University of Bath

UNIVERSITY OF
BATH

PHD

Transfection of human osteoblasts with SV40 DNA: Characterisation of immortalised cell lines

Williams, Meryl Marjory

Award date:
1992

Awarding institution:
University of Bath

Link to publication

TRANSFECTION OF HUMAN OSTEOBLASTS WITH SV40 DNA: CHARACTERISATION OF IMMORTALISED CELL LINES.

Submitted by Meryl Marjory Williams

for the degree of Ph.D. of the University of Bath,

1992

COPYRIGHT

Meryl M. Williams

UMI Number: U602146

UMI U602146

ACKNOWLEDGEMENTS

CONTENTS

ABBREVIATIONS

1,25D	-	1,25-dihydroxyvitamin D_3
BOP	-	Bath Osteoblast Prototype
BRL	-	Buffalo Rat Liver
BSA	-	Bovine Serum Albumin
BSP	-	Bone sialoprotein
cAMP	-	Cyclic adenosine monophosphate
cDNA	-	Complementary DNA
CFU-GM	-	Colony forming unit, Granulocyte-Macrophage
CIF-A and B	-	Cartilage Inducing Factors A and B
DABCO	-	Diazo-bicyclo-octane
DEAE	-	Diethylamino ethyl
DEPC	-	Diethylpyrocarbonate
DIA	-	Differentiation Inhibitory Activity
DNA	-	Deoxyribonucleic acid
E_2	-	17 β oestradiol
FITC	-	Fluorescein isothiocyanate
HILDA	-	Human Interleukin for DA cells
HOBIT	-	Human osteoblast initial transfectant
hrIL-1	-	Human recombinbant Interleukin 1
IGF-I and II	-	Insulin like growth factors I and II
IL-6	-	Interleukin 6
IRMA	-	Immunoradiometric assay
LIF	-	Leukaemia Inhibitory Factor
LT	-	Lymphotoxin
NBT	-	Nitroblue tetrazolium
NRS	-	Normal rabbit serum
OAF	-	Osteoclast Activating Factor
PBS	-	Phosphate buffered saline
PDGF	-	Platelet Derived Growth Factor
PGE_2	-	Prostaglandin E_2
PMA	-	Phorbol Myristate Acetate
PTH	-	Parathyroid hormone
RA	-	Rheumatoid Arthritis
REF	-	Rat Embryo Fibroblasts
RER	-	Routh endoplasmic reticulum
RNA	-	Ribonucleic acid

ROS	-	Rat osteogenic sarcoma
SOD	-	Superoxide Dismutase
SV40	-	Simian Virus 40
TGF	-	Transforming Growth Factor
TNF	-	Tumour Necrosis Factor
TRAP	-	Tartrate resistant acid phosphatase

SUMMARY

There are a limited number of models available suitable for the study of adult human bone cell function. Primary cultures of human osteoblast-like cells derived from explants of trabecular bone display all the hallmarks of mature osteoblasts. The cells express the bone isoenzyme alkaline phosphatase and produce type I collagen and other noncollagenous matrix proteins. The most osteoblast-specific of these is osteocalcin, a matrix protein produced by osteoblasts in response to 1,25-dihydroxyvitamin D_3. Explant cultures consist of heterogeneous populations of cells and are slow growing making them difficult to obtain in large numbers.

Primary cultures of trabecular bone-derived cells were transfected with simian virus 40 DNA and a series of rapidly-growing cell lines were obtained. The cell lines were characterised for expression of alkaline phosphatase, production of bone matrix proteins osteocalcin, osteonectin, bone sialoprotein and thrombospondin, and expression of mRNA for type I collagen. The ability of the cells to produce a mineralised matrix was assessed by von Kossa and Alizerin Red staining.

Seven cell lines were further characterised for the expression of SV40 T antigens and for incorporation of the vector into host cell DNA. The ability of these cell lines to produce interleukin 1, interleukin 6 and tumour necrosis factor was assessed. The effects of oestrogen on cytokine production and on expression of mRNA for transforming growth factor β were monitored.

The cell lines closely resembled the parent cell phenotype with respect to expression of alkaline phosphatase, production of bone matrix proteins, and cytokine responsiveness. The phenotype was stable for fifteen passages and then declined; the cells entered crisis and eventually senesced. Two cell lines obtained by limiting dilution cloning may represent stable post-crisis lines. The cell lines may prove to be a useful new model for studying osteoblast function.

CHAPTER 1

INTRODUCTION

1.1 Bone as an organ

1.1.1 The characteristics and structure of bone.

The skeletal system of vertebrates is composed of two highly specialised connective tissues, namely bone and cartilage. Bone is a highly vascular, mineralised connective tissue remarkable for its hardness, resilience and regenerative capacity. Bone provides structural support for the body and serves to protect vital organs such as the central nervous system, heart and lungs. Cartilage covering the extremities of the bones provides a smooth articular surface permitting frictionless movement of the joints. Bone, in addition to its mechanical role, serves a metabolic function as a vital reserve of calcium and phosphate ions readily mobilised for the maintenance of mineral homeostasis.

Bone consists of an extracellular matrix synthesised and secreted by osteoblasts which are the bone forming cells. The matrix is composed of type I collagen and numerous other noncollagenous proteins. The extracellular matrix of bone, unlike other connective tissue matrices, is physiologically mineralised with crystals of a basic, carbonate-containing calcium phosphate called hydroxyapatite. Deposition of hydroxyapatite crystals occurs following the laying down of collagen fibers. Mineralisation of the extracellular matrix gives bone its characteristic hardness and strength.

1.1.2 Woven and lamellar bone

Three types of bone are identified according to their structure: these are woven bone, cortical bone, and cancellous or trabecular bone. Woven bone is the first type of bone to appear in the developing foetus. Here the collagen fibers are orientated randomly as irregular bundles and calcification occurs in disorganised patches. This is an immature form of bone and is resorbed and replaced by more organised lamellar bone during development. Woven bone may also appear during fracture healing and in

certain pathological conditions such as metabolic bone diseases and tumours.

Lamellar bone constitutes highly organised and mature bone. Collagen fibers are laid down in a preferential direction and the direction alternates from layer to layer forming lamellae. Lamellae are parallel if deposited on a flat surface such as the periosteum and trabeculae, and concentric on the inner surface of the channels through which blood vessels pass. Dense, cortical bone in transverse section is seen to consist of concentric rings of lamellar bone penetrated by blood vessels, forming the characteristic Haversian systems. As bone formation and calcification progress osteoblasts become embedded in the matrix forming osteocytes that lie within lacunae. The osteocytes have long cytoplasmic extensions which lie in channels or canaliculi. These extensions may serve to supply the osteocyte with nutrients from the blood or provide communication channels with osteoblasts on the surface of bone.

Trabecular bone has a less well defined Haversian system. Its structure is that of a honeycomb-like network of bone filaments enclosing spaces that are filled with haemopoietic bone marrow. The trabecular structure provides a vast surface area of bone available for remodeling. Bone is constantly in a dynamic state and is remodelled to provide calcium and phosphate ions essential to the maintenance of mineral homeostasis. Compact cortical bone provides support and because of its relatively slow turnover is probably less important in mineral homeostasis.

1.1.3   Intramembranous and endochondrial ossification.

In the developing embryo the flat bones (mandible, ileum, scapula, skull bones) develop by a process of intramembranous ossification. Cells of mesenchymal origin cluster together, divide, and differentiate into preosteoblasts and osteoblasts. These osteoblasts secrete a matrix of collagen, proteoglycans and glycoproteins which is subsequently

3

mineralised. This initial trabeculum, lined by osteoblasts, branches into other trabeculae by apposition. As trabeculae widen, osteoblasts are included in the matrix as osteocytes. The early woven bone is resorbed by osteoclasts and replaced by mature lamellar bone. Those trabeculae destined to form compact bone continue to expand, incorporating blood vessels into Haversian canals.

The long bones (femur, tibia, humerus) are formed principally by a process of endochondral ossification which involves partial replacement of a cartilagenous model by bone. Clusters of primitive mesenchymal cells divide and differentiate into prechondroblasts and then chondroblasts. These cells produce a cartilagenous matrix composed of type II collagen and mucopolysaccharides. At the external surface (the perichondrium) mesenchymal cells continue to proliferate and differentiate into osteoblasts. Woven bone is formed and calcified and is penetrated by blood vessels and bone resorbing osteoclasts. Chondroblasts that have become embedded in the cartilagenous matrix are termed chondrocytes, they lie within lacunae but are still able to proliferate. New matrix is synthesised interstitially between chondrocytes and ossification occurs transforming cartilage to bone. Intramembranous ossification, not preceded by cartilage, occurs in the periosteal region of the diaphysis of long bones and on the walls of the Haversian canals that carry blood vessels.

1.1.4 Growth of the long bone

A typical long bone consists of a central cylindrical core (diaphysis) of dense and compact cortical bone enclosing the medullary cavity which is filled with haemopoietic bone marrow. The dense cortical bone of the diaphysis is thinner approaching the two wider extremities (epiphysis) and here the internal space is filled with trabecular bone. The diaphysis and epiphysis are linked by an intermediate zone, the metaphysis. The

metaphysis and epiphysis are separated in growing long bones by a layer of cartilage making up the growth plate. The cartilagenous growth plate continues to proliferate, allowing length increases of the bones, and is only replaced when the growth of the bone ceases. As longitudinal growth occurs the epiphysis is resorbed externally to form the diaphysis which grows by new bone formation at the external surface and by resorption to expand the internal cavity. In this way, resorption and formation activities maintain the shape of the long bone during growth (Figure 1.1). The anatomy, ultrastructure, and development of bone are reviewed by Baron (1990) and Teitelbaum (1990).

1.2   The proteins of bone matrix.

The major constituent of connective tissue extracellular matrix is collagen. Bone differs from other connective tissues in its ability to become mineralised under physiological conditions. The organic matrix of bone contains many noncollagenous proteins which may function as instigators and/or regulators of the mineralisation process.

1.2.1 Collagen

Collagen is the most abundant component of bone matrix, comprising 85-90% of total bone protein. This protein has a high tensile strength and gives strength to various tissues and organs. More than ten types of collagen have been identified so far but the collagen of bone is almost exclusively type I. The type I collagen molecule is a supercoiled triple helix composed of two identical α_1 (I) chains and one α_2 (I) chain which has a slightly different amino acid composition. The collagen chains have a unique repeating sequence of three amino acids -Gly-X-Y- where X and Y are often proline or its hydroxylated derivative, hydroxyproline.

