bFGF plays an important role in the induction, whereas TGF-ß is a stimulatory, and the two together are synergistic.

1.14 PRECLINICAL AND CLINICAL APPLICATIONS FOR BMPS

In vivo studies of BMPs have made it possible to extend the knowledge of their *in vivo* morphogenetic potential in rodents to primates which is a prerequisite for the exploration of potential therapeutic applications for the regeneration of bone in man. Johnson *et al.* (1988) reported that upon treating 47 refractory tibial and femoral nonunions with the use of naturally derived BMPs, results showed union with BMP supplementation in patients who had undergone unsuccessful multiple surgical grafting attempts. Ripamonti *et al.* (1992), used the calvarial model of the baboon (*Papio ursinus*) to demonstrate efficacy of native BMPs with both porous hydroxyapatite and collagenous substrata. Native BMPS were also effective in regenerating periodontal ligament, cementum and alveolar bone in surgically created periodontal defects in the baboon (Ripamonti *et al.*, 1994). Native BMP fractions were shown to completely regenerate calvarial defects in adult baboons 90 days after surgical implantation (Ripamonti *et al.*, 1993a). Reconstitution of the collagenous

carrier with recombinant hOP-1 induces massive bone differentiation 30 days after calvarial implantation (Ripamonti and Reddi, 1995; Ripamonti *et al.*, 1996).

Adsorption of BMP-3 and related BMPs onto hydroxyapatite has resulted in the construction of delivery systems using porous hydroxyapatite activated by BMPs with osteogenic activity in extra skeletal sites of baboons (Ripamonti *et al.*, 1992; Ripamonti *et al.*, 1993b) BMP-3, when adsorbed on porous hydroxyapatite substrata, induces rapid bone differentiation (Ripamonti *et al.*, 1993).

1.15 ISOBOLOGRAPHIC ANALYSIS

A convenient graphical way of representing the results of combination studies is by the use of an isobologram which is a Cartesian plot of pairs of doses that in combination yield a specified level of effect. The use of an isobologram was introduced by Loewe and Muischnek (1926). Isobolographic analysis is a mathematical model that provides a fundamental basis for assessing whether biological responses induced by mixtures of agents are greater, equal or smaller than would have been expected on the basis of the individual activities of the component agents and the concept of dose additivity (Gessner, 1995). If the individual effectiveness of agent 1 and agent 2 are denoted by Z1* and Z2*, respectively, the classification of the result of the combination of the agents as additive or non-additive is based on the definition involving the amounts Z1 and Z2 in a mixture that

produces the specified level of effect. This definition is expressed in the equation below:

$$Z1/Z1^* + Z2/Z2^* = A$$

where $A = 1$ denotes additivity

$A < 1$ denotes synergism

$A > 1$ denotes antagonism

In an isobologram, the straight line passing through the points (0, Z1*) and (Z2*, 0), which is called the line of additivity, gives a convenient means of visually discriminating additive from non additive interactions on the basis of whether on not the co-ordinate of the ED50 (the dose that is effective in 50% of the test organisms) value of the combination falls on additive, below (superadditive) or above (subadditive) the line of additivity (Tallarida and Raffa, 1995). Typically, isobolograms have a visual interpretation. A straight line isobole is interpreted as zero interaction; any deviation above or below the straight line is interpreted as antagonism or synergism, respectively (Machado and Robinson 1994). These interactions occur when the effect resulting from the use of the combination exceeds that attributable to the sum of effects of the agents when applied singly. In addition, isobolographic analysis allows one to quantify the intensity of the interaction by calculating the ratio of the distance from the origin to the point of intersection with

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1.16 AIMS

To investigate the cellular and molecular events that are initiated by OP-1 and TGF-ß1 when applied singly and in combination in the rat subcutaneous site and to demonstrate synergistic interaction between them.

the line of additivity, and the distance from the origin to the plotted point as well as the interaction intensity.

If the interaction intensity is greater than 1, synergism is indicated and if it is less than 1, antagonism is indicated (Machado and Robinson, 1994). These concepts are graphically presented in figure 1.

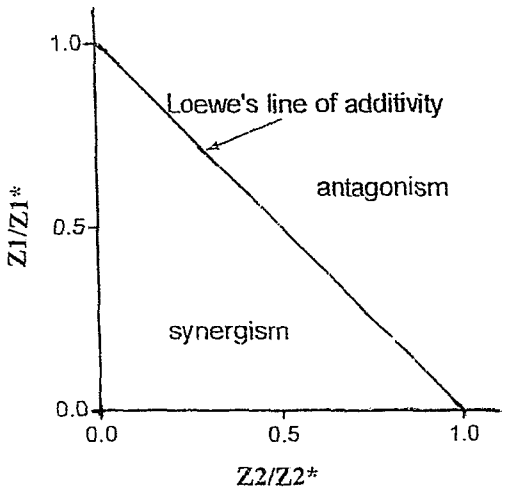


Figure 1. An example of an isobologram.

2 MATERIALS AND METHODS

2.1 Experimental animals: The rat (*Rattus norvergicus*)

The research protocol was cleared by the Animal Ethics Screening Committee of the University (AESC number 97/63/5). There are many advantages for using the rat in the field of regeneration: it has the ability to produce ectopic bone within 12 days in response to the applied morphogenetic signal which provides a quick and economical way to generate tissues for analysis by Northern blots. It also enables rapid generation of data.

The model demonstrating bone formation by induction at heterotopic sites has been used widely in the field. The utilised sites are subcutaneous (Reddi and Huggins, 1972; Reddi, 1981), or intramuscular (Urist, 1965). In this project, the subcutaneous site was used because of its simplicity and ease of tissue harvest at the end of the experiment.

2.2 Selection

The Central Animal Service of the University supplied Long Evans rats of 30-35 days of age. The rats were kept in cages in groups of four at room temperature and fed a standard rat chow.

2.3 Morphogens

2.3.1 rhOP-1

Recombinant human osteogenic protein -1 (rhOP-1) was prepared as previously described (Sampath *et al.*, 1992). Mature OP-1 is a glycosylated 36-kD homodimer of 139 amino acid residue chains. rhOP-1 was a kind gift from Creative Biomolecules, Hopkington, MA, USA. Quantities of OP-1 are available in the laboratory at concentrations of 100 µg/500µl in 5 mM HCl.

2.3.2 TGF-ß1

Transforming growth factor beta-1 (TGF- ß1) was purified from porcine platelets as described (Duneas *et al.*, 1997). Quantities of TGF-ß1 are available in the Bone Research Laboratory.

2.4 Insoluble Collagenous Bone Matrix

Insoluble collagenous bone matrix (ICBM) was prepared from rat tibial diaphyseal bone. The tibias were harvested and the diaphyses were cut. The bone segments were then demarrowed and cleaned with tap water, defatted with ether and dehydrated with ethanol, then crushed to particle size of 75µm to 420µm. The bone matrix was demineralised with repeated additions of 0.5 M HCl until demineralisation was complete. The demineralised bone matrix was washed with distilled water and extracted with 4 M Gnd-HCl in 50mM Tris-Cl pH 7.4, then washed with repeated additions of distilled water, dehydrated with ethanol and freeze-dried. A control was made by implanting the ICBM in the rat subcutis, and there was no bone induction, which confirmed that the matrix was inactivated.

2.5 The rat heterotopic model

2.5.1 Experimental design

The implantation protocol for OP-1 (μg/25 mg ICBM) and TGF-ß$_1$ (μg/25 mg ICBM) singly or in combination was varied as shown in Table 1. Each application had four replicates as shown.

The rats were anaesthetised intramuscularly with a mixture of 4:1 ketamine/rompun in doses of 0.16 ml/100 mg weight of rat. The chests were shaved and scrubbed with hibitane. After a median skin incision, pouches were created by blunt dissection and implants were inserted bilaterally into the pouches (one implant per pouch) in the subcutaneous space above the pectoralis fascia. Skin incisions were closed using resorbable sutures. The implants were harvested on days 7, 12, 21 post implantation and subjected to histological, biochemical and molecular analyses.

Table 1: Implantation protocol for hOP-1 and TGF-ß$_1$

OP-1 TGF-ß$_1$	0	0.1	0.3	1	3
0	4	4	4	4	4
0.01	4	4	4	4	4
0.03	4	4	4	4	4
0.1	4	4	4	4	4

2.6 Preparation of implants

The rat ICBM (25 mg) was weighed into sterile 5 ml polypropylene tubes. Morphogens were added in the correct amounts and the mixture was allowed to soak overnight at room temperature. 100 µl of 10 mg/ml stock of chondroitin sulphate-C (Sigma-Aldrich S.A. Pty. Ltd) and rat tail type I collagen (150 µl of 5 mg/ml,0.5 M acetic acid stock) were added and the mixture was centrifuged and allowed to soak for 30 minutes. Three volumes of ice-cold absolute ethanol were added and the mixture was vortexed. Proteins were allowed to precipitate at 4°C for 15 minutes. The tubes were centrifuged for 10 minutes to pelletise the contents, and the ethanol supernatant discarded. The pellets were washed 3 times with chilled 85% ethanol and centrifuged again then lyophilised at -50°C to -60°C, resulting in solid plano-convex pellets.

2.7 ANALYTICAL PROCEDURES

2.7.1 Tissue harvest, histology and biochemistry

Rats were euthanased with an intramuscular overdose of sodium pentobarbitone (100 mg/ml) on days 7, 12 and 21 post implantation. The implants were retrieved from the rats, freed and trimmed of adhering tissue. The implants were divided into pieces for histological, biochemical, and for Northern blot analyses.

2.7.2 Histology

The implants were fixed for five days in Bouin's fixative, embedded and processed in historesin. Sections were then cut at 4 µm and dried on a hot plate

for 30 minutes, after which they were stained with toluidine blue on the hot plate for 60 seconds. The specimens were then washed in running tap water, dehydrated and mounted.

2.7.3 **Biochemical Markers of Bone Formation**

The activity of alkaline phosphatase was detected by the appearance of a yellow coloured reaction solution that resulted from the enzymatic conversion of the substrate, para-nitrophenyl phosphate (PNPP) at pH 9.3.

2.7.4 **Reagents:**

Substrate solution- 0.01 M PNPP in distilled water

Buffer solution- 0.1 M sodium barbitone in distilled water pH 9.3

Base solution- 0.1 N (Sodium hydroxide) NaOH in distilled water

Homogenising buffer- 0.15 M (Sodium chloride) NaCl, 0.003 M $NaHCO_3$ in distilled water

Sample assay- The harvested tissue was homogenised in 2ml of homogenising buffer and centrifuged. The supernatant was transferred into a new tube and used for the assay.

One ml of substrate solution and 1 ml of buffer solution were added to clean test tubes which were then placed in a 37°C water bath to equilibrate temperature. Blanks were prepared by adding 2 ml of base solution to tubes containing substrate and buffer solutions. The sample assay was then added to all tubes and the reaction was left for 30 minutes after which it was stopped with 2 ml of the base solution. The absorbance of the samples was determined by

spectrophotometer at 400nm. Alkaline phosphatase was determined as units of activity per mg protein. One unit of alkaline phosphatase activity is defined as that which generates 1 µmole of nitrophenyl phosphate in 30 minutes at 37°C. This unit of activity was calculated based on the extinction coefficient value of PNPP that is 218.58.

The amount of solubilised protein was determined by the Lowry assay.

2.8 PROTEIN LOWRY ASSAY

2.8.1 Reagents:

Bovine serum albumin (BSA): 1 mg/ml in distilled water

Protein stock solution: 2% Na_2Co_3 (w/v) in 0.1 N NaOH

Copper Reagent: To 100 ml of protein stock solution, were added 1 ml of 2% Na Tartrate and 1 ml of 1% (w/v) $CuSO_4.5\ H_2O$.

Phenol reagent: 2 N Folin Ciocalteau diluted 1:1 with distilled water.

Standard solutions were prepared with appropriate volumes of BSA. To standards and sample tubes, distilled water was added to give a final volume of 400 µl. Blanks were prepared with distilled water without sample assay. To all tubes, 2 ml of copper reagent were added and the reaction was allowed to proceed for 30 minutes after which 200µl of phenol reagent was added and contents vortexed. The reaction was allowed to proceed for another 30 minutes and the absorbance of the samples was determined at 750 nm by using standard values to obtain a standard curve, and the protein in the sample read from the curve.

2.9 Calcium assay

The pellets from the homogenate were washed with 10 ml of 0.005 M Tris-HCl, stirred with a stirrer bar for 15 minutes, and centrifuged for 15 minutes at 3000 rpm. The wash fluid was discarded and the procedure was repeated. Ten ml of 0.5 N HCl was added to pellets and vortexed thoroughly to resuspend the pellet. The tubes were stirred overnight with magnetic stirrer bars. The tubes were centrifuged at 3000 rpm and the supernatant containing solubilised calcium was assayed for calcium in the presence of 0.118% lanthanum oxide on atomic absorption spectrophotometer at 422.7 nm and band width of 1.0 nm. A standard curve was constructed with calcium chloride ($CaCl_2$) in the range of 0.01 ppm and 0.999 ppm Ca^{2+}.

3 NORTHERN BLOT ANALYSIS

3.1 Preparation of total RNA

Pooled tissues weighing between 100 and 200 mg from replicate specimens were used for Northern analyses. All glassware was baked at 250°C overnight, and plasticware autoclaved at 121°C for 20 minutes in the presence of 0.1% diethylpyrocarbonate (DEPC) (ICN Biochemicals Inc. OHIO). All solutions were prepared in deionised distilled water treated with DEPC. Sample tissues were snap-frozen in liquid nitrogen and stored at -70°C. Samples were crushed to a fine powder with a heat sterilized mortar and pestle pre-cooled to -70°C. Crushed samples were homogenised on ice in Tri- Pure isolation reagent (Boehringer Mannheim Biochemicals), 1 ml per 100 mg of tissue, with an IKA Ultra-Turrax T025 tissue homogeniser at 20000 rpm (Janke and Kunkel, Staifen, Germany).

Following homogenisation, samples were centrifuged at 4000 rpm in a Heraeus megafuge at room temperature for 10 minutes. The supernatant was aliquoted into 2 ml Eppendorf tubes and the pellet was discarded. Chloroform (0.2 ml/ 100 ml of the supernatant) was added and the tubes were shaken for 15 seconds and left at room temperature for 15minutes, then centrifuged at 13000 rpm at 4°C for 20 minutes to separate the phases. The upper layer that contained the RNA was transferred to a new tube and 0.5 ml of 100% isopropanol was added for 1ml of aqueous layer. The tubes were capped and inverted several times to mix, then incubated at room temperature for 15 minutes to allow the RNA to precipitate. The tubes were spun in a microfuge at 13000 rpm at 4°C for 20 minutes to pelletise the RNA. The supernatant was discarded and the pellet was washed with 75% ethanol to reduce the salt concentration. The tubes were vortexed briefly until the pellet floated, and spun to settle the pellet. The ethanol was decanted and the pellets were dried for 5 minutes under vacuum. The pellets were resuspended in 30µl of lauryl sarcosine/EDTA solution. The concentration of the RNA was determined spectrophotometrically at 260 nm by employing an extinction coefficient of 1 representing a concentration of 40 µg/ml. The quality of the RNA was judged by the A260/A280 ratio of absorbance of the sample, the scrutiny of the quality of ribosomal RNA bands visualised on agarose gels and by the quality of gamma actin signals on Northern blots.