The three constituent α chains of collagen are synthesised simultaneously and after translation hydroxylation of certain proline and lysine residues

5
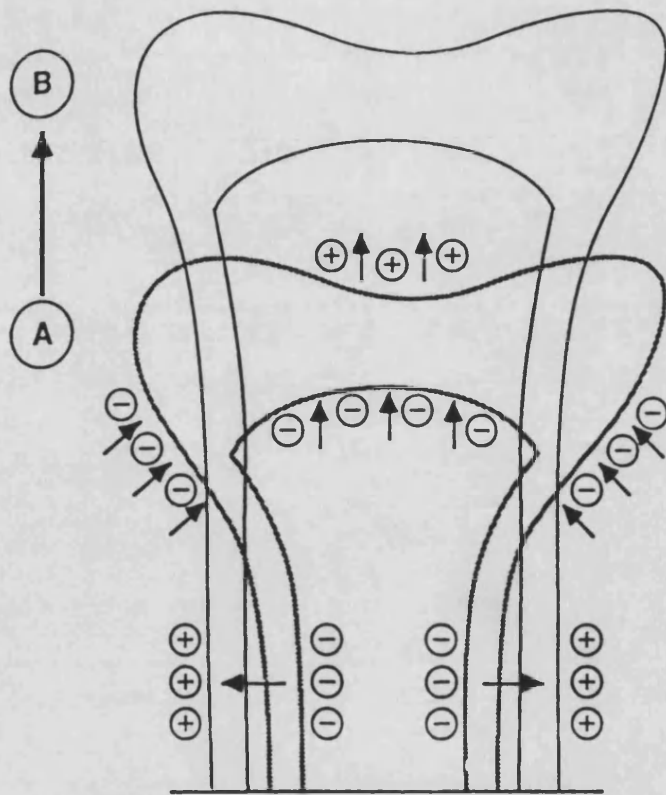
Fig. 1.1   Resorption (-) and formation (+) activities during the longitudinal
growth of bones: During the growth from A to B, the cortex in the diaphysis
must be resorbed inside and reformed outside. The wider parts of the bone
must be reshaped into a diaphysis (from Baron, 1990).

occurs. Hydrogen bonds are formed between hydroxyl groups stabilising the super helix. Collagen is secreted as a precursor molecule procollagen which has non-helical terminal peptides. These telopeptide extensions are cleaved following secretion of the molecule, leaving short non-helical regions of 15-30 amino acids at each terminal of the collagen molecule. When the triple α-chain molecules aggregate to form fibers, strong covalent bonds are formed between the non-helical terminal portions of the α chains and the helical region of an adjacent molecule. The cross-links are formed by oxidative deamination of specific lysine or hydroxylysine residues in the non-helical N- and C- telopeptides by a copper-dependent amino-oxidase enzyme, lysyl oxidase.

In bone, cartilage, tendon, and most other connective tissues except skin, two 3-hydroxypyridinium amino acids, each linking three collagen molecules have been identified (Eyre and Oguchi, 1980). Hydroxylysylpyridinoline is present as the major component in all these tissues. Lysylpyridinoline is present at relatively high concentrations in bone and dentine, and its presence in urine has been used as an indicator of bone resorption in patients with rheumatoid arthritis and osteoarthritis (Robins *et al*, 1986), and osteoporosis (Delmas *et al*, 1991).

In mature, lamellar bone, collagen fibers are arranged in parallel layers and provide the substratum for deposition of hydroxyapatite crystals and calcification of the extracellular matrix. Transmission electron micrographs of individual, mineralised collagen fibrils show that hydroxyapatite crystals are located mainly within the fibrils at the level of the gap regions (Weiner and Traub, 1986).

1.2.2 Noncollagenous proteins of bone

a). Osteocalcin

Price *et al* (1976) and Hauschka, Lian and Gallop (1975) independently described a protein extracted from bone matrix which contained three residues of the calcium-binding amino acid γ-carboxyglutamate (Gla). Sequence analysis of the amino-terminal residues established that the novel protein was not a fragment of Gla-containing blood clotting factors. The protein was demonstrated in bovine dentine, human tibia and swordfish vertebrae, but not in tooth enamel. Bone Gla protein (osteocalcin) has a molecular weight of 5300 daltons and a single disulphide bond; it binds strongly and reversibly to hydroxyapatite but fails to bind collagen or amorphous calcium phosphate.

Osteocalcin is synthesised in a precursor form; cleavage of a 23-residue transmembrane signal peptide yields a 10kDa pre-pro-osteocalcin molecule which is post-translationally modified by the vitamin K-dependent and CO^{2-} requiring carboxylation of glutamic acid residues (Lian and Friedman, 1978). Osteocalcin is a potent inhibitor of hydroxyapatite crystal growth *in vitro* at physiological pH and ionic strength, the inhibitory activity is decreased by thermal decarboxylation of Gla residues (Romberg *et al*, 1986). Rats treated with the vitamin K antagonist warfarin, which reduces bone levels of osteocalcin to less than 2% of normal, displayed an excessive mineralisation disorder characterised by a loss of longitudinal growth and a densely calcified growth plate (Price *et al*, 1982). No other skeletal defects were observed suggesting that osteocalcin may serve an *in vivo* role as a regulator of mineralisation by inhibiting crystal growth.

Northern analysis of extracted mRNA and immunocytochemical studies demonstrate that osteocalcin is mineralised tissue specific and occurs in young osteoblasts and odontoblasts (Bronckers *et al*, 1985; 1987). Several osteosarcoma cell lines and non-transformed cultures of osteoblasts

8

synthesise osteocalcin and its production is transcriptionally regulated by 1,25-dihydroxyvitamin D_3 (1,25D) (Price and Baukol, 1980; Beresford *et al*, 1984). In addition, osteocalcin has been shown to be chemotactic for two rat osteoblastic osteosarcoma cell lines, ROS and UMR (Lucas, Price and Caplan, 1988). Osteocalcin is bound exclusively to mineral and its release during demineralisation may serve to recruit osteoblast precursors, thus initiating a new round of bone formation.

A small fraction of bone osteocalcin is present in the circulation and measurement of serum levels provides a potentially useful indicator of bone formation. Serum osteocalcin values are highly correlated with histomorphometric measurements of bone formation in primary and secondary hyperparathyroidism and postmenopausal osteoporosis (Gundberg-Carpenter and Lian, 1989).

b).   Thrombospondin

Thrombospondin is a trimeric molecule, with a molecular weight of 450kDa, composed of inter and intramolecular disulphide linked monomeric subunits of 150kDa. It is the most abundant protein of platelet α granules and is secreted when platelets are stimulated with thrombin. The protein is a biosynthetic product of a variety of connective tissue cells. More recently, thrombospondin has been identified as a secretory product of human osteoblast-like cells and as a constituent of bone matrix (Gehron Robey *et al*, 1989). The molecule has a stalk region containing a collagen binding site and a carboxy globular domain with a Ca^{2+} binding region.

Thrombospondin is widely distributed in a number of tissues and in general is present during stages of cellular proliferation, migration, and intercellular adhesion. Thrombospondin mediates bone cell attachment, but not spreading *in vitro* (Gehron Robey *et al*, 1989). Proposed functions for this molecule include the organisation of extracellular matrix components and action as an autocrine growth factor.

c). <u>Osteopontin</u>

Osteopontin is an acidic, phosphorylated glycoprotein of 41,500 daltons, rich in aspartic acid, glutamic acid and serine. A study of the tissue specificity of rat osteopontin mRNA demonstrated abundant expression in bone and a high expression in kidney. Much lower levels were observed in brain and lung, and no osteopontin mRNA was detected in heart, intestine, liver, muscle, skin, spleen, cartilage or testes (Yoon, Buenaga and Rodan, 1987). Immunohistochemical studies demonstrate the synthesis of osteopontin by preosteoblasts, osteoblasts and osteocytes, and its localisation in osteoid. Osteopontin is also synthesised by sensory and neural cells of the inner ear, by odontoblasts and hypertrophic cartilage. Strong immunostaining during the early stages of bone formation, prior to the deposition of mineral, was observed in developmental studies of rat foetuses. These findings are reviewed by Butler (1989). 1,25D and transforming growth factor β (TGFβ) increase osteopontin production by ROS 17/2.8 cells and TGFβ increases its production in the murine clonal osteoblast-like cell line MC3T3-E1 (Noda et al, 1988). However, parathyroid hormone (PTH) suppresses osteopontin production and the level of mRNA in ROS 17/2.8 (Noda and Rodan, 1989).

Complementary DNA for osteopontin predicts an amino acid sequence of Gly-Arg-Gly-Asp-Ser which is identical to a cell binding sequence in fibronectin. This sequence has been shown to play a role in cell attachment by binding to cell surface integrins. Oldberg, Franzen and Heinegard (1986) have shown that osteopontin promotes attachment and spreading of ROS 17/2.8 cells and this attachment was specifically inhibited by an RGD-containing peptide. Immunolocalisation studies by Reinholt et al (1990) demonstrated the presence of osteopontin at the clear zone of the osteoclast plasma membrane, much less staining was observed at the ruffled border and deeper within the bone. Highest expression of the vitronectin receptor

was also observed at the clear zone, suggesting that osteopontin may function as an anchor of osteoclasts to bone by binding to the vitronectin receptor and to hydroxyapatite.

d).   Osteonectin

Osteonectin is the most abundant noncollagenous protein present in bone matrix. The molecule is an acidic glycoprotein of molecular weight 32kDa, it is rich in cysteine residues and contains several intramolecular disulphide bonds. The N terminal region is acidic with a clustering of negative charges and is a potential binding site for hydroxyapatite. The predicted amino acid sequence, determined by analysis of cDNA clones from several species, shows greater than 90% homology.

Osteonectin is secreted by a variety of connective tissue cell types and is present in skin and tendon. However, levels in bone are 1-10,000 times higher than in other connective tissues. Northern analysis of RNA extracted from tissues shows expression of osteonectin in bone, periodontal ligament and skin. Immunohistochemical studies demonstrate localisation of the protein to preosteoblasts, osteoblasts, newly deposited osteoid mineralised matrix and osteocytes (Young *et al,* 1990). Osteonectin is also expressed by cells of the expanding decidua of the placenta, indicating the predominance of the protein in tissues which are undergoing active remodelling.

Osteonectin is present in the α granules of platelets and is released as a complex with thombospondin during platelet aggregation. Platelet and bone osteonectin are structurally and immunologically different (Malaval *et al,* 1991). The precise function of platelet osteonectin is not known but Clezardin *et al* (1991) have demonstrated that antiosteonectin F(ab')$_2$ fragments inhibit collagen-induced platelet aggregation in a dose-dependent manner. These authors postulate a role for osteonectin in the maintenance of platelet aggregation. Osteonectin is a potent inhibitor of crystal growth *in vitro* and was five times more potent than osteocalcin in

this respect (Romberg *et al*, 1986). Osteonectin avidly binds collagen and has been reported to promote the deposition of calcium phosphate onto type I collagen *in vitro* and may play a role in mineralisation (Termine *et al*, 1981).

e).   Bone sialoprotein

Bone sialoprotein (BSP) is a glycoprotein of molecular weight 70,000-80,000Da and consists of 50% protein, 12% sialic acid, 7% glucosamine and 6% galactosamine. BSP is synthesised as a pre-protein of 317 amino acids (34,982Da) which is processed to a 33,352Da protein before being made into the highly modified glycoprotein. The molecule contains no cysteine residues or disulphide bonds (Fisher *et al*, 1990).

In situ hybridisation of mRNA and immunostaining showed expression of BSP in osteoblasts, osteocytes and in some hypertrophic cartilage in developing human long bones and calvariae. A few osteoclasts also expressed the protein and BSP was localised to bone matrix (Bianco *et al*, 1989). Like osteopontin, BSP has cell attachment activity and possesses an RGD seqence, however osteopontin is much more active at promoting cell attachment at lower concentrations (Somerman, Fisher and Foster, 1988). The presence of BSP has been demonstrated in platelets and the protein is released from thrombin-activated platelets (Chenu and Delmas, 1992). However, patients with gray platelet syndrome - a disorder characterised by a deficiency of platelet α granules - also demonstrated a release of BSP. Unlike osteonectin and thrombospondin which are released from the α granules, BSP would appear to be endocytosed from plasma by megakaryocytes. The levels of BSP in serum may reflect changes in bone remodelling.

f).   Proteoglycans

Proteoglycans I (biglycan) and II (decorin) make up around 10% of the noncollagenous proteins present in bone matrix. These molecules possess

a protein core to which there is covalent attachment of long chains of repeated disaccharide units, termed glycosaminoglycans. Proteoglycan I (Mr 350,000) contains two chondroitin sulphate chains and proteoglycan II (Mr 200,000) contains one chondroitin sulphate chain. N terminal sequence analysis revealed that the two are homologous yet distinct (Fisher *et al*, 1987). Proteoglycans are synthesised by human osteoblast-like cells *in vitro* (Beresford *et al*, 1987), but are not unique to bone. They are found in articular cartilage, nasal cartilage, skin, tendon, sclera, cornea, and aorta. In soft connective tissues they exist as dermatan sulphate proteoglycans, whereas in bone they bear chondroitin sulphate. In bone, biglycan and decorin are immunolocalised to matrix and cells in areas of new bone formation (Gehron Robey, 1989).

1.3 The maintenance of mineral homeostasis.

The adult human body contains approximately 1100g of calcium of which 99% is stored in the skeleton in the form of hydroxyapatite which has the chemical formula $Ca_{10}(PO_4)_6(OH)_2$. 40% of total serum calcium (normally 2.5 mmol/L) is bound to protein, principally to albumin, and 10% is complexed with diffusible anions. 50% of serum calcium is ionised and maintenance of this fraction in the body fluids is essential for proper bone mineralisation, membrane integrity, cellular biochemical reactions, blood coagulation, muscle contraction, and nerve function. Phosphorous is essential for metabolic reactions that require energy which is supplied by adenosine triphosphate (ATP). Total body phosphorous is 500-800g and 85-90% is found in the skeleton.

The hormones, vitamin D_3 and PTH are the principal regulators of calcium metabolism. Vitamin D_3 (cholecalciferol) is formed by ultraviolet radiation of the precursor molecule 7-dehydrocholesterol which is present in skin. Vitamin D_3 is metabolised in the liver by hydroxylation at carbon 25 to yield

25-hydroxyvitamin D_3, the major circulating form of vitamin D_3 with a serum concentration of 25ng/ml and a half life of 15 days. Final metabolic conversion occurs in the kidney where hydroxylation at carbon 1 yields 1,25-dihydroxyvitamin D_3. 1,25D has a circulating half-life of 15h and a plasma concentration of 20-50pg/ml. PTH is synthesised by chief cells of the parathyroid gland.

The regulation of calcium homeostasis is depicted in figure 1.2. Calcium ingested in the diet is actively transported out of the intestine and some absorption also occurs by passive diffusion. Active transport of both Ca^{2+} and PO_4^{3-} is facilitated by 1,25D, the active metabolite of vitamin D_3. The intestine is the principal target organ of 1,25D although the hormone also facilitates reabsorption of Ca^{2+} in the kidney. The formation of 1,25D is regulated in a feedback fashion by plasma Ca^{2+} and PO_4^{3-}. High plasma PO_4^{3-} levels directly inhibit renal 1α-hydroxylase and levels of 1,25D fall in the presence of increased plasma calcium. When plasma Ca^{2+} is low, secretion of PTH from the parathyroid gland is increased. PTH increases the formation of 1,25D which in turn increases release of Ca^{2+} and PO_4^{3-} from bone, restoring the plasma levels. PTH also mobilises calcium from bone, increases bone resorption, increases Ca^{2+} reabsorption in the kidney, and increases the excretion of phosphate ions.

1.4. The cells of bone

1.4.1 Lining cells.

In the adult, bone lining cells occupy the majority of bone surfaces considered inactive or resting because they are not being remodelled. Lining cells are flattened cells immediately apposed to the bone surface and they generally contain few intracellular organelles. The role of lining cells is unknown but they may serve as nutritional support cells for osteocytes via gap junctions between cell processes (Stanka,1975).
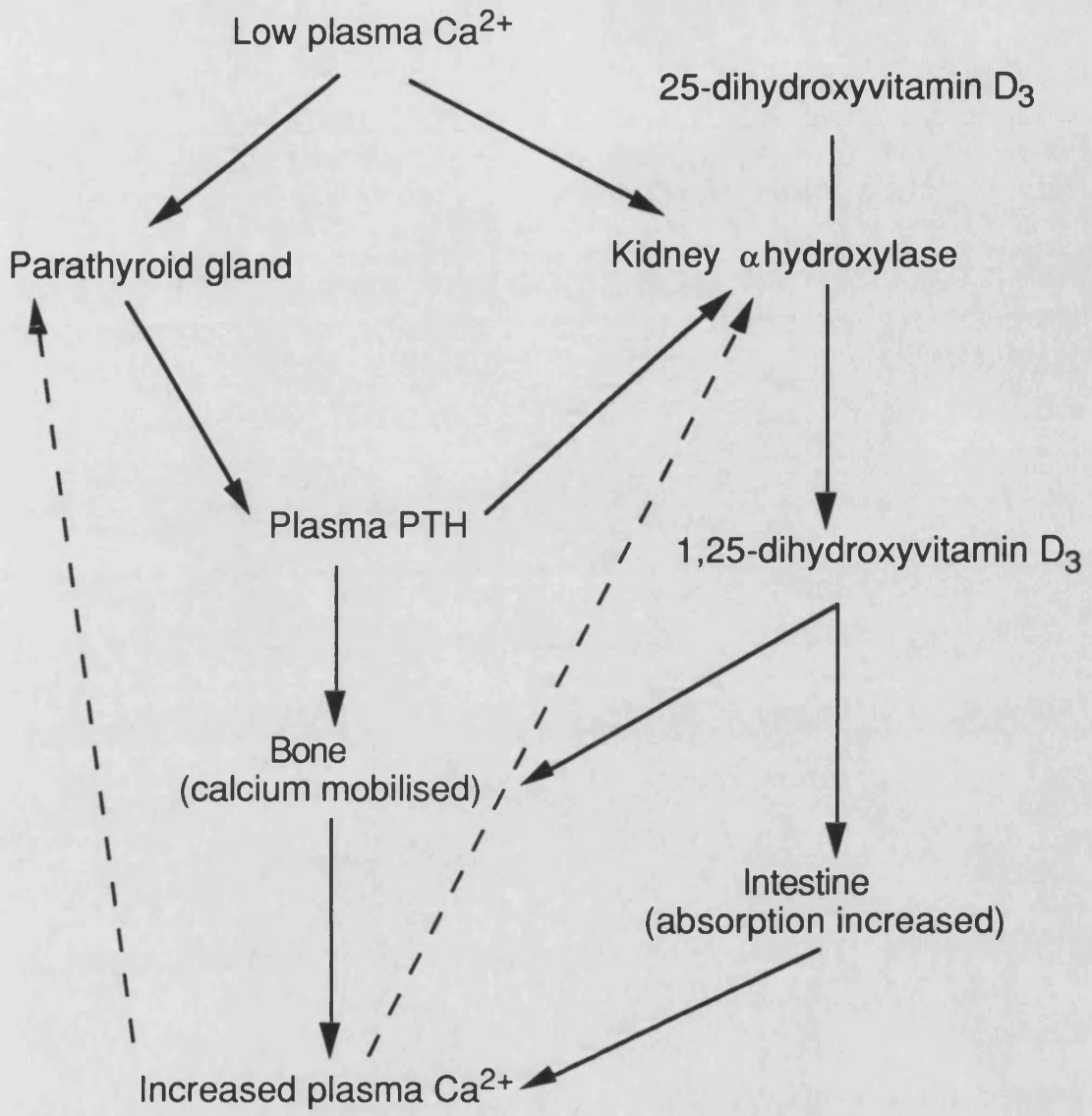
Fig. 1.2   The control of calcium homeostasis by parathyroid hormone and 1,25-dihydroxyvitamin D_3. PTH, secreted in response to low plasma calcium, increases Ca^{2+} reabsorption in the kidney, stimulates bone resorption, and enhances the activity of kidney α hydroxylase. 1,25-dihydroxyvitamin D_3 increases intestinal absorption of Ca^{2+} and also stimulates release of calcium from bone. A dashed line indicates negative feedback control.

1.4.2 Osteoblasts

The osteoblast is the bone-forming cell, it produces and secretes collagen and other noncollagenous proteins providing an organic matrix which is subsequently calcified. In histological sections of bone the osteoblast is recognised by its cuboidal shape and its position directly apposed to the bone surface. The osteoblast is a highly metabolically active cell and this is reflected in its ultrastructure. There is an extensive network of rough endoplasmic reticulum (RER) clustered in the cytosol nearest the bone surface. Between the nucleus and the RER is the Golgi apparatus. This structure is present because of the large amount of type I collagen synthesised and secreted by the cells. The cells also contain numerous mitochondria. At the bone surface, cytoplasmic processes extend from the osteoblast through the osteoid. These processes may serve as a communication link with osteocytes embedded in the matrix. The osteoblast is rich in the enzyme alkaline phosphatase. The precise function of this enzyme is not known. In the genetic disorder hypophosphatasia a deficiency of this enzyme occurs resulting in severe osteomalacia demonstrating that alkaline phosphatase is essential for skeletal mineralisation (Whyte *et al*, 1979).

Osteoblasts that are actively engaged in laying down matrix first synthesise type I collagen. The collagen matrix is subsequently 'decorated' with the noncollagenous proteins of bone. Mineralisation of the osteoid is also under the control of the osteoblast although the precise mechanism is not understood. Mineralisation of collagen occurs at the calcification front, the interface between osteoid and calcified bone. Hydroxyapatite crystals form in association with matrix fibrils that are collagen - noncollagenous protein complexes. More mineral appears at the gap regions that occur between collagen fibers.

In addition to its bone forming activities, the osteoblast plays a major role in regulating osteoclastic bone resorption. The osteoblast possesses surface receptors for parathyroid hormone and nuclear receptors for 1,25D whereas the osteoclast does not (Kream *et al*, 1977). These two hormones are potent stimulators of bone resorption and their effects on resorption may be mediated by the osteoblast (McSheehy and Chambers, 1986; 1987). Osteoblasts also possess receptors for interleukin 1 (Bird and Saklatvala, 1986), and oestrogen receptors (Eriksen *et al*, 1987).

1.4.3 Osteocytes

Osteoblasts that become embedded in the calcified extracellular matrix during bone formation are osteocytes, the end cells of the osteogenic lineage. Osteocytes have long cell processes which are in contact with previously incorporated osteocytes and osteoblasts at the surface of bone (Stanka, 1975; and Nijweide, van der Plas and Scherft, 1981), the cytoplasmic process lie in canals or canaliculi. The osteocyte lies within a lacuna filled with extracellular fluid. Deeper into the bone the osteocytes become smaller and lose many of their cytoplasmic organelles. Osteocytes do not divide but may secrete matrix at the surface of the lacuna, followed by calcification.

1.4.4 The osteoblast lineage

Marrow stromal tissue is a network of cells and extracellular matrix which physically supports the haemopoietic cells and influences their differentiation. When marrow fragments or marrow cell suspensions are implanted into diffusion chambers *in vivo* an osteogenic tissue of bone and cartilage is formed (Rosin, Freiburg and Zajicek, 1963; Friedenstein, Piatetzky-Shapiro and Petrakova, 1966). Cultures of marrow stromal fibroblasts are readily established *in vitro* from single-cell bone marrow suspensions and colonies derived from a single initiating colony-forming unit (CFU-F) can be obtained. CFU-F is capable of proliferating and

differentiating into all the stromal cell lines necessary for the formation of bone and a haemopoietic microenvironment (reviewed by Owen, 1988; Beresford, 1989). CFU-F give rise to fibroblastic, adipocytic, reticular and osteogenic cell lines. As certain CFU-F are capable of forming a bone and marrow organ when grafted under the renal capsule *in vivo,* whereas some colonies produce bone only, it has been postulated that CFU-F contains both stromal stem cells and committed osteogenic precursors. The stages of osteoblastic differentiation, from a committed progenitor to the mature osteoblast, have not yet been elucidated owing to a lack of suitable biochemical or cell-surface markers.