3.2 Formaldehyde Agarose Gel Electrophoresis

3.2.1 Reagents:

Electrophoresis buffer, 5X concentrate

0.1 M Mops

40 mM sodium acetate, pH 7.0

5 mM EDTA

Gel loading buffer:

50% glycerol

1 mM EDTA

0.25% bromophenol blue

0.25% xylene cyanol FF

A 2% stock agarose (electrophoresis grade) gel was made in 0.1% DEPC treated water and autoclaved. The appropriate volume of the gel was melted in the microwave oven, mixed with a volume of concentrated electrophoresis buffer to give a 1x concentration in the final preparation and with formaldehyde to a final gel concentration of 1% agarose.

3.3 Sample preparation and gel electrophoresis

Before electrophoresis, samples of RNA containing 5 and 20µg of RNA were denatured at 65°C in appropriate volumes of formamide, formaldehyde and 5X electrophoresis buffer. Samples were then mixed with gel loading buffer and electrophoresis was started at 5V/cm until the dye had reached 2/3 of the gel length. The gel was stained with ethidium bromide (Boehringer Mannheim

Biochemicals) 0.5μg/ml in 0.1M sodium acetate for 1 hour and then destained for 16 hours with changes of DEPC treated water. The gel was then photographed.

3.4 RNA transfer to membrane

Transfer and hybridization solution: Saline sodium citrate (SSC).

A 20X concentrate of SSC was prepared by adding 175.3g of NaCl and 88.2g of tri-sodium citrate to 1 litre of DEPC treated water, adjusting the pH to 7.0. The solution was sterilized by autoclaving at 121°C for 20 minutes.

RNA was transferred to a nylon membrane (Hybond N^+, Amersham) by capillary blotting using an apparatus specific for the purpose (Life Science Technologies, UK). The transfer was allowed to proceed for 48 hours in the presence of 20X SSC as described (Sambrook *et al.*, 1989). The membrane was baked at 80°C under vacuum to effect the crosslinking of RNA to the membrane.

3.5 Probes and hybridization

The following probes were used:

Type II collagen: this is a 2.2 kb fragment of the gene, coding from amino acid 343 of the triple helix to 70 amino acids inside the C-propeptide (Baldwin *et al.*, 1989).

Type IV collagen-alpha 2 cDNA: this is most of the collagenous domain (Hostikka *et al*,. 1988).

cDNAs for gamma actin and collagens were gifts of Professor de Wet, University of the Witwatersrand, Johannesburg.

OP-1 cDNA: comprises a 679 bp fragment harboured within pÖ320 (Helder *et al.*, 1995) and coveres amino acids 63 to 262 of the pro-region and the first 25 amino acids of the mature polypeptide.

cDNA for TGF-$ß_1$: (pRK5-ß1E) contains the unmodified wild type TGF-$ß_1$ precursor (Derrynck *et al.*, 1985). cDNAs for OP-1 and TGF-ß1 were gifts from Professor S. Vukicevic, University of Zagreb, Croatia.

cDNA BMP-3: contains a 1508 bp insert including the full coding region of hBMP-3 (Wozney *et al.*, 1988). This cDNA was a gift from Professor A. Hari Reddi, John Hopkins University, Maryland, USA.

3.6 **Amplification of Constructs of E. coli**

Escherichia coli strain XL1Blue, a gift from W. de Wet, department of Medical Biochemistry, University of the Witwatersrand, was used as a vector for transformation. An overnight culture was prepared in Luria Bertani (LB) medium (0.5% bacto yeast, 1% NaCl, 1% bactotryptone (Life technologies, UK) in deionised water) which was autoclaved at 121°C for 20 mirutes on liquid cycle. Bacteria were then grown for 16 hours on LB plates that were solidified by 1.5% agar (Sigma Aldrich, Germany). The bacteria were made competent by the $CaCl_2$ method (Cohen *et al.*, 1972). A single bacterial colony was picked from the LB agar plate and inoculated into 10 ml of LB medium, incubated at 37°C with vigorous aeration overnight. One ml of the overnight incubation was transferred to 100 ml of LB medium in a 1 litre flask and incubated at 37°C with vigorous aeration until the OD_{600} was approximately 0.4. The cells were transferred to a sterile ice-cold 50 ml falcon tube and cooled to 0°C by storing on ice for 10

minutes after which they were recovered by centrifugation at 4000 rpm for 5 minutes at 4°C. The medium was decanted and the pellet was resuspended in 10 ml ice-cold 0.1 M $CaCl_2$ by gentle shaking and stored on ice for 30 minutes. The cells were recovered by centrifugation again at 4000 rpm for 5 minutes at 4°C. The pellet was resuspended in 2 ml of ice-cold $CaCl_2$ (0.1 M) for each 50ml of original culture, snap-frozen in liquid nitrogen and stored at -70°C until use.

3.7 Transformation of E.coli

Using a chilled sterile pipette tip, 200µl of the bacterial suspension was transferred to a sterile polypropylene tube. Supercoiled plasmid DNA (100 ng) was added to the tube, mixed and stored on ice for 30 minutes. A control was made with competent bacteria that received no plasmid. The cells were transferred to a water bath at 42°C for 90 seconds, and back to ice for 2 minutes. LB medium (800 µl) was added to the mixture and incubated at 37°C with gentle aeration for 45 minutes to allow the bacteria to express the antibiotic resistance marker encoded by the plasmid. 100 µl of the transformed competent cells was transferred on to 90 mm LB agar plates containing the appropriate antibiotic and, using a sterile bent glass rod, the transformed cells were spread over the surface of the agar plate. The plates were inverted and incubated at 37°C overnight.

3.8 Preparation of plasmid DNA

A kit was used for this purpose (High Pure Plasmid Kit Boehringer Mannheim). Plasmid DNA was isolated from E.coli (XL1 Blue) by lysing the bacterial cells according to the alkaline lysis method (Birnboim and Doily, 1979). An overnight

culture of transformed bacterial cells was grown in 4ml of LB at 37°C. Cells were collected by centrifugation in the Heraeus megafuge. The cells were lysed and bacterial RNA was removed by the addition of RNAse A supplied with the kit. The lysate was neutralised and adjusted to high salt binding conditions after which chromosomal DNA was precipitated and the supernatant, which contained the plasmid was purified from the salts, proteins and other cellular impurities and eluted in 10mM Tris-HCl buffer.

3.9 Preparation of probe Labelled with ^{32}P dCTP

The vector DNA was treated with suitable restriction enzymes to cleave the inserts. Restriction digests of plasmid vectors and molecular weight markers were run on 1% agarose gel in Tris borate (TBE) buffer at 5 volts per centimetre. The DNA was visualised by UV transillumination of the gel. The inserts were identified according to molecular weight. The DNA concentration was then determined by spectrophotometric analysis of the sample absorbance at a wavelength of 260nm, where an absorbance of 1 unit represents a DNA concentration of 50µg/ml. 25ng of DNA was labelled with alpha-^{32}P dCTP using a random prime labelling kit (DNA megaprime labelling kit RPN 1606, Amersham Life Science). A DNA purification kit (QIA quick, White Head Scientific) was used to remove unincorporated label and the purified probe was used for hybridization.

3.10 Hybridisation of probes and detection

Conical propylene tubes of 50ml capacity were used for the hybridization procedure which was carried out in a hybridization oven (Hybaid Corporation, UK). The probe was mixed with 100µl of sonicated fish sperm DNA (10mg/ml) and boiled for 2 minutes to denature the DNA. A hybridization solution (QuikHYB, White Head Scientific) supplied commercially for this purpose was used to prehybridize membranes first at 37°C for 10 minutes, then at 68°C for 20 minutes. Probe was added and hybridization was allowed to continue for 2 hours at 68°C except for type IV collagen detection, which was carried out at 72°C because of the non-specific binding of the probe to GC rich sequences of ribosomal RNA at lower temperatures. Membranes were washed twice for 15 minutes at room temperature with 150ml of 2X SSC, 0.1% SDS, followed by another wash at 68°C with 0.1X SSC, 0.1%SDS. The blot for type IV collagen was washed at 72°C to achieve a higher stringency. Membranes were dried on blotting paper and covered with a saran wrap. Autoradiography was performed with Kodak Biomax MS film at -70°C for given time periods, ranging between 16 hours to 5 days. Films were developed and photographed.

3.11 Signal quantitation

The signal intensity for each lane was quantitated by scanning densitometry (Gel Documentation and Analysis system, Syngene, U.K) and normalized against values obtained for the gamma actin signal. Values were adjusted to relative densitometric units onto a scale of 1 to 100.

3.12 Statistical analysis

The data were analyzed with the Statistical Analysis System (version 6.12). An Analysis of Variance procedure (ANOVA) was performed using the General Linear Models procedure with multiple interactions. For each treatment, there were 4 specimens available for biochemical analysis representing 4 alkaline phosphatase activity determinations and 4 calcium content determinations per group. Comparison of the mean values was obtained using Bonferroni (Dunn) procedure on the dependent variables that were included in the analysis. The critical level of statistical significance chosen was $P<0.05$.

4 RESULTS

Single applications of TGF-ß1 were inactive (compared to the controls, which contain ICBM solo) at all doses tested at days 7, 12 and 21 as reflected by very low activity of alkaline phosphatase (range 0.03 to 0.09 U/mg protein; Figures 2, 8 and 13) and calcium levels (range 0.05 to 0.6 µg/mg tissue; Figures 3, 9 and 14). Histological sections of implants containing single applications of TGF-ß1 did not show any sign of cartilage or bone formation, only fibrous tissue as shown in the control sections (Figure 4).

4.1 Seven Days Implantation

OP-1 applications at doses of 0.1 to 3 µg gave rise to a dose dependent response in alkaline phosphatase activity at levels of 0.1 to 0.3 U/mg protein and calcium levels of 0.4 to 0.5 µg/mg tissue. Addition of TGF-ß1 in amounts of 0.01-0.1 µg to 1 and 3 µg OP-1 elicited a slightly higher alkaline phosphatase activity dose dependently (0.2 - 0.5 U/mg protein) except at 0.01µg TGF- ß1/1µg OP-1 where the activity was lower compared to the control. Addition of 0.1µg TGF-ß1 to 1µg OP-1 elicited a significant difference of alkaline phosphatase activity at 5% level compared to single application of OP-1. The other combinations did not have a significant effect. Below 1µg OP-1 there was negligible changes in the activity of alkaline phosphatase activity (Figure 2). OP-1 comparison that had a significant difference at 5% level in alkaline phosphatase activity were 3-1, 3-0.3, 3-0.1, 3-0, 1-0, 1-0.3, 1-0.1 and 0.3-0 µg. Alkaline phosphatase activity was found to be significantly different (5% level)

between day 7 and day 12 and day 7 and day 21 at the combinations of 0.1TGF-β1/3.0 μg OP-1, 0.03 TGF-β1/3.0 μg OP-1, 0.03TGF-β1/1.0 μg OP-1 and 0.01TGF-β1/1.0 μg OP-1.

The calcium content was slightly higher at levels of 0.2 to 1 μg/mg tissue as compared to activity due to single applications of OP-1. Significant changes (at 5% level) were only shown where 0.03 and 0.1μg of TGF-ß1 were added to 0.3, 1.0 and 3.0 μg of OP-1. Calcium accumulation however, tends to suggest an antagonistic effect of TGF- ß1 at 0.01 μg on OP-1 at doses of 0.3 and 1.0 μg (Figure 3). Besides the antagonism mentioned, addition of TGF-β1 to OP-1 resulted in a synergistic effect, at least at the combinations of 0.03 μg TGF-β1 with 0.3, 1.0 and 3.0 μg OP-1; and those of 0.1 μg TGF-ß1 with 0.3, 1.0 and 3.0 μg of OP-1. Synergy is an interaction between two agents which results in an effect greater than the sum of the effects attributable to each agent when applied singly (Greco *et al.*, 1995). Significant differences (5%) in calcium content between day 7 and 12 and day 7 and 21 were observed at the combinations of 0.01, 0.03 and 0.1 μg TGF-ß1 with 0.3, 1.0 and 3.0 μg OP-1.

Histological examination showed that OP-1 implants did not exhibit any sign of bone formation yet (Figure 5 A). A similar result was observed with TGF-ß1 implants singly (Figure 4 B). Addition of 0.03 μg TGF-ß1 to 3 μg of OP-1 and 0.1μg of TGF-

ß1 to 3μg of OP-1 showed the beginning of chondroblast formation in a dose dependent manner in the implanted matrix (Figures 5 B and C).

Gamma-actin was expressed as a major band at 1.9 kb (figures 6a, 11a and 16a). The minor bands that appear in the blots could be due to RNA splicing. OP-1 was expressed at 4.0 kb (figures 6b, 11b and 16b), TGF-β1 was expressed at 2.4 kb (figures 6c, 11c and 16c), BMP-3 was expressed at 3.0 kb (figures 6d, 11d and 16d), type II collagen was expressed at 4.3 kb (figure18a) and type IV collagen was expressed at 5.1 kb (figure 18b). The minor bands that appear in the blots could be due to differential RNA splicing.

mRNA expression showed that TGF-β1 singly increased the expression of OP-1, TGF-β1 and BMP-3 with 0.03 μg resulting in a high expression of TGF-ß1, OP-1 and BMP-3. OP-1 singly increased the expression of both TGF-β1 and OP-1 but decreased the expression of BMP-3. OP-1 increased its own expression and TGF-β1 mRNA but decreased the expression of BMP-3. The TGF-β/OP-1 combinations containing 0.03 μg TGF-β1 decreased the expression of OP-1, TGF-β1 and BMP-3 while those containing 0.1 μg of TGF-β1 increased the expression of all three genes. Collagen type II and IV were not detected . These results were confirmed by densitometric analysis (figure 7). The signal intensity for the mRNAs was quantitated by scanning densitometry (Gel Documentation and Analysis system, Syngene, U.K) and normalised against values obtained for the gamma-actin signals. Values were adjusted to relative densitometric

units onto a scale of 100 (figure 7). There was a significant difference (5% level) in the BMP-3 mRNA expression between day 7 (figure 7 c) and 12 (figure 12 c) as well as between day 7 and 21 (figure 17 c). There is no significant difference on the mRNA expression of OP-1 and TGF- β1 between the time periods (figures 7, 12, 17 and 19).