1.4.5 Osteoclasts

The osteoclast is a highly differentiated, specialised, multinucleate cell that has the capacity to resorb bone. Osteoclasts are not observed on quiescent bone surfaces but seen at sites of actively remodeling bone, at the metaphysis of growing bones, or in pathological states. Such a distribution implies that osteoclasts are formed from precursors only as and when required. The regulation and mechanisms of osteoclast formation are poorly understood but local factors produced by osteoblasts can stimulate both the proliferation and differentiation of osteoclast progenitors.

The osteoclast is a giant cell formed by fusion of precursors. Osteoclasts contain around 10-20 nuclei which are centrally placed and contain 1-2 nucleoli. An actively resorbing osteoclast contains many primary lysosomes, numerous mitochondria, a RER where lysosomal enzymes are synthesised, and prominant Golgi stacks. Osteoclasts are rich in the enzyme tartrate-resistant acid phosphatase (TRAP). Elevated plasma levels of TRAP are associated with increased bone turnover in metabolic bone diseases. Inhibition of TRAP activity by a specific antibody, or the specific phosphatase inhibitor molybdate, causes an inhibition of bone resorption (Zaidi *et al,* 1989). TRAP is a pyrophosphatase and pyrophosphate is a potent inhibitor

of apatite solubilisation, the first step in bone resorption. TRAP may serve to remove pyrophosphate in the early stages of resorption.

At the interface of the osteoclast and the bone the resorbing cell has a ruffled border where the plasma membrane has numerous folds and invaginations allowing intimate contact with the bone surface. The ruffled border is entirely surrounded by an annular zone rich in contractile proteins. This so-called sealing zone, where the plasma membrane is closely apposed to the bone surface, mediates attachment of the osteoclast to the bone matrix and seals off a resorption compartment. Resorption occurs at the ruffled border forming a pit or Howship's lacuna.

Bone is resorbed by the action of proteolytic enzymes which are transported via primary lysosomes and are secreted into the resorption lacuna. Baron *et al* (1985) have demonstrated that the plasma membrane of the ruffled border expresses a 100kDa lysosomal membrane protein. No expression of this protein was observed at the plasma membrane of the sealing zone or facing the bone marrow compartment. The 100kD protein is characteristic of the limiting membrane of secondary lysosomes and its distribution at the ruffled border suggests that the resorption lacuna functions as a secondary lysosome. The osteoclast is rich in the enzyme carbonic anhydrase II, an enzyme associated with acid-secreting epithelia, which generates hydrogen ions. H^+ ions are pumped across the membrane of the ruffled border by a proton pump identical to the kidney H^+ ATPase (Blair *et al*, 1989). The acidic microenvironment so formed is sufficient to dissolve the mineral phase of bone matrix and provides optimal conditions for the degradative action of acid hydrolases.

A further mechanism whereby osteoclasts may resorb bone is via the production of oxygen-derived free radicals. Generation of free radicals by injection of xanthine/xanthine oxidase over the calvariae of 5 week old mice caused bone resorption, characterised by an increase in osteoclast numbers

and activity, which was inhibitable by superoxide dismutase (SOD) (Garrett *et al*, 1990). PTH and interleukin 1-induced resorption was also inhibited by SOD but not by catalase, indicating that peroxide anions and not hydroxyl anions were responsible for the resorptive effect. The generation of free radicals by osteoclasts was visualised with the dye nitroblue tetrazolium (NBT) and calcitonin decreased the number of NBT positive osteoclasts. The generation of oxygen-derived free radicals by activated phagocytes and osteoclasts may be responsible for the bone resorption and tissue damage that occurs in chronic inflammatory conditions such as rheumatoid arthritis.

Gottesburen and co-workers, using polyclonal antibodies to bone matrix proteins, have shown the presence of osteocalcin, decorin and biglycan within cytoplasmic vesicles of the osteoclast (Gottesburen *et al*, 1991). In addition, workers in this laboratory raising monoclonal antibodies to primary cultures of human osteoblasts have demonstrated antibodies that bind to the cytoplasm of osteoclasts (S. Walsh, unpublished observations). These studies would suggest that as bone is resorbed matrix particles are engulfed by the osteoclast.

Osteoblasts *in vitro* have been shown to secrete plasminogen activator which can activate collagenase (Hamilton *et al*, 1984). It has been suggested that osteoblasts prepare the surface of the bone for osteoclastic resorption by degrading the non-mineralised layer of collagen and thereby exposing the mineralised matrix for attack by the osteoclast (Chambers, 1988). However, scanning EM studies show that osteoclasts do not require preparation by osteoblasts for resorption to occur and isolated osteoclasts can resorb bone without the apparent support of other cells (Boyde *et al*, 1986).

1.4.6   The osteoclast lineage

Osteoclasts are thought to be derived from haemopoietic precursors in the bone marrow (Mundy, 1990). Evidence suggests that the osteoclast is

derived from a colony forming unit for the granulocyte-macrophage series (CFU-GM), which can give rise to osteoclast precursors, monocytes and granulocytes. Mononuclear precursors, which proliferate, differentiate, and fuse to form the mature osteoclasts, respond to colony stimulating factors which act only on cells derived from CFU-GM (Mundy, 1990). In addition, monocytes and macrophages have been shown to degrade devitalised bone (Mundy *et al*, 1977). However, other workers report that osteoclasts do not express common leucocyte antigen or monocyte markers (Horton *et al*, 1985). The true ontogeny of the osteoclast is as yet uncharacterised.

1.5   The bone remodeling cycle

Throughout life the skeleton is constantly remodelled disynchronously at numerous sites. Bone remodelling maintains mineral homeostasis and serves to resorb effete bone replacing it with new matrix (Parfitt, 1984). The activities of osteoblasts and osteoclasts are tightly coupled and since bone remodelling occurs regardless of the endocrine status of the individual it is likely that locally produced growth factors play the major role in this coupling process. The process of bone remodelling can be described as a series of events (Baron, Vignery and Horowitz, 1983), depicted in figure 1.3.

Bone that is not being actively remodelled is in a state of quiescence. Osteoclasts are not seen and the cells lining the surface of the bone are flattened and apparently in a resting state. The first stage of the cycle is activation; mononuclear cells are recruited to the bone surface and osteoblasts that were lining the bone surface stop synthesising matrix and become flattened. The activated mononuclear cells reach the bone surface by sending long pseudopods between the now flat lining cells. The plasma membranes of the mononuclear cells become ruffled along the bone surface. The cells fuse to form multinucleate giant cells with ruffled borders and tight sealing zones, and begin to resorb bone.

Bone resorption involves the removal of both mineral and organic constituents of bone and occurs in areas where osteoclasts are attached to the bone surface. During the resorptive phase osteoclasts resorb a quantum of bone, forming a Howship's lacuna.

Reversal follows the resorption phase. The osteoclasts are replaced by large phagocytic cells that may be fission products of the osteoclasts. During reversal a "cement line" is laid down which contains glycoproteins, glycosaminoglycans and acid phosphatase. The cement layer components may be chemoattractants for osteoblasts which colonise the Howship's lacuna. Mundy *et al* (1982) have demonstrated that conditioned media from resorbing bone cultures are chemotactic for osteoblast-like osteosarcoma cells. Factors released during resorption may thus recruit osteoblast precursors to the remodelling site. Reversal is followed by a new round of bone formation; osteoblasts lay down new osteoid which matures and is subsequently mineralised.
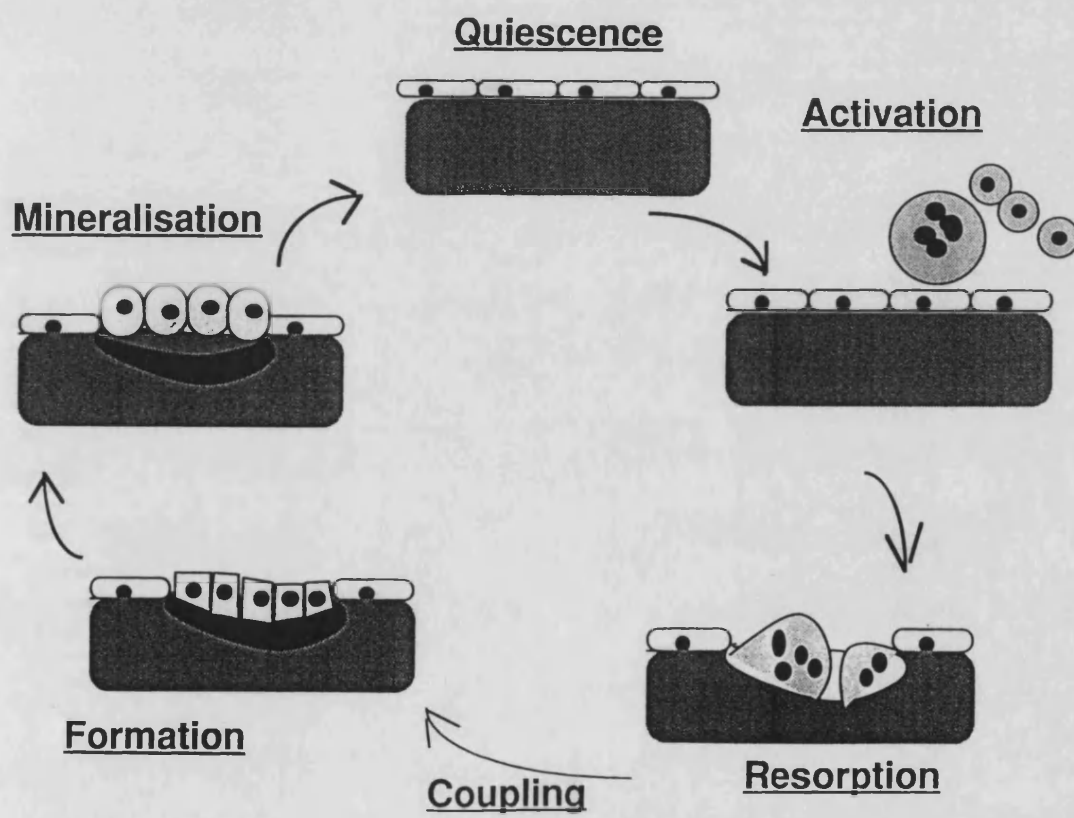
Fig.1.3   The cellular events of the bone remodeling cycle.

1.6   Systemic and local factors affecting bone remodeling.

In the bone microenvironment bone cells are in intimate association with both neighbouring cells and the osteoid matrix; signals from a whole plethora of systemic hormones, local factors, cytokines and adhesion molecules regulate the resorption of effete bone and its subsequent renewal. The characteristics and actions of some of the major factors are described below. The effects of cytokines interleukin 1, interleukin 6 and tumour necrosis factor α on human osteoblast-like cells are more fully described in Chapter 5 of this thesis.

1.6.1 Systemic factors

a).   Parathyroid hormone

PTH is an 84 amino acid peptide synthesised by chief cells of the parathyroid gland. The hormone is synthesised as part of a larger precursor molecule containing a 25 amino acid hydrophobic residue-rich signal sequence and a 6 amino acid prosequence. These portions of the precursor are sequentially removed within the cell and 84 amino acid PTH is released from storage granules. After secretion PTH is cleaved between residues 33 and 34. The amino terminal fragment has the full biological activity of the intact molecule, the carboxy-terminal fragment is inactive. The synthesis and secretion of PTH are regulated by serum Ca^{2+} and PTH is essential to the maintenance of serum calcium concentrations. Bone and kidney are the two principal target organs of PTH. Cell surface receptors for PTH are coupled to adenyl cyclase and cAMP is the intracellular mediator of PTH action.

PTH stimulates bone resorption in organ cultures and increases the number and activity of osteoclasts (Holtrop and Raisz, 1979). McSheehy and Chambers (1986) report that PTH had little effect on isolated osteoclasts, the bone resorptive effect of PTH required the presence of accessory cells such as osteoblasts. PTH stimulates the fusion of osteoclast

precursors but as this can occur in the presence of DNA synthesis inhibitors this effect is apparently not dependent on proliferation of the precursors (Lorenzo, Raisz and Hock, 1983).