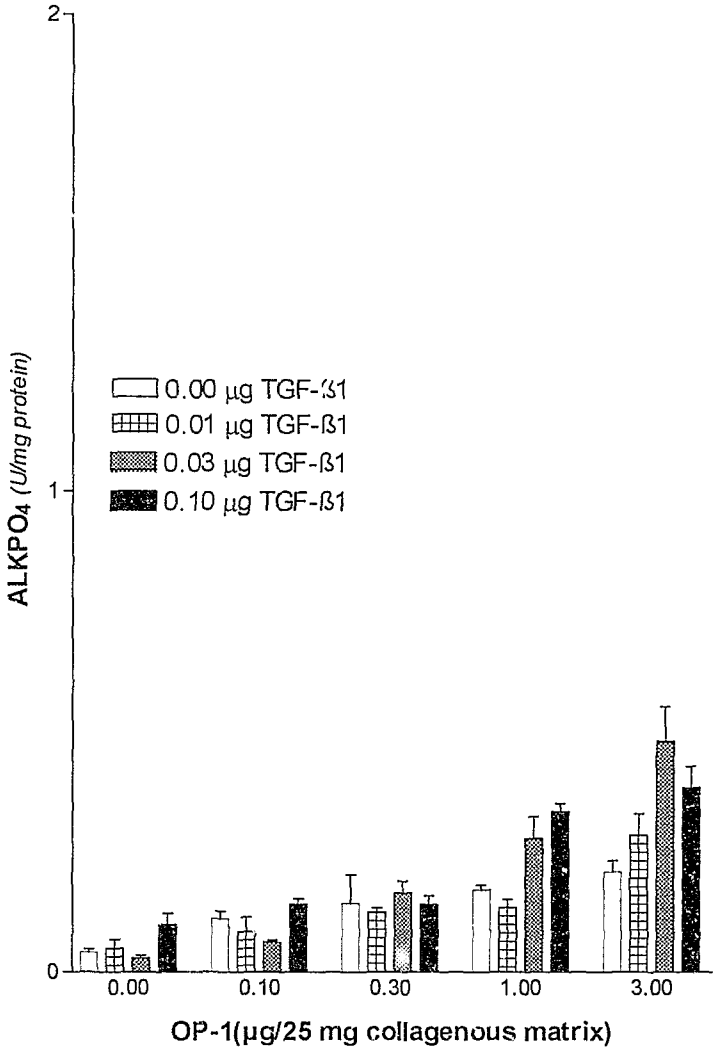


Figure 2. Biochemical results of implantation of OP-1 and TGF-β1, both singly and in combination in the rat subcutis. Generated tissues were harvested at 7 days post implantation and alkaline phosphatase activity was determined as described in the methods. n=4. Values are means ± (Standard error of the mean) SEM.

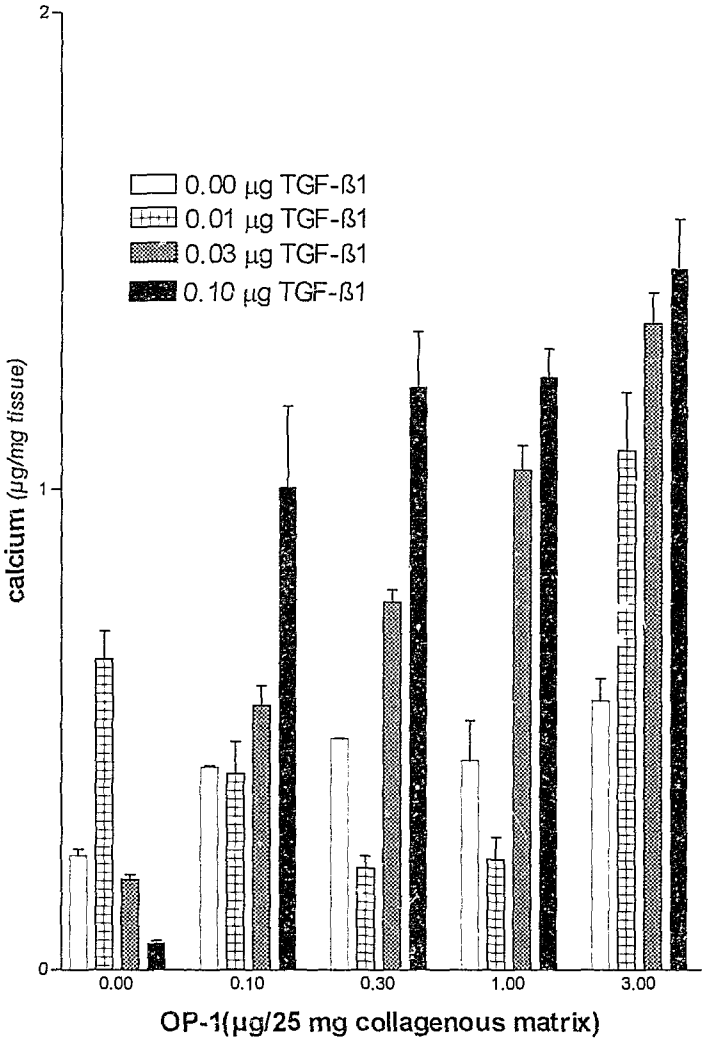
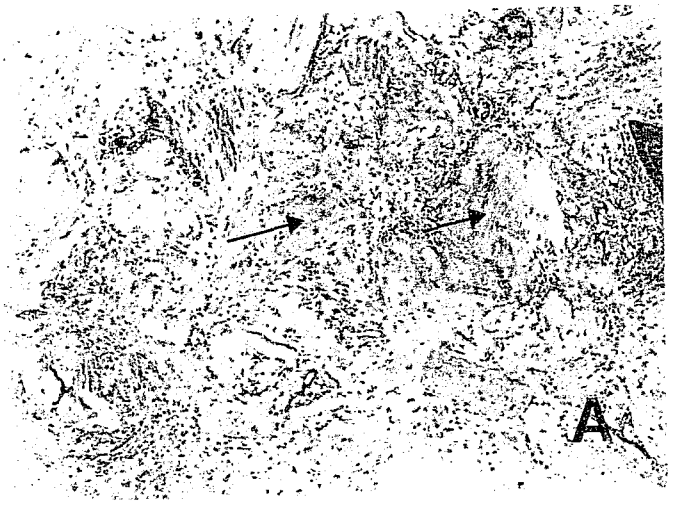


Figure 3. Calcium content of tissues generated by OP-1 and TGFβ1, both singly and in combination in the rat subcutis. Generated tissues were harvested at 7 days post implantation and calcium content was determined as described in the methods. n=4. Values are means ± SEM.

Figure 4. Toluidine blue stained sections of tissues generated in the subcutaneous space of the rat by ICBM and TGF-β1 solo. The tissues were processed as described in the methods.

A: ICBM (solo). Arrows indicate insoluble collagenous bone matrix (ICBM).

B: TGF-β1 (solo). Filled arrow indicates the ICBM. Unfilled arrow indicates mesenchymal cells localised between particles of the ICBM.


A

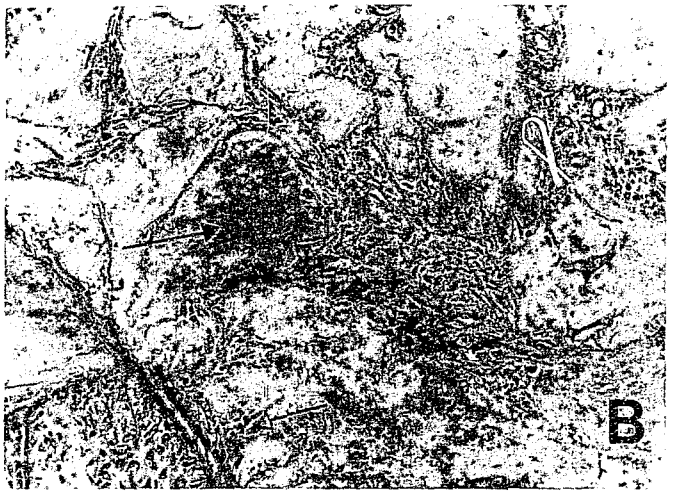
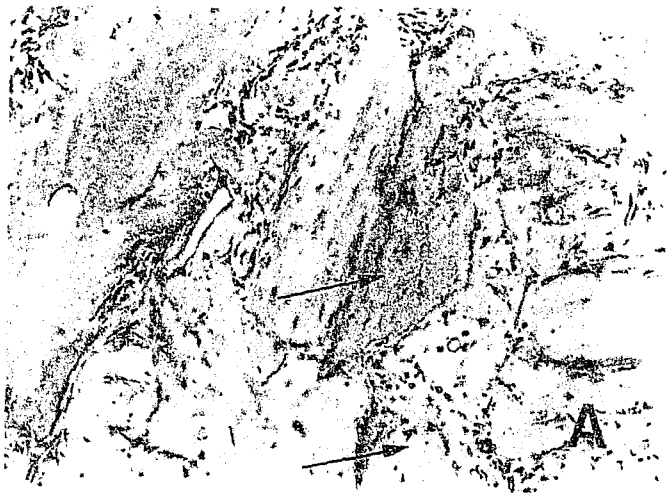

B

Figure 5. Toluidine blue stained sections of tissues generated in the subcutaneous space of the rat 7 days post implantation. The tissues were processed as described in the methods.

A: 3 μg OP-1 solo. Arrows indicate ICBM.

B: 3 μg OP-1 with 0.03 μg TGF-β1. Filled arrow indicates ICBM. Unfilled arrows indicate invading mesenchymal cells between the particles of the ICBM.

C: 3 μg OP-1 with 0.1 μg TGF-β1. Filled arrow indicates ICBM. Unfilled arrow indicates the *de novo* induction of chondrogenesis achieved by the binary application of the morphogens.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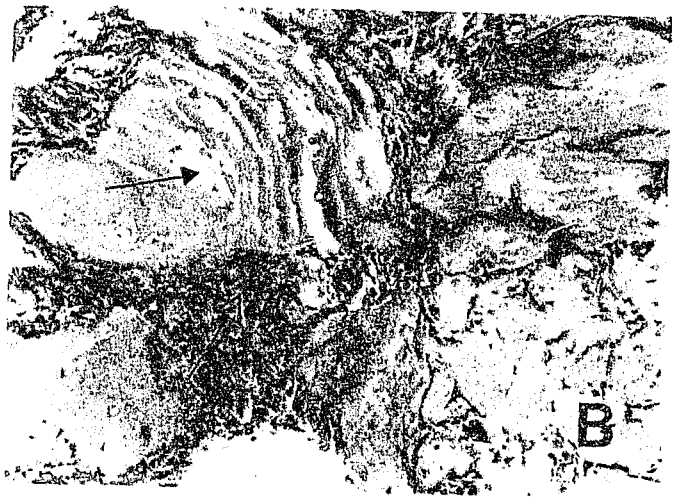
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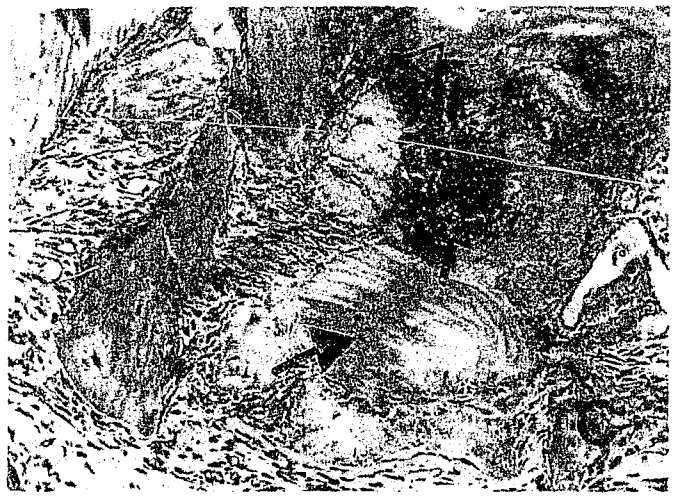


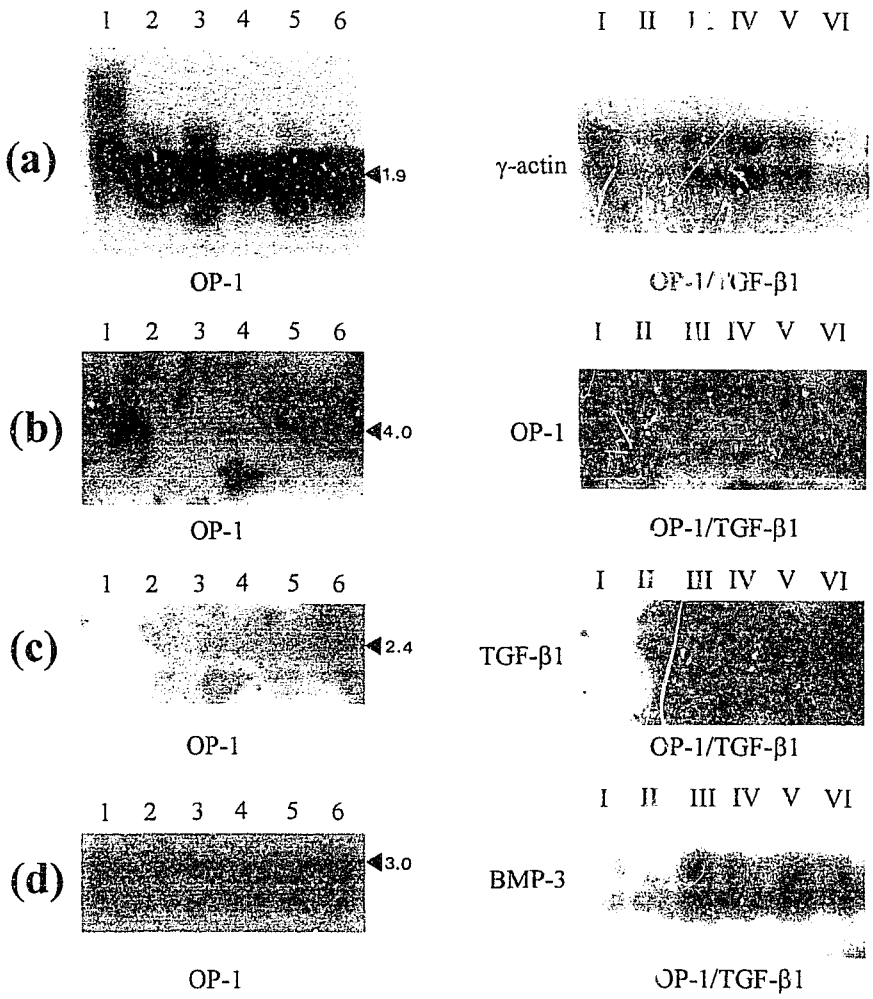
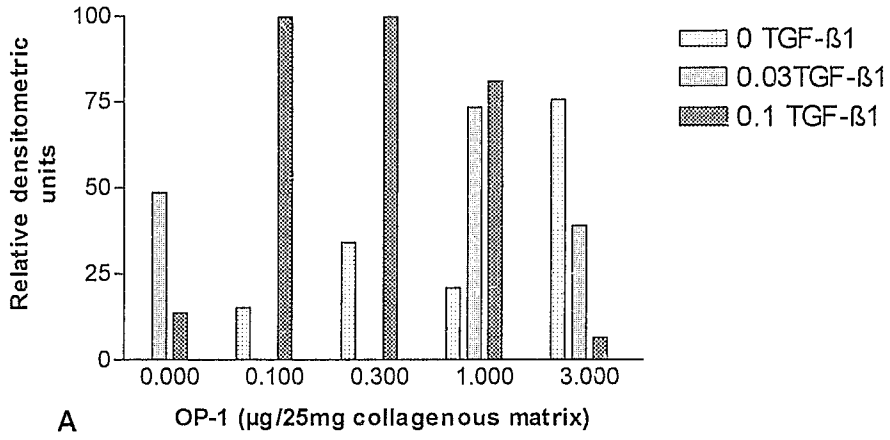