PTH stimulates the production of cAMP in osteoblasts, however cyclic nucleotide analogues which block the PTH stimulation of cAMP do not inhibit bone resorption. In addition, PTH analogues which have little effect on cAMP can stimulate bone resorption suggesting that other messengers are involved (Raisz, 1988a). PTH has also been reported to have anabolic effects in bone; Tam *et al* (1982) report that PTH stimulates the bone apposition rate in thyroparathyroidectomised rats. Intermittent doses of PTH stimulated the rate of mineralised matrix production at individual formation loci. Endo *et al* (1980) observed an enhancement of calcification of osteoid matrix in chick embryonic femur treated with PTH and 3 vitamin D_3 metabolites in organ cultures *in vitro*. Conversely, PTH inhibits collagen synthesis in the rat osteosarcoma cell line ROS 17/2. The anabolic effects of PTH may be due to an increase in osteoblast cell number as the hormone stimulates proliferation of human trabecular bone cells (MacDonald *et al*, 1984).

b).   1,25-dihydroxyvitamin D_3

1,25D binds with high specificity and affinity to an intracellular receptor protein present in bone (Kream *et al*, 1977). 1,25D is a potent stimulator of bone resorption (Raisz *et al*, 1972) and stimulates formation and activity of multinucleate giant cells in long term human bone marrow cultures (Roodman *et al*, 1985). 1,25D stimulated the alkaline phosphatase (ALP) activity of rat osteogenic sarcoma cells (ROS 17/2) in a dose dependent manner and inhibited cell growth (Manolagos, Burton and Deftos, 1981). The hormone also enhanced ALP activity in two human osteogenic osteosarcoma cell lines, SAOS and TE85 (Mulkins *et al*, 1982) and

enhanced ALP activity and inhibited growth in human trabecular bone derived cells (Beresford, Gallagher and Russell, 1986).

1,25D has been reported to specifically enhance synthesis of type I collagen in the human osteosarcoma cell line MG-63 (Franceschi, Romano and Park, 1988) and in human osteoblast-like cells (Beresford, Gallagher and Russell, 1986). However, 1,25D inhibited collagen synthesis in ROS 17/2 cells (Kream *et al*, 1986). 1,25D stimulates the production of osteocalcin by human osteoblast-like cells (Beresford *et al*, 1984) and regulates the production of other bone matrix proteins (Gehron Robey *et al*, 1986).

Vitamin D$_3$ may also influence cells of the immune system. Monocytes and activated T cells possess receptors for 1,25D (Peacock *et al*, 1982). 1,25D inhibits T cell proliferation and production of IL-2 (Rigby, Stacy and Fanger, 1984), and can enhance expression of interleukin 1 (IL-1) in monocytes (Bhalla, Amento and Krane, 1986). Activated macrophages from the synovial fluid of patients with inflammatory arthritis can convert 25-hydroxyvitamin D$_3$ (25-(OH)D) to the active metabolite 1,25D, and these patients respond to a 25-(OH)D challenge with an increase in serum concentration of 1,25D (Mawer *et al*, 1991). Mononuclear cells at the surface of bone may respond to 1,25D by enhanced production of interleukin 1 (IL-1), thus contributing to local bone remodelling. Extrarenal production of 1,25D in pathological conditions such as arthritis may contribute to bone loss by directly enhancing bone resorption and via stimulation of the potent resorbing agent IL-1.

c).   Calcitonin

Calcitonin is a 32 amino acid polypeptide synthesised by neuroendocrine cells located within the thyroid gland. The hormone is synthesised as a portion of a larger precursor protein. Circulating levels of calcitonin are <100pg/ml and its half-life is 10 minutes. The synthesis and secretion of

calcitonin are controlled by the concentration of serum ionised Ca^{2+}. An increase in serum Ca^{2+} suppresses PTH secretion and the synthesis of calcitonin is increased. Calcitonin decreases bone resorption by inhibiting osteoclast function, resulting in lowered serum Ca^{2+} and PO_4^{2-} concentrations. Calcitonin increases urinary excretion of Ca^{2+}, PO_4^{2-}, K^+, Na^{2+}, and Mg^{2+} but this effect persists only as calcitonin concentrations remain elevated.

The physiological effects of calcitonin on serum Ca^{2+} in humans are minor. The effects of this hormone are most marked when rates of bone turnover and osteoclastic activity are highest as in the young or in Paget's disease. Administration of calcitonin reduces accelerated bone turnover in Paget's disease of bone.

d).   Oestrogen

The dramatic reduction of circulating oestrogen levels following the menopause is thought to be a major factor affecting the onset of osteoporosis, a disease characterised by increased bone turnover and trabecular bone loss (Raisz, 1988b). At the menopause there is an increase in bone resorption accompanied by a smaller increase in bone formation so that bone mass decreases. Reduction of bone mass causes an increased susceptibility to fractures. Treatment with oestrogen, as hormone replacement therapy, leads to an overall gain in bone mass. This would suggest a role for oestrogen in maintaining normal bone mass via anabolic effects on osteoblasts.

Ernst, Schmid and Froesch (1988) describe the effects of 17β-oestradiol (E_2) on osteoblast-like cells cultured from neonatal rat calvariae. Using this model, oestradiol stimulated proliferation of the cells and increased steady-state levels of mRNA for the $\alpha 1$ chain of type I procollagen. The effect of oestrogen was inhibitable by tamoxifen, an agent that competes with E_2 for binding to the receptor, and no effect was observed with the stereoisomer

17α-oestradiol. In further studies Ernst, Heath and Rodan (1989) report that E_2 enhanced the proliferation of osteoblasts derived from rat long bones and of the Simian Virus 40 (SV40) immortalised rat osteoblast-like cell lines RCT-1 and RCT-3. Alkaline phosphatase activity was not affected by E_2 in these cells. E_2 decreased PTH responsive cAMP in both these cell types and enhanced mRNA levels for insulin-like growth factor I (IGF-I). When cultures of primary rat calvarial osteoblasts were exposed to E_2 with a monoclonal directed against IGF-I, the E_2 induced proliferation was inhibited.

Further evidence of a role for IGFs in mediating the effects of oestrogen is supplied by Gray et al (1989). These workers report an increase in the production of both IGF-I and II stimulated by E_2 in the rat osteosarcoma cell line UMR106. 1,25D also enhanced production of IGF-I but pretreatment with E_2 blocked this response.

Egrise et al (1990) studying cultured osteoblasts from mature female rats observed a decrease in ^{3}H-thymidine incorporation in response to E_2. E_2 enhanced alkaline phosphatase activity in these cells, but decreased the osteocalcin produced in response to 1,25D. Such differences in responsiveness of different model systems may reflect different stages of differentiation represented by these cultures. The small effects observed with oestradiol may be due to a subpopulation of cells displaying oestrogen responsiveness.

Keeting et al (1991b) studied the effects of 17β-oestradiol on primary cultures of human osteoblasts derived from trabecular bone. These workers report no direct effects of E_2 on either proliferation or differentiation of adult human osteoblasts. No interaction of E_2 with insulin, 1,25D or dexamethasone was observed. Oestradiol displayed equivocal effects on mRNA for pro-α (I)-collagen, increasing steady state levels in 6/11 cell

strains, having no effect in 3 cell strains and decreasing the mRNA levels in 2 cell strains.

Specific binding sites for oestrogen have been demonstrated in ROS 17/2.8 rat osteosarcoma cells and in the human osteosarcoma line HOS TE85 (Komm *et al*, 1988). The number of high affinity binding sites per cell was low (~200 detectable) compared to uterus which possesses several thousand high-affinity oestrogen receptors per cell. The low number of receptors present in the osteoblast-like cells were sufficient to elicit a response to oestrogen. The hormone increased mRNA for type I procollagen by 2 fold and enhanced mRNA for TGF-β by 2.5 fold in the cell lines studied. Oestrogen receptors have also been demonstrated on primary cultures of human osteoblasts (Eriksen *et al*, 1988).

The precise role of oestrogen in the regulation of bone metabolism remains unclear. Riggs *et al* (1972) describe the effects of oestrogen therapy in postmenopausal osteoporotic subjects. Treatment decreased the values for bone resorbing surfaces as determined by microradiography of iliac crest biopsies. Long-term treatment however resulted in a secondary decrease in bone formation. The principal effect of oestrogen therapy may be a decrease in the progression of bone loss.

1.6.2   Locally derived factors

a).   Interleukin 1

In 1972, Horton *et al* described bone resorbing activity in conditioned medium from human peripheral blood mononuclear cells. This so-called Osteoclast Activating Factor (OAF) has since been demonstrated to be due to a variety of cytokines, including interleukin 1. IL-1 is a potent stimulator of bone resorption both *in vivo* (Sabatini *et al*, 1988) and *in vitro* (Gowen *et al*, 1983; Gowen and Mundy, 1986; Stashenko *et al*, 1987). IL-1 stimulates the proliferation of osteoclast precursors and their fusion to form multinucleate giant cells in human long term bone marrow cultures (Pfeilschifter *et al*,

1989). IL-1 stimulates the production of prostaglandin E_2 (PGE$_2$) which in turn is a potent stimulator of bone resorption (Gowen, 1988). IL-1 failed to stimulate resorption by isolated rat osteoclasts maintained on slices of human cortical bone; the presence of calvarial osteoblasts as accessory cells was necessary for the resorptive effect of IL-1 (Thomson, Saklatvala and Chambers, 1986).

Interleukin 1α (IL-1α) and IL-1β are acidic and neutral products of two separate genes with limited homology in their primary structures but apparently identical bioactivities. Both proteins have a molecular weight of 17kDa and are derived by complex proteolytic cleavage from larger precursors. Interleukin 1 is produced by peripheral blood monocytes and tissue macrophages; it induces fever and the acute phase response, enhances lymphocyte proliferation and stimulates haemopoiesis. IL-1 can stimulate synovial cells, chondrocytes and fibroblasts to secrete proteinases such as collagenase and plasminogen activators thus contributing to connective tissue breakdown (Gowen, 1992).

IL-1 may also have anabolic effects on bone by stimulating the proliferation of osteoblasts (Gowen, Wood and Russell, 1985; Evans, Bunning and Russell, 1990). However, IL-1 antagonises 1,25D induced osteocalcin synthesis by human osteoblast-like cells and inhibits alkaline phosphatase expression in these cells (Evans, Bunning and Russell, 1990). IL-1 in low doses inhibits collagen synthesis whereas high doses or prolonged exposure stimulate collagen synthesis (Canalis, 1986).

In a study by Pacifici *et al* (1987), monocytes from the peripheral blood of patients with osteoporosis were shown to have significantly higher levels of IL-1 than monocytes from normal subjects. Patients whose monocytes secreted high levels of IL-1 also had a higher rate of bone formation. A marked increase in blood monocyte IL-1 production was observed following the menopause and this increase was suppressed by ovarian steroid

therapy (Pacifici *et al*, 1989). Increased IL-1 activity may reflect the rapid bone turnover that occurs after the menopause, and may play a role in the pathogenesis of postmenopausal osteoporosis.