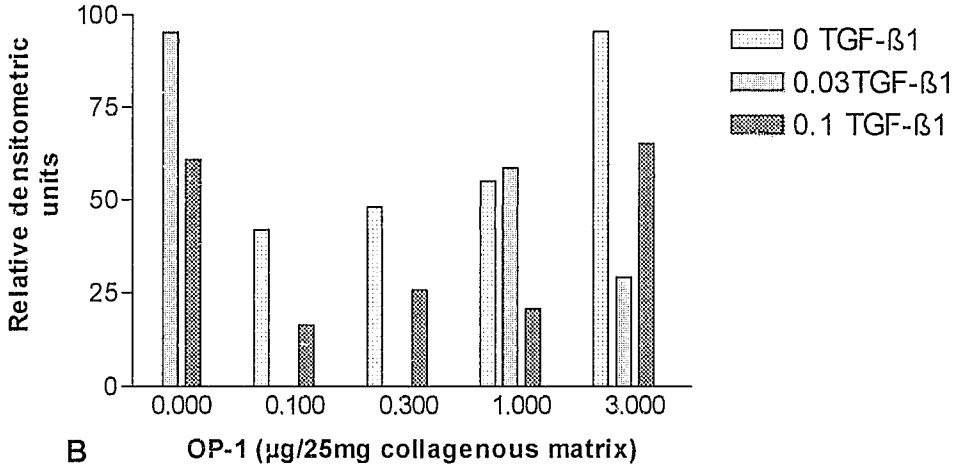
Figure 6. Northern blot analyses of gamma-actin, OP-1, TGF-ß1, and BMP-3 mRNA species generated in the rat subcutaneous space on day 7. Replicate generated tissues (n=4) were pooled and total RNA was extracted and hybridized as described in the methods. Samples of total RNA were subjected to Northern blot analysis for gamma-actin, OP-1, TGF- ß1 and BMP-3 (20μg per lane). **1**: 0.03 μg TGF- ß1, **2**: 0.1 μg TGF- ß1, **3**: 0.1μg OP-1, **4**: 0.30 μg OP-1, **5**: 1.00 μg OP-1, **6**: 3.00 μg OP-1, **I**: 0.03μg TGF-ß1/1.00 μg OP-1, **II**: 0.03μg TGF- ß1/3.00 μg OP-1, **III**: 0.1μg TGF- ß1/0.1μg OP-1, **IV**: 0.1μg TGF- ß1/: 0.30 μg OP-1, **V**: 0.1μg TGF- ß1/1.00 μg OP-1 and **VI**: 0.1μg TGF- ß1/3.00 μg OP-1.

Figure 7. mRNA signal quantitation at day 7. Replicate generated tissues (n=4) were pooled and total RNA was extracted and hybridized as described in the methods. Samples of total RNA were subjected to Northern analysis for gamma-actin, OP-1, TGF- ß1 and BMP-3 (20μg per lane). The signal intensity for each lane was quantitated by scanning densitometry (Gel documentation and Analysis system, Syngene. U.K) and normalized against the values obtained for the gamma-actin signals. Values were adjusted to relative densitometric units onto a scale of 100 (figure 7).

A: Gamma-actin, B: OP-1, C: BMP-3 , D: TGF-β1,


Relative densitometric units
100
75
50
25
0
0.000
0.100
0.300
1.000
3.000
OP-1 (µg/25mg collagenous matrix)
0 TGF-ß1
0.03TGF-ß1
0.1 TGF-ß1

A


Relative densitometric units
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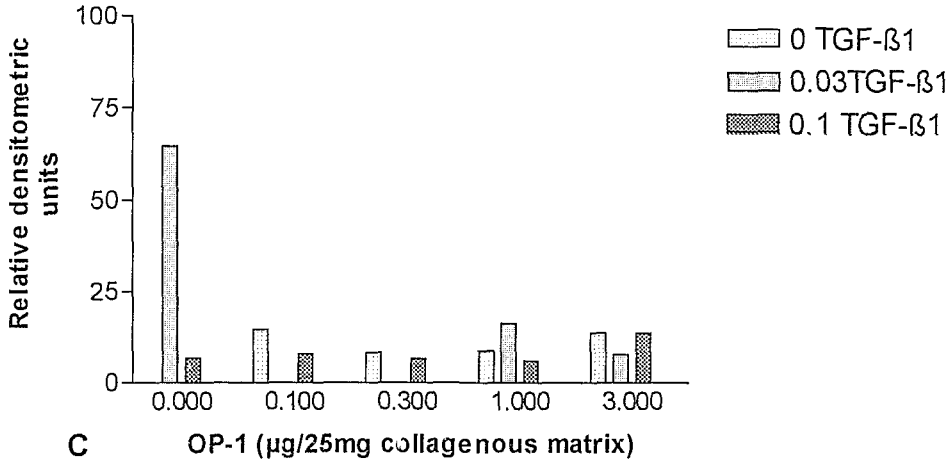
3.000
OP-1 (µg/25mg collagenous matrix)
0 TGF-ß1
0.03TGF-ß1
0.1 TGF-ß1

B


100
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Relative densitometric units
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0.100
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OP-1 (µg/25mg collagenous matrix)
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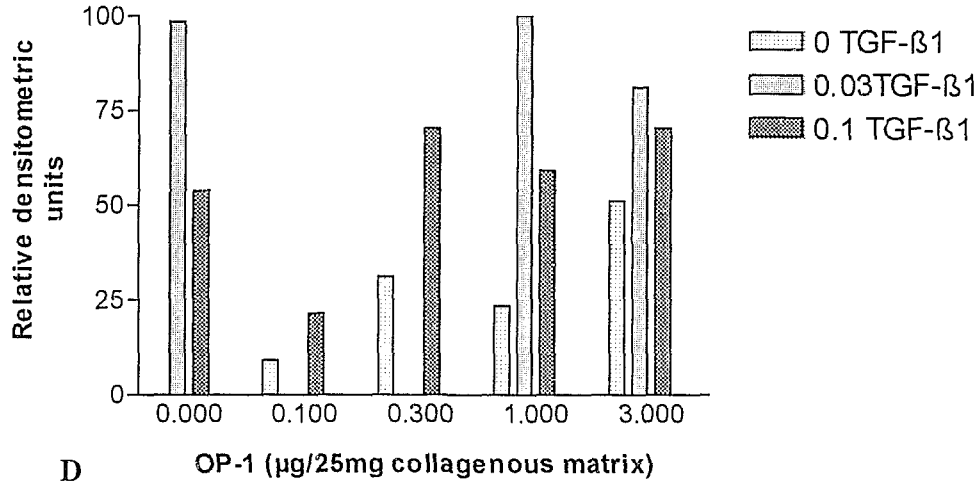
0.03TGF-ß1
0.1 TGF-ß1

C


100
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Relative densitometric units
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0.100
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1.000
3.000
OP-1 (µg/25mg collagenous matrix)
0 TGF-ß1
0.03TGF-ß1
0.1 TGF-ß1

D

4.2 Twelve Days Implantation

A dose dependent response in alkaline phosphatase activity was observed upon single applications of OP-1 at doses of 0.1-3 μg. The activity ranged from 0.1 to 1.2 U/mg protein (Figure 8). The application of TGF-ß1 at the given doses (0.01-0.1μg) to OP-1 at the doses of 1 μg and 3 resulted in a high activity of alkaline phosphatase in a dose dependent manner (0.8-7 U/mg protein) compared to the applications of OP-1 singly. Significant differences (5% level) were observed when applying 0.3 and 0.1 μg TGF- ß1 to 3 μg OP-1. Synergy was shown in 1.0 and 3.0 μg OP1 groups. OP-1 comparisons that showed a significant difference in alkaline phosphatase activity at 5% level were 3-1, 3-0.3, 3-0.1, 3-0, 1-0.3, 1-0.1 and 1-0 μg.

Calcium accumulation increased dose dependently at a range of 0.1 to 17 μg/mg protein (Figure 9). The calcium levels also increased dose dependently in the range 5-20 μg/mg tissue on addition of TGF-ß1 to OP-1. However, at the OP-1 dose of 1μg, there appears to be a biphasic response to TGF- ß1 resulting in a lower level of calcium on addition of increasing amounts of TGF-ß1. A significant difference (5% level) in calcium level was observed at the combination of 0.1μg TGF- ß1/0.1 μg OP-1. OP-1 comparisons that were significant at 5% level were 3-1, 3-0.3, 3-0.1, 3-0, 1-0.3, 1-0.1, 1-0, 0.3-0.1 and 0.3-0. Histological analyses showed that OP-1 implants exhibited a dose dependent response in the formation of cartilage upon addition of 0.03 and 0.1 μg of TGF-ß1 to 3 μg of OP-1 (Figure 10). Synergy was shown at 3 μg OP-1 with the combinations of TGF-β1.

mRNA expression showed that TGF-β1 singly increased the expression of OP-1 as well as its own expression to levels almost similar to those elicited by 3μg of OP-1 singly but decreased the expression of BMP-3 (figure 11). OP-1 increased the expression of all the three genes. The TGF-β/OP-1 combinations containing 0.03 μg TGF-β1 decreased the expression of OP-1 and increased that of TGF-β1 and BMP-3 while those containing 0.1 μg TGF-β1 increased OP-1, TGF-β1 and BMP-3 expression. OP-1 expression was high in pellets containing 0.03 μg TGF-β1 singly and in those containing 3μg OP-1 singly (figure 11). Collagen type II was expressed at high levels (figure 19). The results were verified using densitometric analysis (figure 11). The signal intensity for the mRNAs was quantitated by scanning densitometry (Gel Documentation and Analysis system, Syngene, U.K) and normalised against values obtained for the gamma-actin signals. Values were adjusted to relative densitometric units onto a scale of 100 (figure 12).

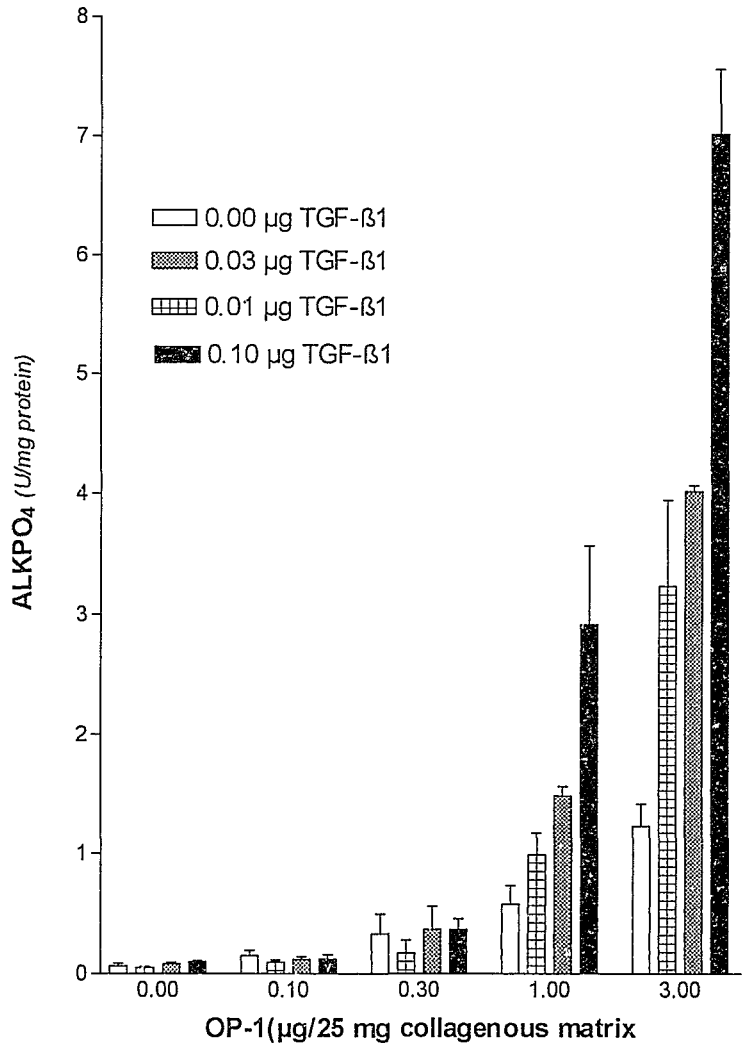


Figure 8. Biochemical results of implantation of OP-1 and TGF-β1, both singly and in combination in the rat subcutis. Generated tissues were harvested at 12 days post implantation and alkaline phosphatase activity was determined as described in the methods. n=4. Values are means ± SEM.