Bone matrix constituents released during bone resorption may stimulate IL-1 release from blood mononuclear cells. Pacifici *et al* (1991) demonstrated the ability of bone fragments, collagen, and hydroxyapatite to stimulate IL-1 release from peripheral blood mononuclear cells. In this way, IL-1 could initiate a cascade of events that leads to further production of IL-1 and to an amplification of bone resorption (Figure 1.4). 1,25D may further potentiate this effect by enhancing IL-1 production by monocytes (Bhalla, Amento and Krane, 1986). IL-1 may be a potent mediator of bone loss in conditions of chronic inflammation.

b). Tumour necrosis factor

Tumour necrosis factor (TNF) was originally identified on the basis of its ability to cause haemorrhagic necrosis of tumours in patients with bacterial infections. A separate activity called cachectin, responsible for tissue wastage and weight loss in tumour-bearing animals, was subsequently shown to be identical to TNF (Beutler and Cerami, 1987). Cachectin is an endogenous pyrogen, capable of inducing fever both by a direct effect on hypothalamic neurones and by induction of IL-1. TNF exists as two proteins, TNFα and TNFβ, which share approximately 30% amino acid homology and have a similar spectrum of activity. TNFα is released by activated macrophages whereas TNFβ (lymphotoxin) is lymphocyte-derived.

Bertolini *et al*, (1986) demonstrated that both TNFα and lymphotoxin (LT) stimulate bone resorption in a dose-dependent manner in foetal rat bones and cause an increase in the number of multinucleated osteoclasts. The resorptive effect of both cytokines was inhibited by calcitonin, demonstrating the involvement of osteoclasts. TNFα and β inhibited the percentage
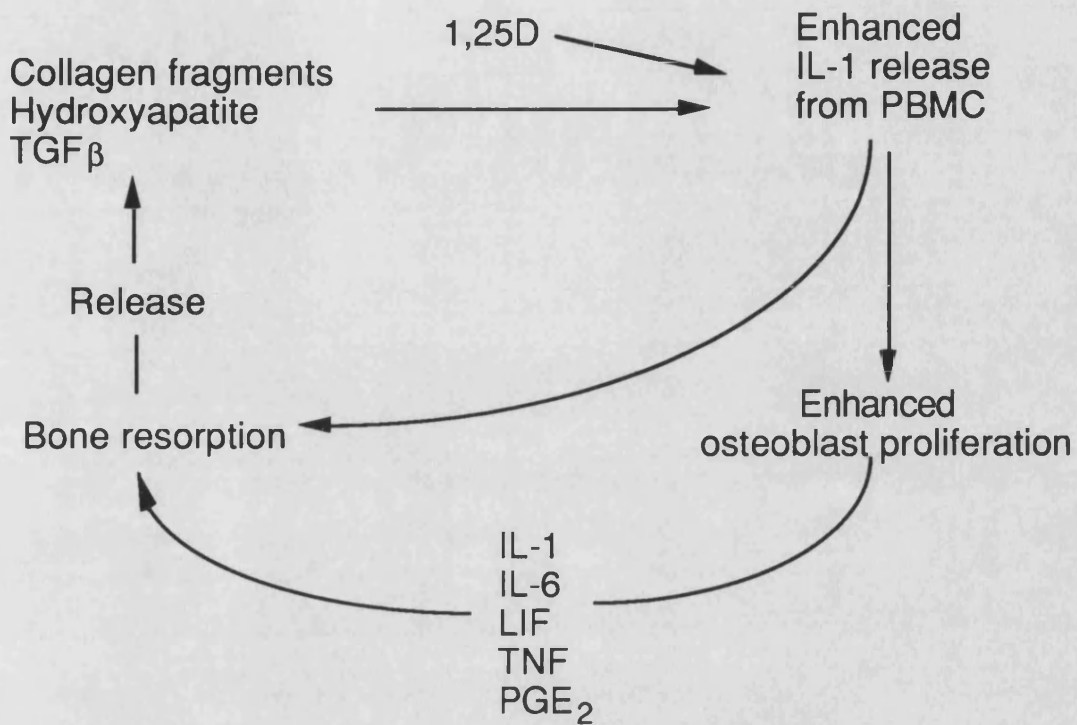
Fig.1.4   Interleukin 1 and bone resorption. IL-1 is a potent stimulator of

bone resorption and stimulates the release of other potent resorbing agents

(e.g. TNF, PGE$_2$) from osteoblasts . The subsequent release of bone

fragments may further augment IL-1 production. 1,25D also increases

production of IL-1 by monocytes, enhancing the resorptive signal.

LIF - Leukaemia Inhibitory Factor

PBMC - Peripheral blood mononuclear cells

collagen synthesis of foetal rat bone explants, and also inhibited alkaline phosphatase expression in the rat osteogenic sarcoma cell line ROS 17/2.8. The resorptive effect of the TNFs is 1,000 fold less potent than IL-1. Suboptimal concentrations of TNF and lymphotoxin produce a synergystic effect when co-incubated with suboptimal concentrations of IL-1, resulting in a twofold increase in activity of IL-1 and a 100 fold increase in the resorptive activity of TNF and LT (Stashenko *et al,* 1987). TNF also causes the release of proteoglycan from cartilage and inhibits formation of new cartilage by inhibiting proteoglycan production (Saklatvala, 1986).

The precise mechanism of TNF and LT induced bone resorption is unknown. Thomson, Mundy and Chambers (1987) demonstrated that isolated osteoclasts incubated with TNFα and β failed to respond to the cytokines by enhanced resorption of cortical bone unless calvarial osteoblasts were also present. These data would suggest that osteoblasts mediate the effect of TNF by producing a resorbing factor. However, such a factor has not yet been identified. The heterogeneous populations of cells used by these authors would not permit identification of the specific cell types responding to resorptive agents.

Transient (24h) treatment with TNFα caused an increase in DNA synthesis in cultured rat calvariae, an increase in alkaline phosphatase, and an increase in ^{3}H-proline incorporation which was DNA synthesis dependent (Canalis, 1987). Continuous treatment of the calvariae (48-96h) with TNF caused an inhibition of collagen synthesis and an inhibition of alkaline phosphatase activity. TNFα stimulates the proliferation of primary cultures of human osteoblasts (Gowen,1992). In the course of bone remodelling, TNF may enhance the proliferation of newly recruited osteoblast precursors prior to the deposition of new matrix.

c). Interleukin 6

Interleukin 6 (IL-6) was originally characterised by several laboratories owing to its many actions on a wide variety of cell types. IL-6 has been designated as B-cell differentiation factor due to its ability to induce terminal differentiation of B cells into immunoglobulin secreting cells (Hirano *et al,* 1986). IL-6 induces the differentiation of thymocytes and splenic T cells (Lotz *et al,* 1988) and is a potent growth factor for hybridoma cells (Aarden *et al,* 1987). IL-6 has strikingly similar activities to IL-1, notably induction of acute phase proteins in hepatocytes (Gauldie *et al* 1987) and induction of fever (Helle *et al* 1988). IL-6 is a glycoprotein of 184 amino acids derived by removal of an N-terminal hydrophobic leader peptide from a 212 amino acid precursor. IL-6 is produced by stimulated monocytes, endothelial cells and fibroblasts.

Conflicting results have been reported as to whether IL-6 induces bone resorption. Lowik *et al* (1989) and Ishimi *et al* (1990) report that IL-6 induced bone resorption in foetal rat metacarpals and foetal mouse calvariae respectively. However, Al-Humidan *et al* (1991) failed to observe a resorptive effect of IL-6 in neonatal mouse calvariae, in this study IL-6 had no effect on the bone resorption induced by IL-1 but inhibited both PTH and 1,25D induced resorption. Such differences may be due to the different stages of differentiation displayed by these organ culture models. Addition of IL-6 to 17day-old foetal mouse metacarpals caused a dose-dependent increase in ^{45}Ca release but had no effect on 17-day-old radii (Lowik, 1992). 17day-old foetal mouse radii have a mineralised matrix that contains mature resorbing osteoclasts, metacarpals from the same animals possess a mineralised matrix that does not contain mature osteoclasts. IL-6 may act by enhancing the differentiation of haemopoietic precursors of osteoclasts, but may have no effect on the mature osteoclast.

Primary rat osteoblast-like cells and the rat osteogenic sarcoma cell lines

UMR-106 and ROS 17/2.8 constitutively produce IL-6 and this production is

enhanced by PTH (Lowik,1992; Littlewood *et al*, 1991b). It has been

postulated that PTH may induce bone resorption by stimulating the

production of IL-6 by osteoblasts. However, Garrett, Black and Mundy

(1990) report no effect of IL-6 neutralising antibodies on PTH or 1,25D

stimulated resorption in neonatal mouse calvariae, whereas the antibody

inhibited the resorptive effect of IL-1 and TNF. Furthermore, human

osteoblast-like cells failed to respond to PTH by enhanced IL-6 production

(Littlewood *et al*, 1991b).

Patients with rheumatoid arthritis (RA) and other inflammatory arthritides

were found to have significantly more IL-6 in synovial fluid than patients with

osteoarthritis (Houssiau *et al*, 1988). IL-6 activity was detected in the serum

of patients with rheumatoid arthritis but not in normal controls; in patients

with RA IL-6 levels correlated with values for the acute phase protein C-

reactive protein. In conditions of chronic inflammation IL-6 is clearly

involved as an inducer of the acute phase response, a role for IL-6 in the

joint destruction associated with RA is less clear.

d).   Transforming Growth Factor β

Transforming growth factor β (TGF-β), a 25kDa homodimeric peptide

originally purified from human platelets, derives its name from its ability to

induce anchorage - independent growth in non-neoplastic rat kidney

fibroblasts (Pircher, Lawrence and Jullien, 1984). TGF-β has been isolated

from a variety of normal and neoplastic cells, and bone is the most abundant

source of TGF-β after platelets (Sporn *et al*, 1987).

Two distinct forms of TGF-β were identified first in bovine bone. These

proteins were described as cartilage inducing factors A and B (CIF-A and

CIF-B) due to their ability to induce the formation of cartilage proteoglycan

and type II collagen in undifferentiated mesenchymal cells *in vitro* (Seyedin

et al, 1985). CIF-A was found to be equivalent to the form of TGF-β purified

from platelets, that is TGF-β1, and CIF-B is the same as TGF-β2. TGF-β is

produced by fibroblasts, mesothelial cells, osteoblasts and transformed cells

and is an important regulator of growth and differentiation. High affinity

receptors for TGF-β have been identified in almost every cell type studied

(Wakefield *et al,* 1987) and TGF-β has dramatic effects on cells possessing

the receptor.

TGF-β appears to be an important immunosuppressive agent. B

lymphocytes possess high affinity receptors for TGF-β and the protein

suppresses factor-dependent IL-2 B cell proliferation and immunoglobulin

secretion (Kehrl *et al,* 1986). B lymphocytes also synthesise and secrete

TGF-β. TGF-β inhibits the proliferation of mitogenically stimulated T

lymphocytes and also suppresses the proliferation of a number of human

tumour cell lines. TGF-β may also exert its actions as a potent inhibitor of

cell growth, particularly on epithelial cells and mesenchymal cells such as

embryonic fibroblasts. (Reviewed by Sporn *et al,* 1987).

TGF-β is secreted by virtually all cell types in a latent form. Latent TGF-β

can be activated by transient acidification, by incubation with cathepsin D

and by plasmin. Latent TGF-β fails to bind to the TGF-β receptor suggesting

that extracellular activation of the molecule is essential for its actions

(Wakefield *et al,* 1987). The necessity for extracellular activation of the

molecule may be important in regulating the actions of TGF-β, which are so

wide ranging. Little is known about the cellular activation of TGF-β.