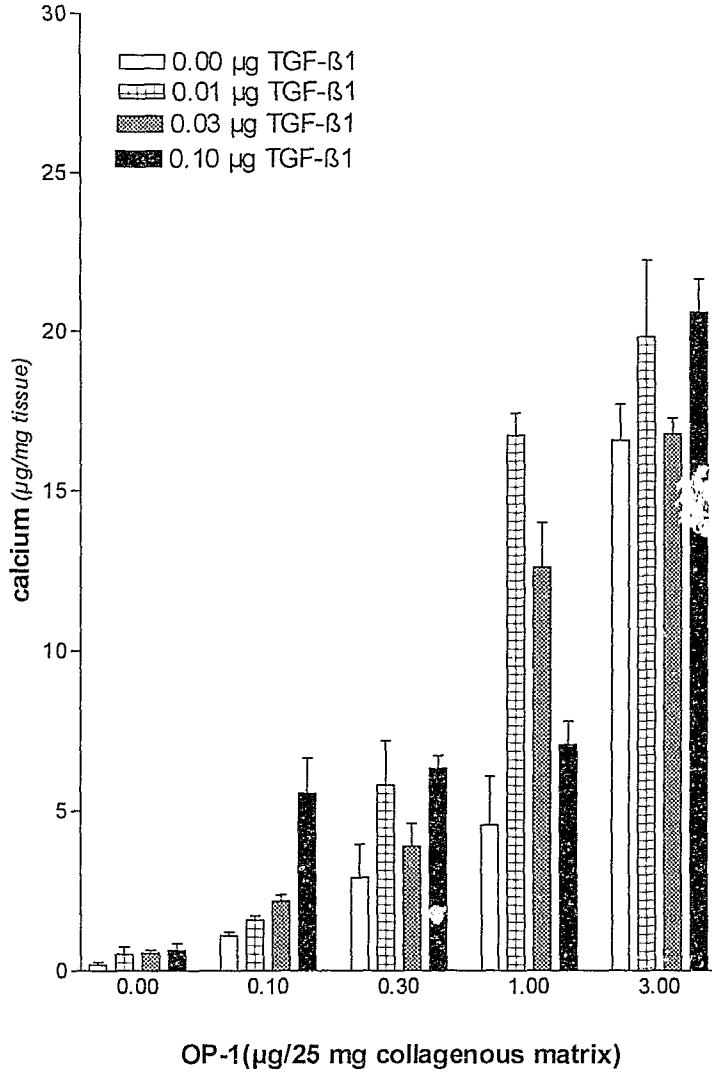


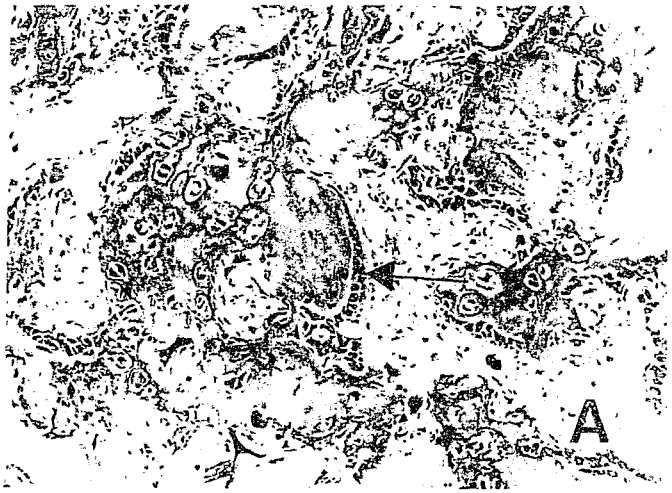
Figure 9. Calcium content of tissues generated by OP-1 and TGFβ1, both singly and in combination in the rat subcutis. Generated tissues were harvested at 12 days post implantation and calcium content was determined as described in the methods. n=4. Values are means ± SEM.

Figure 10. Toluidine blue stained sections of tissues generated by in the subcutaneous space of the rat 12 days post implantation. The tissues were processed as described in the methods.

A: 3 μg OP-1. Arrow indicates islands of chondrocytes and newly formed bone with contiguous osteoblasts .

B: 3 μg OP-1 with 0.03 μg of TGF-β1. Arrows indicate scattered islands of chondrogenesis.

C: 3 μg OP-1 with 0.1 μg of TGF-β1. Arrows indicate foci of cartilage and bone formation.


A


B



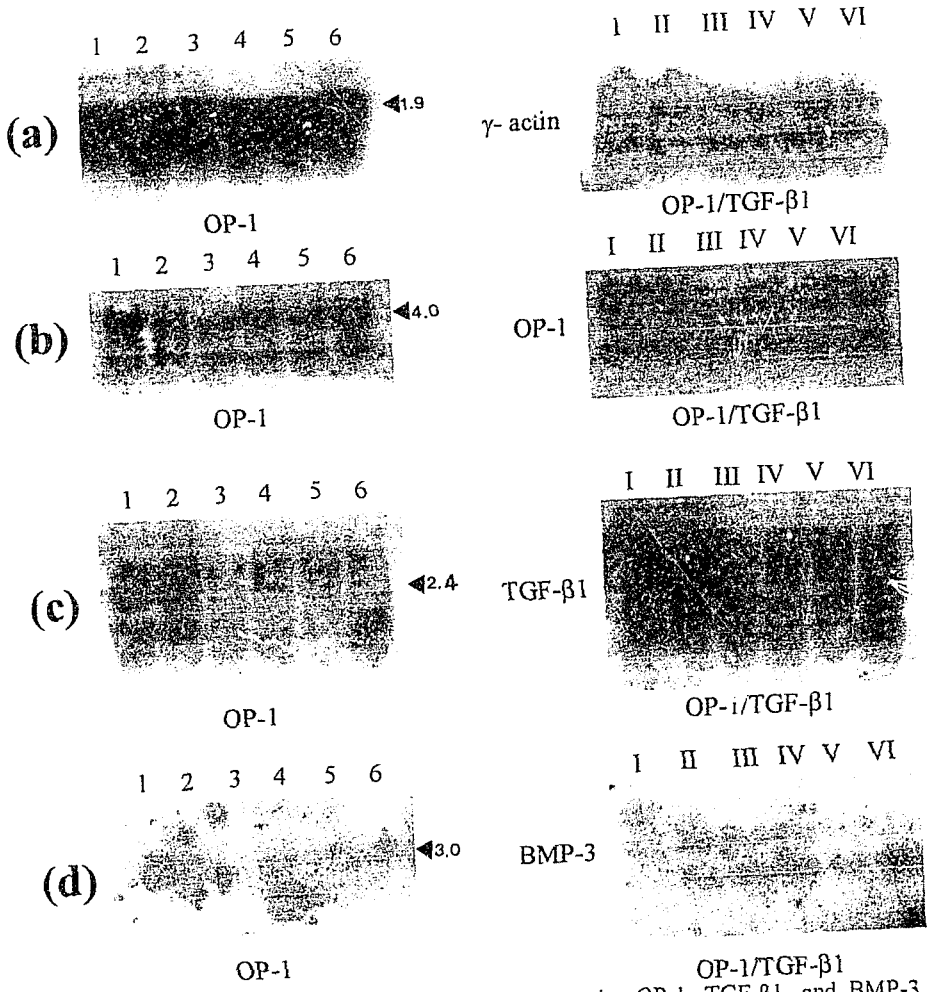
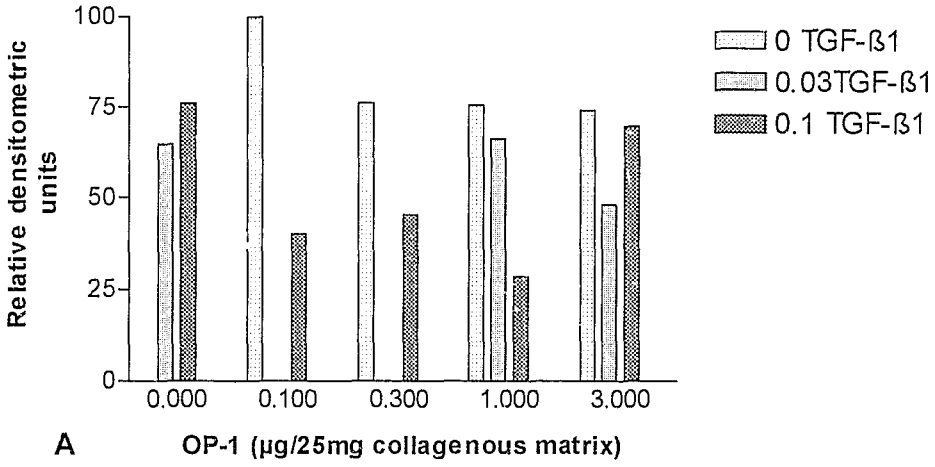
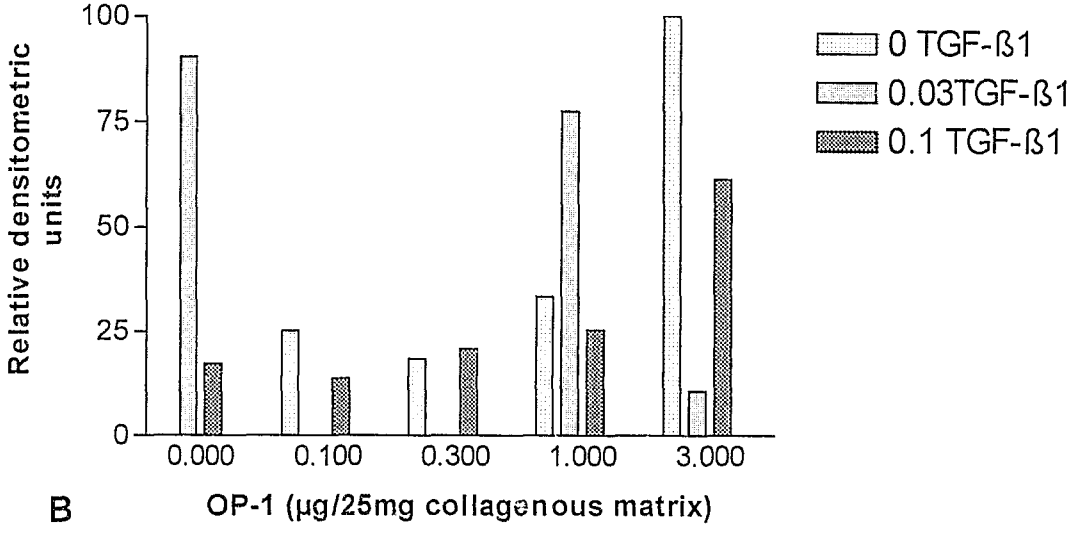


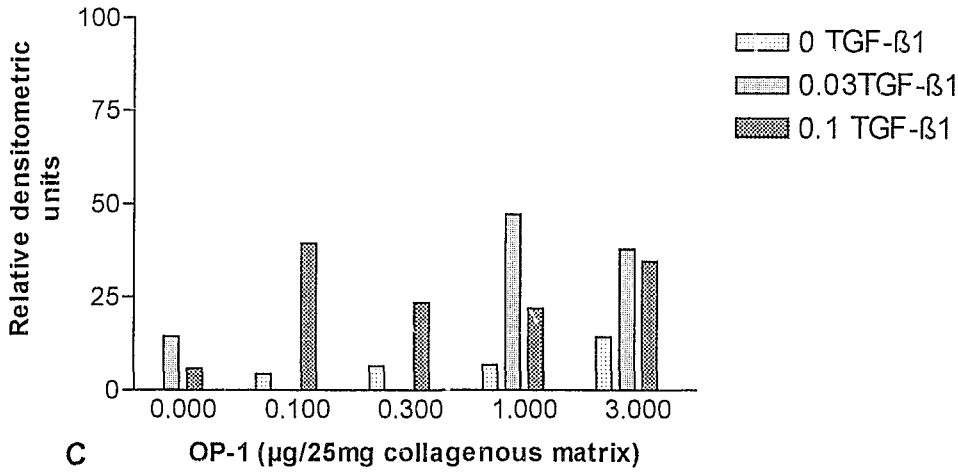
Figure 11. Northern blot analyses of gamma-actin, OP-1, TGF-ß1, and BMP-3 mRNA species generated in the rat subcutaneous space on day 12. Samples were pooled and mRNA was extracted and hybridized as described in the methods Replicate generated tissues (n=4) were pooled and total RNA as described in the methods. Samples of total RNA were subjected to Northern analysis for gamma-actin, OP-1, TGF- ß1 and BMP-3 (20µg per lane). 1: 0.03 µg TGF- ß1, 2: 0.1 µg TGF- ß1, 3: 0.1µg OP-1, 4: 0.30 µg OP-1, 5: 1.00 µg OP-1, 6: 3.00 µg OP-1, I: 0.03µg TGF-ß1/1.00 µg OP-1, II: 0.03µg TGF- ß1/3.00 µg OP-1, III: 0.1µg TGF- ß1/0.1µg OP-1, IV: 0.1µg TGF- ß1/: 0.30 µg OP-1, V: 0.1µg TGF-ß1/1.00 µg OP-1 and VI: 0.1µg TGF- ß1/3.00 µg OP-1.

Figure 12. mRNA signal quantitation at day 12. Replicate generated tissues (n=4) were pooled and total RNA was extracted and hybridized as described in the methods. Samples of total RNA were subjected to Northern analysis for gamma-actin, OP-1, TGF- ß1 and BMP-3 (20μg per lane). The signal intensity for each lane was quantitated by scanning densitometry (Gel Documentation and Analysis system, Syngene, U.K) and normalized against the values obtained for the gamma-actin signals. Values were arbitrarily adjusted to relative densitometric units onto a scale of 100.

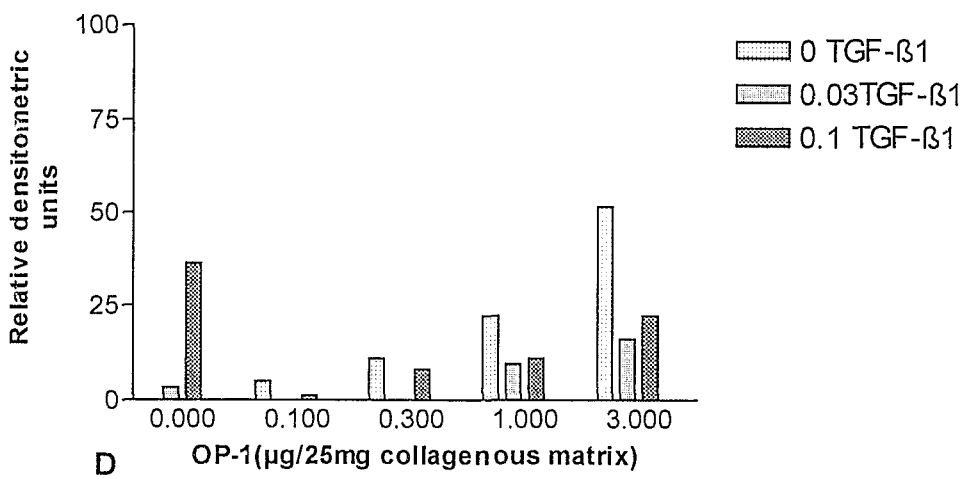
A: Gamma-actin, B: OP-1, C: BMP-3, D: TGF-β1,


Relative densitometric units
100
75
50
25
0
0.000
0.100
0.300
1.000
3.000
0 TGF-ß1
0.03TGF-ß1
0.1 TGF-ß1
A
OP-1 (µg/25mg collagenous matrix)


Relative densitometric units
100
75
50
25
0
0.000
0.100
0.300
1.000
3.000
0 TGF-ß1
0.03TGF-ß1
0.1 TGF-ß1
B
OP-1 (µg/25mg collagenous matrix)


100
75
50
25
0
Relative densitometric units
0.000
0.100
0.300
1.000
3.000
OP-1 (µg/25mg collagenous matrix)
0 TGF-ß1
0.03TGF-ß1
0.1 TGF-ß1

C


100
75
50
25
0
Relative densitometric units
0.000
0.100
0.300
1.000
3.000
OP-1(µg/25mg collagenous matrix)
0 TGF-ß1
0.03TGF-ß1
0.1 TGF-ß1

D

4.3 Twenty one days Implantation

Generally, compared to the 12 day activity, the alkaline phosphatase activity was highly reduced (5% significance level) at the combinations of 0.01,0.03 and 0.1 μg TGF- ß1 with 1.0 and 3.0 μg OP-1 whilst the calcium levels were elevated. Applications of OP-1 solo at doses of 0.1-3 μg elicited a dose dependent response in alkaline phosphatase activity at a range of 0.1-0.5 U/mg protein (Figure 13). OP-1 comparisons that were significantly different (5% level) were 3-1, 3-0.3, 3-0.1, 3-0 μg. Addition of TGF-ß1 at amounts of 0.01-0.1μg to OP-1 doses resulted in a decreased alkaline phosphatase activity in a dose dependent manner (no significance at 5% level).

Calcium content accumulation at the doses of 0.1-3 μg OP-1 were 10 to 68 μg/ mg tissue. However, with increasing amount of TGF-ß1 to OP-1 the accumulation was negligible. Addition of 0.1μg TGF- ß1 to 0.1 and 0.3μg OP-1 elicited a sharp decrease in calcium content that was significant at 5% level (Figure 14).

Specimens of 3 μg of OP-1 were shown histologically to have almost complete chondrolysis (Figure 15). The bone matrix was exhibiting signs of resorption and remodelled bone showed the formation of hematopoietic bone marrow. With combinations of 0.03 and 0.1 μg of TGF-ß1 and 3 μg of OP-1, histological examination showed also formation of bone marrow.

mRNA expression indicates that TGF-β1 singly decreased OP-1, TGF-β1 and BMP-3 expression. OP-1 singly increased the expression of all three genes. All the TGF-β1/OP-1 combinations used decreased BMP-3 expression and increased OP-1 and TGF-β1 expression (figure 16 and 17). Collagen type IV was detected at high levels (figure 18 a). These results were confirmed by densitometric analysis (figure 17). The signal intensity for the mRNAs was quantitated by scanning densitometry (Gel documentation and Analysis system, Syngene, U.K) and normalised against values obtained for the gamma-actin signals. Values were adjusted to relative densitometric units onto a scale of 100 (figure 19).

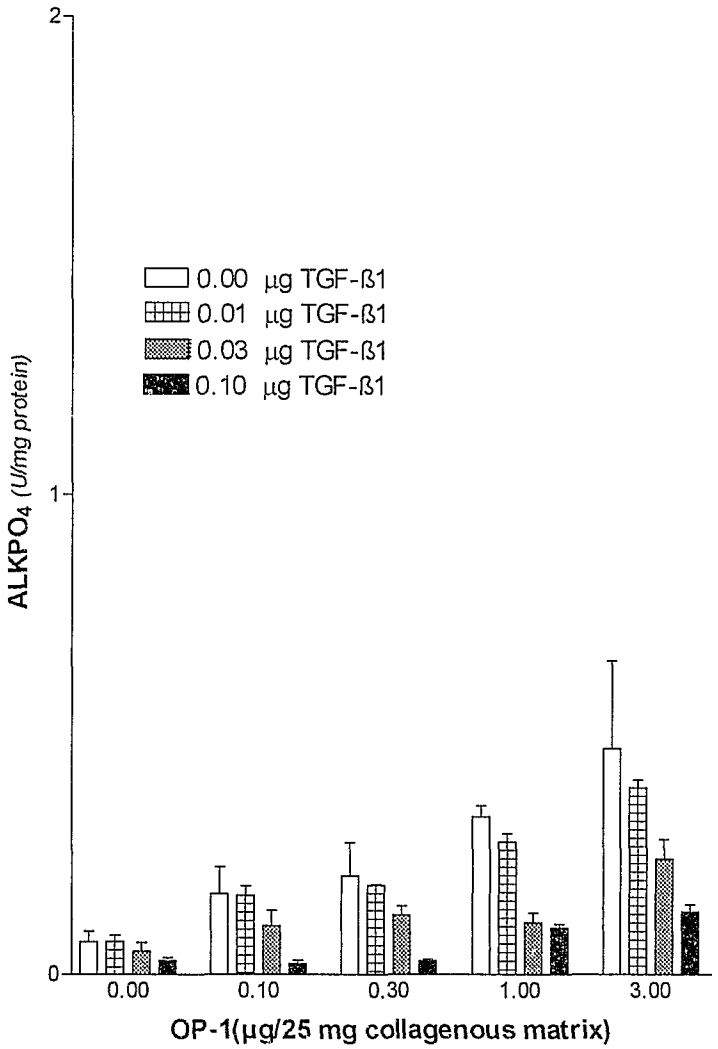


Figure 13. Biochemical results of implantation of OP-1 and TGF-β1, both singly and in combination in the rat subcutis. Generated tissues were harvested at 21 days post implantation and alkaline phosphatase activity was determined as described in the methods. n=4. Values are means ± SEM.

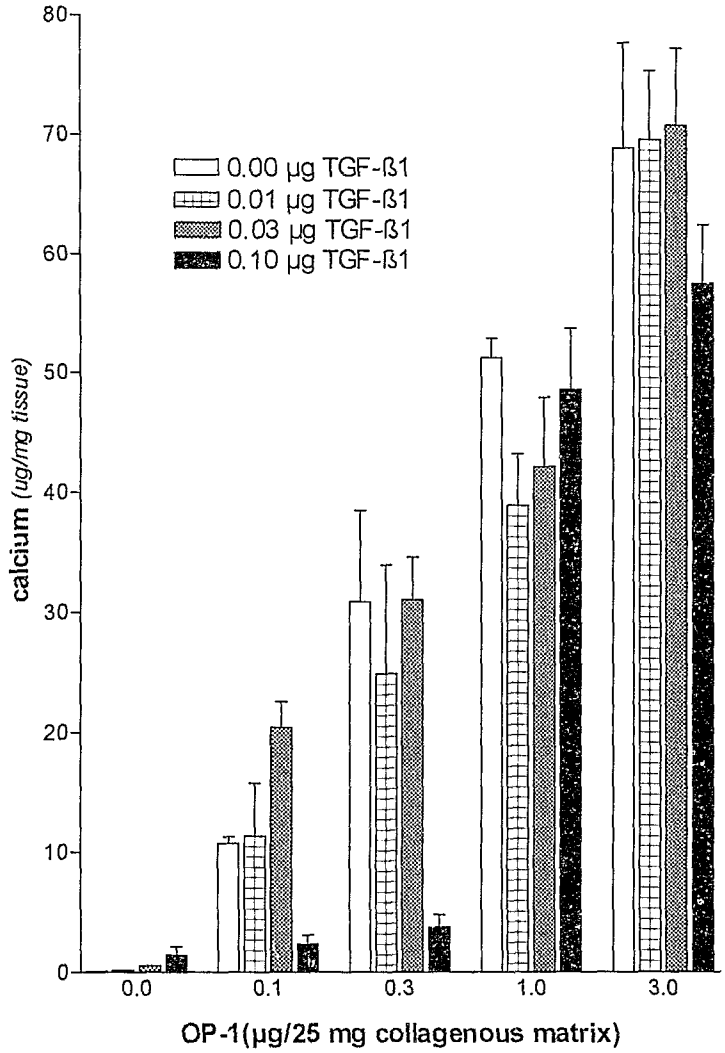


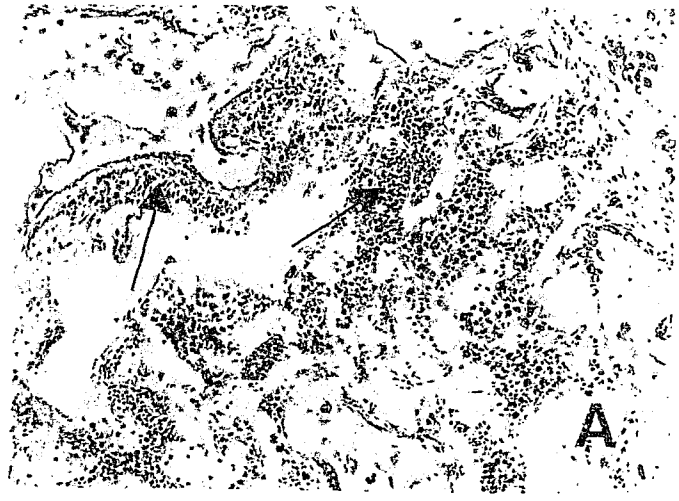
Figure 14. Calcium content of tissues generated by OP-1 and TGFβ1, both singly and in combination in the rat subcutis. Generated tissues were harvested at 21 days post implantation and calcium content was determined as described in the methods. n=4. Values are means ± SEM.

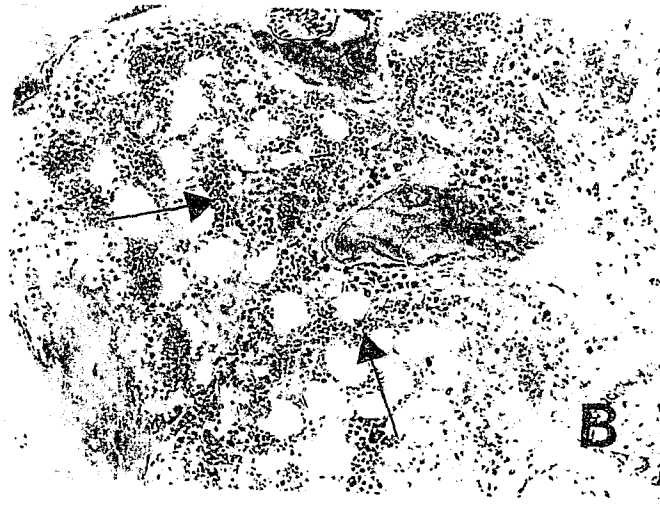
Figure 15. Toluidine blue stained sections of tissues generated in the rat subcutaneous space 21 days post implantation. The tissues were processed as described in the methods.

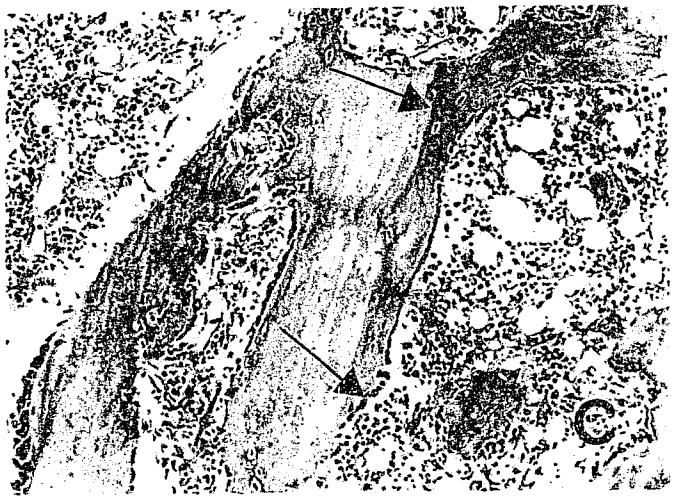
A: 3 μg of OP-1 solo. Arrows indicate hematopoietic bone marrow formation.

B: 3 μg of OP-1 with 0.03 μg of TGF-β1. Arrows indicate hematopoietic bone marrow formation.

C: 3 μg of OP-1 with 0.1 μg of TGF-β1. Arrows indicate osteoblasts facing newly formed hematopoietic bone marrow.


A


B



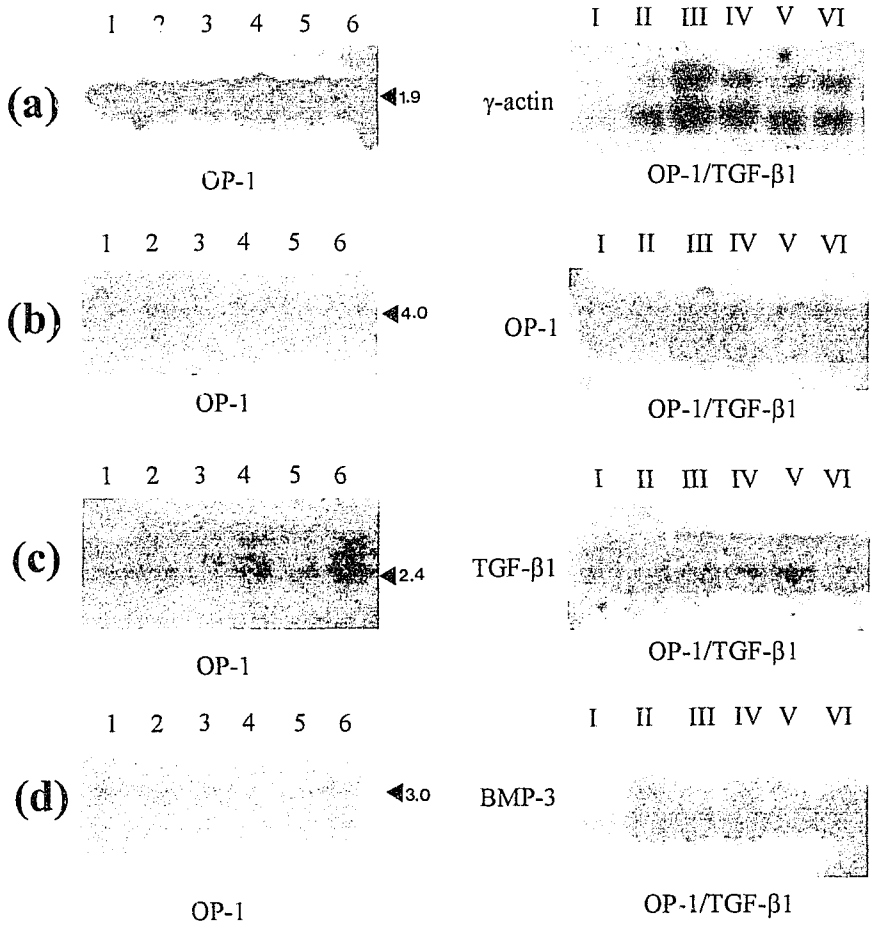
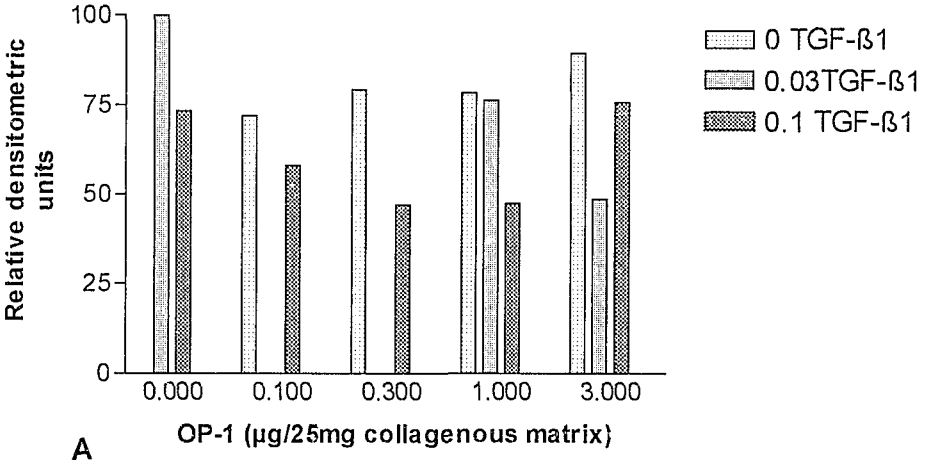