TGF-β is produced by bovine bone cells and the osteoblast-like cell lines

ROS 17/2.8 and UMR-106 (Gehron Robey *et al,* 1987) and has growth

inhibitory or stimulatory activities depending on the osteoblast model

studied. TGF-β increases type I collagen expression and decreases alkaline

phosphatase activity in both fibroblastic and osteoblastic populations of

foetal rat calvarial digests. TGF-β decreases cellular proliferation, and

increases collagen and alkaline phosphatase expression in ROS 17/2.8 cells, UMR-106 cells and in the human osteosarcoma cell line MG-63. However, TGF-β decreases alkaline phosphatase in the murine clonal osteoblast-like cell line MC3T3-E1 and in the human osteosarcoma cell line SaOS$_2$. TGF-β stimulates bone resorption in neonatal mouse calvariae via the synthesis of prostaglandins (Tashjian et al, 1985). TGF-β inhibits bone resorption in foetal rat long bone cultures (Pfeilschifter, Seyedin and Mundy, 1988) and inhibits the resorption induced by IL-1 and 1,25D in this model. TGF-β inhibited DNA synthesis in a similar manner to hydroxyurea in rat long bone cultures but neither TGF-β or hydroxyurea affect PTH induced resorption. The suppressive action of TGF-β on resorption in these cultures may have been due to an inhibition of osteoclast precursor proliferation. For a review of the actions of TGF-β on bone cells see Bonewald and Mundy (1989).

TGF-β is a potent osteoinductive factor in vivo. In soft tissues TGF-β accelerates healing of dermal wounds. Single applications of TGF-ß$_1$ were sufficient to induce bone formation and complete healing in skull defects in rats (Beck et al, 1991b). TGF-β also induced bone formation in rabbit ear full thickness wounds; the early phase of formation was observed overlying the intact cartilage (Beck et al, 1991a). In both these studies TGF-β appeared to stimulate bone formation without a cartilagenous precursor. The bone formed was characterised by a mineralised matrix interspersed with marrow cavities that contained blood vessels. In rabbit ear full thickness wounds, no bone formation occurred if the perichondrium was left intact, suggesting that disruption of the perichondrium exposes chondroblasts and mesenchymal cells to the osteoinductive actions of TGF-β. TGF-β increases the synthesis of type II collagen in bovine periosteal cells but decreases ALP mRNA suggesting that the factor may promote chondrogenesis during fracture repair thus aiding fracture healing (Izumi et al, 1992).

TGF-β is released from cells as an inert complex bound to one or more binding proteins which mask its biological activity. The TGF-β in bone culture media is in an inactive form. Isolated avian osteoclasts, stimulated to resorb by vitamin A, activate latent TGF-β (Oreffo *et al*, 1989). The area resorbed by the osteoclast (Howship's lacuna) under the ruffled border has a low pH which may be sufficient to activate latent TGF-β. Thus TGF-β is a likely candidate for the coupling of bone resorption and formation phases. TGF-β released as a result of resorption, and activated in the acidic environment of the resorption pit, may recruit osteoblast precursors to the resorption site and stimulate bone formation.

e). Leukaemia Inhibitory Factor

A novel cytokine, leukaemia inhibitory factor (LIF) was purified from Krebs II ascites cell-conditioned medium, following the observation that the factor induced macrophage differentiation of the murine MI myeloid leukaemia cell line (Hilton *et al*, 1988). Moreau *et al* (1988) subsequently isolated a cDNA clone encoding a novel human haemopoietic growth factor called human interleukin for DA cells or HILDA. This factor supports the proliferation of the interleukin 3 dependent murine leukaemic cell line DA-1a. HILDA is produced by lectin stimulated C10-MJ2 T cells and both lectin and phorbol myristate acetate (PMA) stimulate the expression of mRNA for HILDA. The cDNA for HILDA encodes a 202 amino acid protein that is essentially identical to LIF (Moreau *et al* 1988). Recombinant LIF/HILDA secreted by COS-1 cells is a glycoprotein of Mr 40,000.

A third factor, differentiation inhibitory activity (DIA) was purified from the medium conditioned by Buffalo rat liver (BRL) cells (Smith *et al*, 1988). This factor suppressed the spontaneous differentiation of murine embyronic stem (ES) cells, thereby maintaining the pluripotentiality of these cells *in vitro* . Purified DIA was found to be related in structure and function to LIF/HILDA. DIA purified from BRL cell conditioned medium is a single chain glycoprotein

Mr 43,000 consisting of an extensively glycosylated central protein core of Mr 20,000. ES cells, in the absence of DIA, spontaneously differentiate and show an altered morphology. Incubation with DIA inhibited this differentiation. Purified recombinant LIF was shown to have a similar effect (Williams *et al*, 1988), and to substitute for DIA in the maintenance of totipotent ES cells. DIA, LIF and HILDA are multifunctional, regulatory molecules with both growth promoting or differentiation inducing activities, depending on the target cell type. The presence of receptors for LIF on ES cells was demonstrated by the binding of ^{125}I labelled LIF, which was inhibited by DIA (Smith *et al*, 1988).

Evidence of a role for LIF in the regulation of bone turnover was suggested by the observation that LIF promoted bone resorption in a prostaglandin mediated manner in neonatal mouse calvariae (Abe *et al*, 1986). In contrast, Metcalf and Gearing (1989) demonstrated a potent effect of LIF on bone formation in mice injected with cells producing high levels of the cytokine. The mice exhibited ectopic calcification in muscle tissue and liver and a fatal cachexia developed. Tumour necrosis factor is the only agent previously shown to induce cachexia (Rosenblum and Donato, 1989). LIF may be an equally potent agent or may act via the induction of TNF.

The presence of receptors for LIF in osteoblasts, but not in osteoclasts, was demonstrated by specific binding of ^{125}I-LIF (Allan *et al*, 1990). Osteoblasts obtained by digestion of rat calvariae, and the rat osteosarcoma cell line UMR106-06, produced LIF when treated with retinoic acid or TGF-α. LIF mRNA was detected in TGF-α treated UMR-201 cells, and was detected in unstimulated cells following polymerase chain reaction amplification of DNA copies of LIF transcripts. The rat osteoblasts responded to exogenous LIF by an increase in the mRNA and protein production of plasminogen activator inhibitor. LIF inhibited plasminogen activator induced by parathyroid hormone, 1,25D, PGE_2, TNFα and TNFβ.

LIF has been reported to stimulate proliferation of osteoblasts obtained by digestion of rat calvariae. However, LIF decreased proliferation in the transformed osteosarcoma cell line UMR-106 (Lowe et al, 1991). LIF also suppresses proliferation in the murine clonal cell line MC3T3-E1 (Noda et al, 1990), and inhibits platelet derived growth factor (PDGF) and IGF-I induced proliferation in these cells. LIF production is increased by TGF-β and TGF-α in murine and rat osteoblast-like cells (Marusic, Kalinowski and Lorenzo, 1990). LIF suppressed ALP activity, and mRNA for type I collagen in MC3T3-E1 cells, but enhanced osteopontin mRNA (Noda et al, 1990).

LIF may be an important paracrine factor in the local control of bone remodelling. LIF produced by osteoblasts in response to other cytokines (IL-1 and TNF) may act to increase bone resorption by enhancing the proliferation of osteoclast precursors. It is unlikely that LIF is the mediator responsible for the resorptive actions of PTH and 1,25D as both these hormones failed to stimulate LIF production by human osteoblast-like cells (Evans et al, 1990). The anabolic actions of LIF in vivo are consistent with the effects of this cytokine as an enhancer of DNA synthesis in osteoblasts. The inhibition of plasminogen activator by LIF would potentiate the anabolic actions of this cytokine.

The variation in responses of different populations of osteoblasts to LIF possibly reflects the heterogeneity of the cultures, and their different stages of differentiation. LIF has growth stimulatory or differentiation inducing activities depending on the target cell type. The inhibition of osteosarcoma cell lines by LIF may be a function of the transformed nature of these cells. In the context of bone remodelling, LIF may act as both a growth promoter and an inducer of differentiation at different stages of bone formation and resorption. LIF may regulate bone cell function by acting in concert with other growth factors that are produced by osteoblasts or released from the matrix by resorbing osteoclasts.

f). Prostaglandins

Prostaglandins are 20-carbon unsaturated fatty acids containing a cyclopentane ring. The essential fatty acid arachidonic acid is released from the 2-position of plasma membrane phospholipids by the action of phospholipase A_2, and serves as a substrate for the synthesis of prostanoids and leukotrienes. Prostanoid synthesis involves the consumption of two molecules of oxygen catalysed by prostaglandin endoperoxide synthase, an enzyme with cyclooxygenase and peroxidase activities. The product of the cyclooxygenase pathway, an endoperoxide (PGH), is converted to prostaglandins D, E and F as well as to the thromboxane TXA_2 and prostacyclin (PGT_2). Leukotrienes are synthesised by the action of the enzyme lipoxygenase on arachidonate.

The prostaglandins are potent biologically active substances and exist in virtually every mammalian tissue. Prostaglandins cause contraction of smooth muscle, aggregation of platelets, and are important inflammatory mediators. The synthesis of the eicosanoids can be inhibited by the action of anti-inflammatory corticosteroid hormones which stimulate the production of lipocortins which in turn inhibit the activity of phospolipase A_2. Non steroidal anti-inflammatory agents such as aspirin and indomethacin block the activity of cyclooxygenase thus inhibiting the production of group 2 prostanoids. Prostaglandins are rapidly inactivated and in most mammalian tissues this rapid metabolism is caused by the enzyme 15-hydroxyprostaglandin dehydrogenase. Indomethacin can inhibit prostaglandin inactivation, and blocking the activity of the catabolic enzyme prolongs the half life of prostaglandins. Prostaglandins are not stored in the cell but are continuously released to the cell exterior where they act locally in a paracrine manner to increase cAMP.

Prostaglandins are potent stimulators of bone resorption (Klein and Raisz, 1970). PGE_1 and PGE_2 are the most potent whereas PGA_1 and $PGF_{1\alpha}$ also

increase bone resorption but higher doses are necessary. The stimulation of bone resorption is similar to that observed with parathyroid hormone and both these agents stimulate cAMP in bone. However, adrenalin also stimulates cAMP but has no effect on resorption, suggesting that prostaglandins enhance bone resorption by some other mechanism.

The bone resorbing actions of IL-1, TNF and PTH can be inhibited by indomethacin but the level of inhibition varies depending on the model used. Gowen *et al* (1983) reported no inhibition of IL-1 induced bone resorption by indomethacin using neonatal mouse calvariae, whereas Akatsu *et al* (1991) observed a decrease in the number of osteoclasts and IL-1 induced resorption in murine foetal long bones coincubated with indomethacin. An explanation for this discrepancy may be that murine foetal long bones are at an earlier stage of development than calvariae. Dieudonne *et al* (1991) observed that 17day calvariae contain many multinuclear, TRAP positive cells that are seen actively resorbing mineralised matrix. 17day metatarsal rudiments were less advanced, osteoclasts were not present and excavation of a marrow cavity had not yet started. Prostaglandins may be involved in the early stages of osteoclast formation but other factors may affect the resorptive function of mature osteoclasts. Collins and Chambers (1991) report that prostalandins E_1 and E_2 increased the number of calcitonin receptor positive cells in murine bone marrow cultures and enhanced bone resorption by these cells when incubated on dentine slices. Similarly Akatsu *et al* (1991) report that IL-1α and IL-1β stimulated osteoclast-like cell formation in murine bone marrow and this could be completely inhibited by indomethacin. These authors observed a correlation between the number of TRAP positive cells formed and the concentration of PGE_2 in the culture media. In cultures of foetal mouse long bone, indomethacin only partially suppressed IL-1 induced bone resorption.

Prostaglandins mediate the stimulation of resorption by platelet derived growth factor, epidermal growth factor, TNFα and TGF-β. Prostaglandins are also reported to strongly inhibit resorption by isolated osteoclasts and PGE_2 decreases the bone resorption stimulated by 1,25D using mouse bone marrow cultures (Collins and Chambers, 1991).