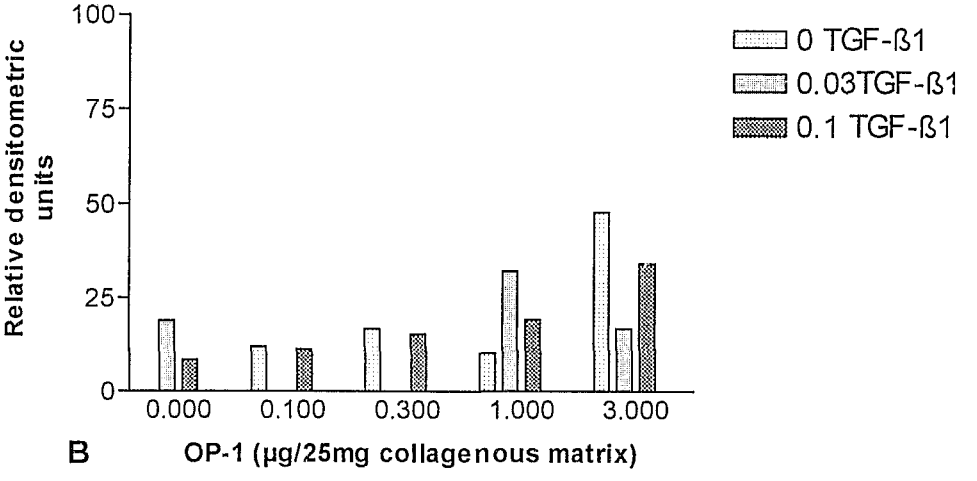
Figure 16. Northern blot analyses of OP-1, TGF-ß1, and BMP-3 mRNA species generated in the rat subcutaneous space on day 21. Replicates generated tissues (n=4) were pooled and total RNA was extracted and hybridized as described in the methods. Samples of total RNA were subjected to Northern blot analysis for gamma-actin, OP-1, TGF- ß1 and BMP-3 (20μg per lane). **1**: 0.03 μg TGF- ß1, **2**: 0.1 μg TGF- ß1, **3**: 0.1μg OP-1, **4**: 0.30 μg OP-1, **5**: 1.00 μg OP-1, **6**: 3.00 μg OP-1, **I**: 0.03μg TGF-ß1/1.00 μg OP-1, **II**: 0.03μg TGF- ß1/3.00 μg OP-1, **III**: 0.1μg TGF- ß1/0.1μg OP-1, **IV**: 0.1μg TGF- ß1/: 0.30 μg OP-1, **V**: 0.1μg TGF- ß1/1.00 μg OP-1 and **VI**: 0.1μg TGF- ß1/3.00 μg OP-1.

Figure 17. mRNA signal quantitation at day 21. Replicate generated tissues (n=4) were pooled and total RNA was extracted and hybridized as described in the methods. Samples of total RNA were subjected to Northern analysis for gamma-actin, OP-1, TGF- ß1 and BMP-3 (20μg per lane). The signal intensity for each lane was quantitated by scanning densitometry (Gel Documentation and Analysis system, Syngene, U.K) and normalized against the values obtained for the gamma-actin signals. Values were arbitrarily adjusted to relative densitometric units onto a scale of 100.

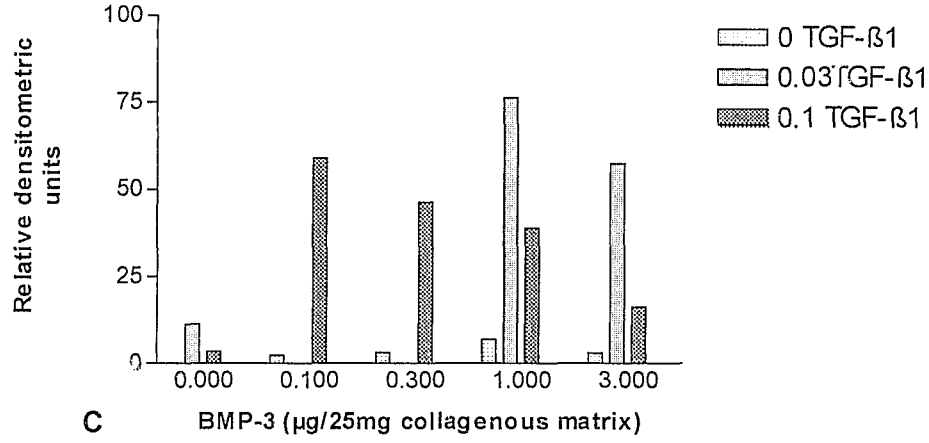
A: Gamma-actin, B: OP-1, C: BMP-3, D: TGF-β1.

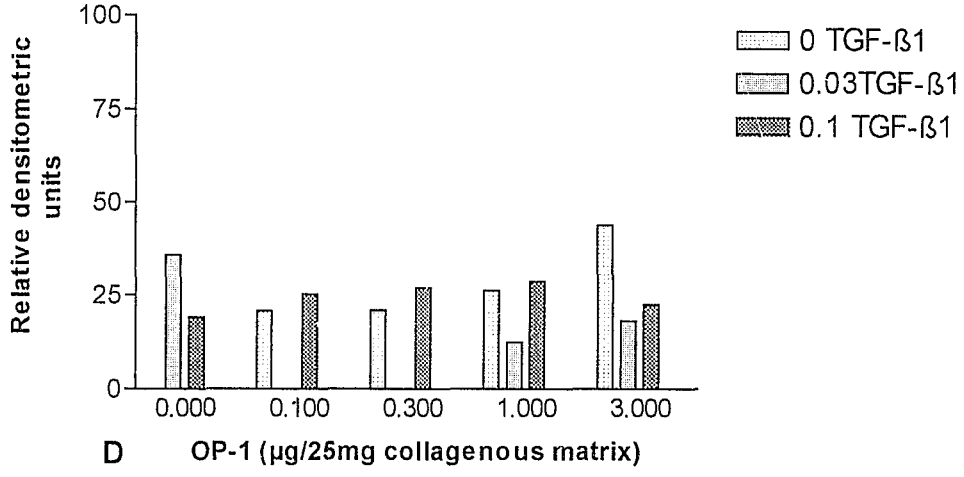

100
75
50
25
0
Relative densitometric units
0.000
0.100
0.300
1.000
3.000
OP-1 (µg/25mg collagenous matrix)
0 TGF-ß1
0.03TGF-ß1
0.1 TGF-ß1

A


100
75
50
25
0
Relative densitometric units
0.000
0.100
0.300
1.000
3.000
OP-1 (µg/25mg collagenous matrix)
0 TGF-ß1
0.03TGF-ß1
0.1 TGF-ß1

B


Relative densitometric units
100
75
50
25
0
0.000
0.100
0.300
1.000
3.000
0 TGF-ß1
0.03 TGF-ß1
0.1 TGF-ß1
C
BMP-3 (µg/25mg collagenous matrix)


Relative densitometric units
100
75
50
25
0
0.000
0.100
0.300
1.000
3.000
0 TGF-ß1
0.03TGF-ß1
0.1 TGF-ß1
D
OP-1 (µg/25mg collagenous matrix)

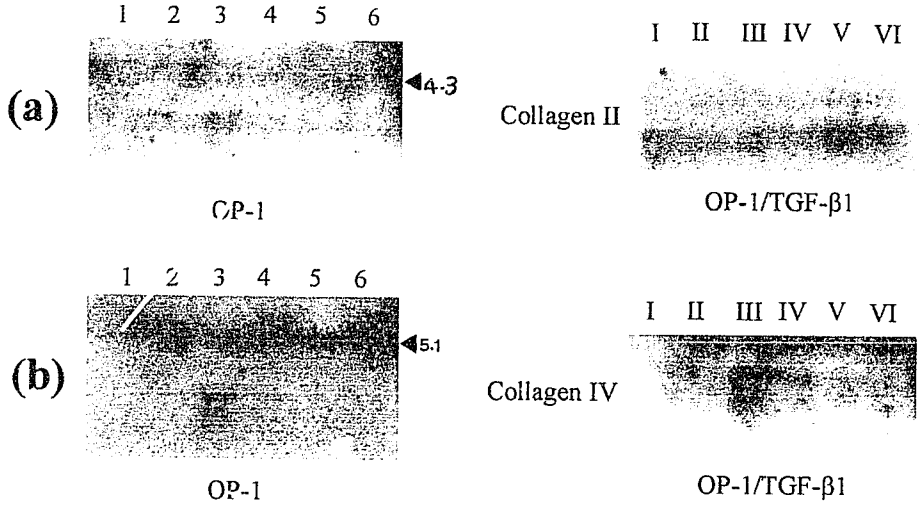
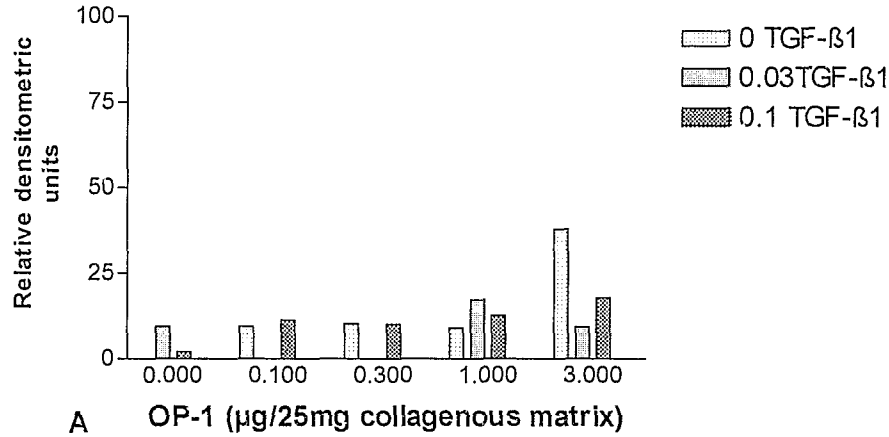


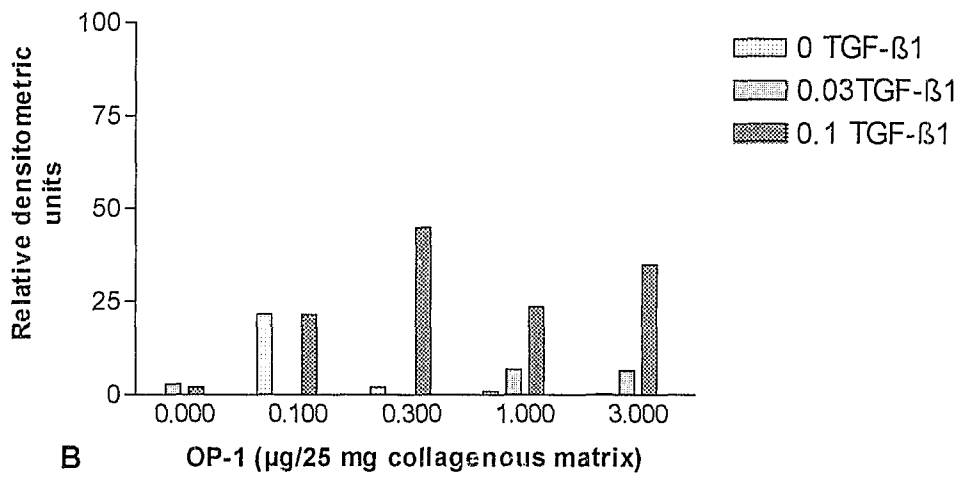
Figure 18. Northern blot analyses of Collagen types II and IV generated in the rat subcutaneous space on days 12 and 21 respectively. Replicate generated tissues (n=4) were pooled and total RNA was extracted and hybridized as described in the methods. Samples of total RNA were subjected to Northern blot analysis for collagen types II and IV (10μg per lane). 1: 0.03 μg TGF- ß1, 2: 0.1 μg TGF- ß1, 3: 0.1μg OP-1, 4: 0.30 μg OP-1, 5: 1.00 μg OP-1, 6: 3.00 μg OP-1, I: 0.03μg TGF-ß1/1.00 μg OP-1, II: 0.03μg TGF- ß1/3.00 μg OP-1, III: 0.1μg TGF- ß1/0.1μg OP-1, IV: 0.1μg TGF- ß1/: 0.30 μg OP-1, V: 0.1μg TGF- ß1/1.00 μg OP-1 and VI: 0.1μg TGF- ß1/3.00 μg OP-1.

Figure 19. mRNA signal quantitation of collagen II and type IV at days 12 and 21. Replicate generated tissues (n=4) were pooled and total RNA was extracted and hybridized performed as described in the methods. Samples of total RNA were subjected to Northern analysis collagen Type II and type IV (10μg per lane). The signal intensity for each lane was quantitated by scanning densitometry (Gel Documentation and Analysis system, Syngene, U.K) and normalized against the values obtained for the gamma-actin signals. Values were adjusted to relative densitometric units onto a scale of 100.

A: Collagen type II

B: Collagen type IV


Relative densitometric units
100
75
50
25
0
0.000
0.100
0.300
1.000
3.000
0 TGF-ß1
0.03TGF-ß1
0.1 TGF-ß1
A
OP-1 (µg/25mg collagenous matrix)


Relative densitometric units
100
75
50
25
0
0.000
0.100
0.300
1.000
3.000
0 TGF-ß1
0.03TGF-ß1
0.1 TGF-ß1
B
OP-1 (µg/25 mg collagenous matrix)

5 DISCUSSION AND CONCLUDING STATEMENTS

The bone-forming activity elicited by OP-1 and TGF-β1, both singly and in combination, was monitored by alkaline phosphatase activity and quantitated by calcium content levels at 7, 12 and 21 days post implantation in the rat subcutaneous site. Histological analysis was performed on the generated tissues to confirm the osteogenic activity of the two morphogens both singly and in combination. mRNA analysis was also performed to study the gene expression during endochondral bone formation induced by the morphogens. Various quantities of OP-1 and TGF-β1 both singly and in combination were used to develop a dose response curve, and the bone inducing activity was reproducible and dose-dependent.

BMPs were discovered by their ability to singly initiate bone formation in heterotopic sites, mimicking events that occur in the normal course of embryonic bone development (Wozney,1992; Reddi, 1994). The developmental cascade of matrix-induced endochondral bone formation consists of chemotaxis and attachment of mesenchymal stem cells to the matrix and multiplication of progenitor cells followed by differentiation of cartilage, of bone and finally hematopoiesis (Reddi, 1981). Local differentiation of endochondral bone in rodents after subcutaneous implantation of demineralised bone has been extensively demonstrated (Urist, 1965; Reddi and Huggins, 1972; Reddi, 1981). Between day 1 and day 7, there is fibronectin deposition onto the matrix, facilitating cell attachment. These events are followed by differentiation of mesenchymal cells resulting in chondroblastic

differentiation leading to cartilagenous matrix deposition. Day 8 marks the beginning of angiogenesis and cartilage resorption followed by formation of new bone by day 12, then extensive bone remodelling with hematopoietic marrow formation follows at day 21 (Reddi, 1981).

TGF-β1 is found in high concentrations in platelets (Assoian *et al.*, 1993) but bone represents the most abundant source (Seyedin *et al.*, 1985). The presence of TGF-β isoforms in bone suggests that they may play important roles in bone formation and maintenance. *In vivo* and *in vitro* studies have indicated that TGF-β has stimulatory effects on proliferation and synthesis of extracellular matrix in many types of skeletal tissue cells. *In vivo*, TGF-β1 has the ability to enhance a repair process and callus formation in uninjured bone (Sumner *et al.*, 1995). Also local injection of TGF-β induces subperiosteal chondrogenesis and osteogenesis as well as enhancing and strengthening healing of tibial fractures (Nielsen *et al.*, 1994). *In vitro*, TGF-β may enhance chemotaxis of osteoblasts since it has a strong proliferative effect on osteoblast precursors, and can also enhance chondrogenesis in periosteal explants (Miura *et al.*, 1994). Si *et al.* (1997) studied the expression of BMP-2 and TGF-β1 mRNA during healing of the rabbit mandible. The results showed higher levels of BMP-2 mRNA in undifferentiated mesenchymal cells, differentiating osteoblasts and chondroblasts during intramembranous bone formation and early chondrogenesis. The level of TGF-β1 mRNA, on the other hand, was higher in chondrocytes and active differentiated osteoblasts during chondrogenesis and and endochondral

ossification. These results suggested that TGF-β1 regulates the proliferation and active synthesis of chondrocytes and osteoblasts while BMP-2 plays critical roles in bone induction and early chondrogenesis, therefore, the combined effects of these two factors may lead to improvement of fracture healing (Si *et al.*, 1997). The main difference between TGF-βs and BMPs is that TGF-βs appear to fail the induction of bone formation in heterotopic sites.

Synergy is the interaction between two agents that results in an effect greater than the sum of the effects attributable to each agent when applied singly (Greco *et al.*, 1995). Single applications of TGF-β1 were inactive at all doses tested as confirmed histologically by lack of cartilage and bone formation. Therefore, all cases for which binary combinations of the two morphogens caused an increase in alkaline phosphatase activity when compared to OP-1 singly were classified as synergistic interactions. This is a classic example of a synergistic interaction between two agents where one is active and the other is inactive when applied singly (Greco *et al.*, 1995). Duneas et al. (1998), reported this type of synergistic interaction in calvarial defects of the adult baboon (*Papio ursinus*) where hTGF-β1 failed to induce bone formation, but instead resulted in limited chondro-osteogenesis at the margins of the calvarial defects, and in increased fibrosis and vascular invasion when compared to controls of collagenous matrix only. OP-1 on the other hand, has been shown to induce complete regeneration of calvarial bone in the baboon (Duneas *et al.*, 1998; Ripamonti *et al.*, 1996). When combinations of different doses of both hOP-1 and porcine-derived

(p)TGF-β1 were administered in calvarial defects of the baboon, the outcome was a marked proliferative and inductive phase at the periphery of the implanted matrix, more so at the level of the pericranium and temporalis muscle, ensued by an abundant osteogenic effect that resulted in the loss of cohesiveness between the pericranial and endocranial osteogenic sites on day 30 (Duneas *et al.*, 1998). On day 90, the defects treated with a single application of hOP-1 (20 μg) were considerably thinner when compared to those attained by the combination of pTGF- ß1 (5 μg) and hOP-1 (20 μg). Defects treated with a combination of 100 μg hOP-1 with 15 μg pTGF-ß1 resulted in marked and exuberant osteogenesis, persistence of pericranial lifting with temporalis muscle displacement and of zones of fibrivascular tissue influx with scattered collagenous matrix particles. Histomorphometric results showed that on day 30, morphogen combinations resulted in greater amounts of osteoid volumes when compared with specimens treated with 100 μg hOP-1 alone. On day 90, greater amounts of osteoid and bone were found in specimens treated with 100 μg hOP-1 with 15 μg pTGF-ß1. Tissue area was also greater in specimens generated by morphogen combinations than in those generated by hOP-1 alone. Alkaline phosphatase activity was also higher in tissues generated by morphogen combinations than in tissues generated by hOP-1 singly Duneas *et al.*, 1998). Although TGF- ß1 has been shown to induce bone formation in the heterotopic sites of the baboon, it is not inductive in the orthotopic sites which suggests that the activity of this morphogen is site specific (Duneas *et al.*, 1998). Si *et al.* (1998), also reported synergistic interaction between hBMP-2 and TGF-ß1. They used TGF-ß1

and rhBMP-2 both singly and in combination in ceramic bovine bone as a carrier, to induce bone formation in the thigh muscle pouches of mice. The histological analysis of the implanted areas was performed at 3, 5, 7, 14 and 21 days. TGF-β1 alone resulted in proliferation of fibroblasts by day 3 and day 5, and in a thin condensed layer of fibrous connective tissue by day 21. Both BMP-2 singly and in combination with TGF-β showed aggregation and proliferation of mesenchymal cells by day 3 and day 5, formation of new cartilage and bone by day 7 and 14 respectively, and formation of new bone marrow by day 21. Comparatively, the newly-formed bone trabeculae were thicker in the tissues generated by the combination of BMP-2 and TGF-β than in those generated by BMP-2 alone. A morphometric study was perfomed which showed that the average area of newly formed bone in the tissues generated by the combination of the two morphogens was significantly higher than that of tissue generated by BMP-2 alone, while TGF-β alone did not promote any bone formation.

Synergistic interaction has been reported in the adult baboon (*Papio ursinus*) where hTGF-ß1 and OP-1 were used both singly and in combination to induce heterotopic endochondral bone formation (Ripamonti *et al.*, 1997). The results showed that TGF-ß1 singly induced endochondral bone formation in the heterotopic site of the baboon. However, binary applications of OP-1 and TGF-ß1 induced tissue morphogenesis to a greater extent than the sum of the effects of both morphogens implanted singly. Previous studies have also shown that a combination of bovine bone-derived BMP

fractions and bovine bone-derived TGF-β2 resulted in enhancement of cartilage and bone formation in the subcutaneous space of the rat when combined with collagen and a ceramic carrier (Bentz *et al.*, 1991; Ogawa *et al.*, 1992). The present study demonstrates bone induction by OP-1 in the rat subcutaneous site and that TGF- ß1 synergises with OP-1 to induce bone formation, whereas it is not inductive in the subcutaneous site of the rat, in agreement with previous studies (Roberts *et al.*, 1986; Sampath *et al.*, 1987; Bentz *et al.*, 1989; Bentz *et al.*, 1991).

In the present study, single applications of OP-1 with rat collagen as carrier induced cartilage and bone by day 12 and bone and bone marrow by day 21 as determined by alkaline phosphatase activity, calcium content and histological examination of subcutaneous implants in the rats. On day 7, OP-1 implants showed a dose-dependent increase in alkaline phosphatase activity and calcium content. Addition of comparatively low amounts of TGF-ß1 to the given doses of OP-1 increased the levels of these markers of bone formation (alkaline phosphatase activity and calcium content) to slightly higher levels than those elicited by OP-1 singly. These results were confirmed histologically.

Addition of TGF-ß1 to OP-1 treatments resulted in a dose-dependent increase in the alkaline phosphatase activity at higher doses of OP-1, for example, 1 and 3 μg OP-1 on day 12. Strong synergistic interactions were shown when 0.1 μg of TGF-ß1 was added to 1 μg of OP-1 and at combinations of 3 μg of OP-1 with 0.01, 0.03 and 0.1

μg of TGF-ß1, for which the binary combinations of both morphogens resulted in alkaline phosphatase activities higher than those of each morphogen when applied singly. Calcium contents at combinations of 1μg of OP-1 with 0.01 μg of TGF-ß1 also reflected strong synergistic interactions by day 12. An interesting feature is that TGF-ß1 showed a biphasic mode of action as at the combination of 1μg OP-1 with 0.03 μg and 0.1μg of TGF-ß1 the calcium level is lower than that induced by 1μg OP-1 with 0.01 μg of TGF- ß1. This biphasic mode of action could be similar to that reported by Rosen and co-workers (Rosen *et al.*, 1990), which authors suggested that TGF-ßs may stimulate bone formation at low levels, cartilage formation at higher levels and fibrosis at yet higher levels. On the other hand, these results could mean that the tissue growth generated exceeds the mineralisation rate, causing a drop in the ratio of calcium to tissue mass. By combining 3 μg of OP-1 with 0.01 μg of TGF-ß1, a slight increase in the calcium content was found, followed by a decrease at the higher concentration of TGF-ß1 (0.03 μg). The combination of 3 μg OP-1 with 0.1 μg of TGF-ß1 showed an increase compared to the level of OP-1 applied singly.

By 21 days, the alkaline phosphatase activity was observed to increase dose dependently with increasing doses of singly applied OP-1. However, addition of TGF-ß1 to the OP-1 implants resulted in a decrease in alkaline phosphatase activity in a dose dependent manner. This, as expected, was most likely due to the fact that at day 21 of the cascade, there is mostly resorption with remodelling and formation of bone marrow. OP-1 solo elicited a very high calcium content and the increase was dose dependent at the range of 10 -70 μg/mg tissue. The addition of TGF-ß1 to OP-

at the given doses resulted in a negligible increase in the calcium level. The biphasic mode of action of TGF-ß1 was observed again, as for the combinations of 0.1μg of TGF-ß1 with 0.1 and 0.3 μg of OP-1, the calcium content was lower compared to the response elicited by combinations of 0.01 and 0.03 μg TGF- ß1 with 0.1 and 0.3 μg OP-1. Single applications of 3 μg OP-1 elicited the formation of cartilage at day 7, followed by differentiation of bone at day 12, and remodelling of bone with formation of bone marrow by day 21. These are the typical steps of matrix- induced endochondral bone formation in rats (Reddi, 1981).

There may be several mechanisms by which TGF-β1 synergizes with OP-1. As previously suggested for a combination of bovine bone-derived TGF-β2 and BMP fractions (Bentz *et al.*, 1989; Bentz *et al.*,1991), TGF-β1 may act as a chemotactic and mitogenic factor for responding precursor cells for subsequent induction by OP-1. Exposure to BMP-2 alters TGF-β binding profiles, prompting bone cells toward the next step of phenotypic expression, simultaneously enhancing TGF-β-induced collagen synthesis and alkaline phosphatase activity, depending on the current state of bone cell differentiation. TGF-β receptor expression is furthermore regulated by exposure to BMP-2 (Centrella *et al.*, 1995). The synergistic activity observed *in vivo* is possibly the result of superior cell migration and recruitment at the site of implantation, resulting in the observed temporal enhancement of bone formation.

Synergistic interactions among morphogens could be a general principle adopted in embryonic development as exemplified by experiments showing a synergistic interaction of TGF-β1 with basic fibroblast growth factor in the regulation of chondrogenesis during the murine optic capsule formation (Frenz *et al.*, 1994). Thus, evidence for synchronous embryonic expression of BMP/OP and TGF-β family members can aid the design of molecular therapeutic approaches based on recapitulation of embryonic development for which the present results may have important therapeutic implications.

The temporal expression of OP-1, BMP-3 and TGF-ß1 (which are cartilage and bone matrix genes associated with the developmental sequence of endochondral bone formation), as well as types II and IV collagen was examined by Northern analysis of tissues generated in the rat subcutaneous space. Type II collagen is a chondrogenic specific marker (Reddi *et al.*, 1977). Type IV collagen is a marker for angiogenesis (Reddi *et al.*, 1977). The results indicate that morphogen-induced endochondral bone formation involves the expression of several members of the TGF-ß superfamily. The high amount of type IV collagen mRNA in the combinations of 0.1, 0,3, 1.0 and 3.0 μg OP-1 with 0.1 μg TGFβ1 over OP-1 single application suggests that the two morphogens interact synergistically to induce angiogenesis and vascular invasion. Since angiogenesis is a prerequisite for osteogenesis, enhanced vascularisation may be part of the mechanism whereby OP-1 and TGF- ß1 synergize in heterotopic bone

induction. Specimens generated by single applications of either morphogen showed expression of their own mRNAs indicating an autoinductive effect, which, for TGF-ß1, is in agreement with previous results obtained *in vitro* (Robey et al., 1987) and *in vivo* (Joyce *et al.*, 1990). The detection of type II collagen mRNA transcripts in specimens generated by single applications of TGF-ß1 confirms the chondrogenic potential of the morphogen (Centrella *et al.*, 1994). It was shown previously that in the demineralised bone matrix-induced bone forming model (Reddi, 1981), cartilage development takes place between day 7 and day 12 and is correlated with the appearance of type II collagen mRNA.

The expression of OP-1, BMP-3, TGF-ß1 mRNAs observed in tissues generated both by single applications of OP-1 and combinations of OP-1 and TGF-ß1 suggest that bone formation induced by the morphogens both singly and in combination requires synthesis of some members of the BMP/OP family. In general, both in tissues generated by OP-1 singly as well as those generated by combinations of OP-1 and TGF-ß1, the mRNA expression of OP-1 peaked by day 12, TGF-ß1 expression decreased temporally and BMP-3 mRNA expression increased temporally. More importantly, the results also showed a stronger signal intensity of gene expression in the tissues generated by combinations of OP-1 and TGF-ß1. Duneas *et al.* (1998) reported similar trends of gene expression in a non-human primate model. The present study has, however, extended further and highlighted the temporal nature of the expression of these genes.

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