Prostaglandins may act by stimulation of the formation of osteoclast-like cells which are then induced to resorb by other factors. Failure of indomethacin to completely block resorption in organ cultures suggests that prostaglandins are only partly responsible for the resorptive response to IL-1. Numerous steps in the induction of bone resorption are not prostaglandin mediated - osteoid degradation mediated by collagenase, recruitment of osteoclasts and activation of newly formed osteoclasts. Resorption induced by 1,25D and formation of TRAP positive multinuclear cells are not affected by indomethacin. Collagenase production induced by IL-1 is also reported to be independent of prostaglandins.

It is possible that osteoclast progenitors at advanced stages can be differentiated into osteoclasts by a mechanism independent of prostaglandin synthesis, whereas immature osteoclast progenitors need prostaglandin for IL-1 mediated osteoclast differentiation.

PGE_2 also enhances bone formation by stimulating the replication and differentiation of osteoblast progenitors (Raisz, 1988a). Osteoblasts release PGE_2 in response to interleukin 1 (Tatakis, Schneeberger and Dziak, 1988), parathyroid hormone (McDonald et al, 1984) and tumour necrosis factor α (Sato et al, 1987) but gamma interferon depresses PGE_2 production by the murine clonal MC3T3-E1 cells (Hoffmann et al, 1987). MacDonald et al (1984) report that 1,25D depresses basal production of PGE_2 in human osteoblast-like cells, whereas Klein-Nulend, Pilbeam and Raisz (1991) report a delayed and small stimulatory effect of 1,25D on PGE_2 production in mouse parietal bones. This latter effect was blocked by the IL-1 receptor

antagonist (IL-1Ra) although IL-1Ra did not block the resorptive effect of 1,25D. Possibly 1,25D may increase the IL-1 produced by monocyte/macrophages in the bone marrow, which in turn causes an increase in the release of PGE_2.

PGE_2 may be involved in the pathological bone loss associated with rheumatoid arthritis as prostaglandins are released at sites of inflammation. A physiological role for prostaglandin in the control of bone remodelling is difficult to assess. Better identification of early and late ostoclast progenitors may assist in defining the role of prostaglandins in enhancing osteoclast formation.

g)   Integrins

In addition to the numerous soluble factors regulating bone turnover and the function of bone cells, it is likely that the bone extracellular matrix itself can influence the sequence of events of the remodelling cycle. Certain noncollagenous proteins of bone possess the amino acid sequence -RGD- which is the recognition site of adhesion molecules for the cell surface integrins. Integrins are heterodimeric molecules that function as cell-substratum or cell-cell adhesion receptors.

Integrins are composed of two noncovalently associated α and β subunits that are integral membrane glycoproteins. Six different β subunits have been described and certain α subunits can combine with more than one β subunit. The α subunit contains areas thought to bind Ca^{2+}. Most integrins bind to more than one ligand. A typical β subunit possesses one membrane-spanning domain and a short cytoplasmic domain at the carboxy terminus of the molecule. On the cytoplasmic side of the plasma membrane, the receptors contain regions capable of binding to cytoskeletal proteins such as talin, vinculin and actinin and serve to link the extracellular matrix to the cytoskeleton. The structure and function of integrins are reviewed by Albelda and Buck (1990).

Integrins recognise specific (RGD) amino acid sequences in their ligands (Humphries, 1990). Adhesive molecules fibronectin, fibrinogen, thrombospondin, vitronectin, laminin and type I collagen all possess the RGD sequence. Each receptor displays specificity, for example the fibronectin receptor binds fibronectin but not vitronectin. Little is known about how such specificity is maintained but the confirmation of the RGD tripeptide and its surrounding sequences may determine the recognition of specific proteins by receptors.

Integrin-mediated attachment of cells to the extracellular matrix may stimulate cell migration or promote differentiation. The matrix may act as a competence-inducing factor making cells capable of responding to hormones or other soluble factors. In addition, binding of cells to the matrix may result in conformational changes that in turn provide inductive signals. Interaction of the fibronectin receptor with fibronectin results in the induction of metalloproteinase and collagenases are released following the binding of the laminin receptor with a synthetic peptide derived from laminin (Humphries, 1990). Thus the integrins may also play a role in tissue breakdown.

The attachment of rat osteoclasts to a number of adhesion molecules was studied by Helfrich *et al* (1992). Osteoclasts adhered well to the proteins osteopontin, BSP, fibronectin, vitronectin, fibrinogen and von Willebrand factor, and adhered to a lesser extent to collagen types I and III. The cells failed to adhere to laminin, thrombospondin, elastin, osteonectin and osteocalcin. A monoclonal antibody recognising the rat β_3 chain inhibited attachment to all proteins with the exception of type I collagen. The attachment of osteoclasts to osteopontin, von Willebrand factor and vitronectin could be inhibited with RGD peptides. These results demonstrate that attachment of osteoclasts is mediated in part by the binding of RGD-containing proteins to β_3. Binding of cells to type I collagen may be

mediated by other receptors. The failure of osteoclasts to attach to thrombospondin, which also contains RGD, may reflect the specificity of ligand-integrin interactions.

Osteoblasts, in cryostat sections of human bone, have been shown to express the α_1, α_3, and β_1 integrin subunits, while osteoclasts express α_2 and β_1 units and possess the $\alpha_v\beta_3$ vitronectin receptor (Clover and Gowen, submitted). The adhesion properties of the two cell types would appear to be differentially regulated, by binding to different adhesion proteins - a property that may reflect the very different functions of the two cell types.

1.7   In vitro models for studying osteoblastic function.

Organ cultures of calvariae and long bones from neonatal or foetal rats and mice have been used extensively to study the effects of osteotropic hormones and cytokines on bone resorption and formation. Using these models, information regarding the overall effects of test agents on bone resorption (usually measured by release of ^{45}Ca) and bone formation (as assessed by incorporation of ^3H-proline into collagen) have been obtained. With respect to certain agents (e.g. TGFβ, IL-6, PGE$_2$, E$_2$, described above) conflicting data from different model systems serves to highlight the differences of differentiation status of the models used, and the heterogeneity of the cell populations involved.

For the study of osteoblast-like cell function, cells prepared from foetal bovine, murine and rat calvariae have been described and characterised (Globus, Patterson-Buckendahl and Gospodarowicz, 1988; Wong and Cohn, 1975; Bellows *et al*, 1986). Sequential collagenase digestion techniques yield populations of cells enriched for the osteoblast phenotype (Sodek and Heersche, 1981). These cell cultures are heterogeneous and do not permit the identification of the cell types responding to specific agents.

A clonal cell line (MC3T3-E1) expressing alkaline phosphatase activity, responsive to 1,25D and able to mineralise *in vitro* has been obtained from neonatal murine calvariae (Sudo *et al*, 1983). Osteosarcoma cell lines of both rodent and human origin have been described (for a review see Rodan *et al*, 1988), which provide large numbers of cells for study and have the advantage of being clonal in origin but are transformed, fail to express the full osteoblast phenotype (Table 1.1) and are unlikely to represent normal function. Very few models are available for the study of adult human bone cell function. Cultures of human osteoblast-like cells derived from trabecular bone express all the hallmarks of the mature osteoblast phenotype such as alkaline phosphatase activity, PTH responsive adenylate cyclase, type I collagen production and osteocalcin production in response to 1,25D (Beresford, Gallagher and Russell, 1986). Table 1.1 shows a summary of certain osteoblast models. The models vary in their expression of osteoblastic characteristics (e.g. osteocalcin production) which may indicate that they represent different stages of the osteoblast lineage.

Heath and co-workers have produced two new bone cell lines by immortalisation of rat calvarial osteoblasts with SV-40 DNA (Heath *et al*, 1989). RCT-3 cells express alkaline phosphatase enzyme which is enhanced by 1,25D, and respond to PTH with the production of cAMP. mRNA for osteopontin is present and is enhanced by 1,25D, whereas mRNA for type I procollagen is downregulated by 1,25D. RCT-1 cells had undetectable ALP activity and failed to respond to PTH. However, treatment of RCT-1 cells with retinoic acid induced both of these osteoblastic characteristics, suggesting that RCT-1 cells represent an osteoblast precursor. Use of such immortalised cell lines may yield interesting information concerning the control of differentiation in osteoblastic cells.

Table 1.1. OSTEOBLAST MODEL SYSTEMS

MURINE

MC3T3 - E1	High basal alkaline phosphatase activity
(Sudo *et al*, 1983)	Functional responses to 1,25D
	No elevation of cAMP in response to PTH.

RAT

UMR Series	Numerous clones and subclones:
(Partridge *et al*, 1981)	PTH responsive adenylate cyclase,
	Alkaline phosphatase activity
	Receptors for 1,25D
ROS Series	High alkaline phosphatase activity
(Majeska, Rodan and Rodan, 1980)	PTH responsive, produce osteocalcin
RCT -1 and -3	Rat calvarial cell lines produced by
(Heath *et al*, 1989)	immortalisation with SV-40 large T antigen.
RCT -1	Osteoblast characteristics induced by retinoic acid: mRNA for osteopontin and type I collagen, inducible PTH responsiveness.
RCT -3	Constitutive osteoblast characteristics: Respond to IL -1 by increased prostaglandin production. Do not produce osteocalcin in response to 1,25D.

HUMAN

SaOS2	Human Osteosarcoma cell line.
(Mulkins *et al*,1982)	High alkaline phosphatase
	PTH responsive adenylate cyclase
	Receptors for 1,25D
	Produce osteonectin but do not produce osteocalcin in response to 1,25D.
MG-63	Low constitutive ALP enhanced by 1,25D.
(Franceschi and Young 1990, Lajeunesse *et al*, 1990)	Produce osteocalcin in response to 1,25D.

1.8 Objectives of study

The study of osteoblast function *in vitro* relies heavily on the availability of model systems representing the normal osteoblast phenotype. Murine cells have the capacity to form continuously growing cultures spontaneously permitting the isolation of clonal osteoblast -like cells from neonatal mouse calvariae. Human cells fail to form spontaneously growing cell lines, thereby limiting the number of model systems available for the study of adult human bone cell function.

Human osteoblast-like cells obtained as outgrowths from trabecular bone express many of the classical characteristics of osteoblasts. These cells produce type I collagen, express alkaline phosphatase (ALP) activity and display PTH-responsive adenylate cyclase. In addition, the cells produce osteocalcin in response to 1,25-dihydroxyvitamin D_3. However, these cultures are heterogeneous and may include not only osteoblastic cells at various stages of differentiation but also other cell types such as fibroblasts and marrow stromal cells. Such heterogeneity limits the identification of specific cells responsive to osteotropic hormones and cytokines.

Osteosarcoma cell lines express many of the characteristics of osteoblasts but these cells are transformed and may not represent normal function. The objectives of this study were to attempt to immortalise human osteoblast-like cells in order to obtain discrete cell lines expressing the osteoblast phenotype and representing different stages of the osteoblast lineage.

CHAPTER 2

GENERAL METHODS

2.1   Materials

Minimum essential medium (MEM), Roswell Park Memorial Institute

Medium (RPMI), Dulbecco's modification of Eagle's medium (DMEM), L-

glutamine, penicillin and streptomycin were obtained from GIBCO BRL

(Paisley, UK). Foetal calf serum (FCS) was obtained from Imperial

Laboratories (Andover, UK). 1,25-dihydroxyvitamin D_3 (1,25D) was a kind

gift from Dr. M. R. Uskokovic, Hoffmann La Roche (Nutley, NJ). Simian Virus

40 (SV40) DNA was a gift from Dr. Vittoria Marini, University College and

Middlesex School of Medicine (London, UK). A calcium phosphate

transfection kit was purchased from 5 prime, 3 prime Inc. (West Chester, PA).

Recombinant human interleukin 1α (rhIL-1α) was a gift from Glaxo Group

Research (Middlesex, UK). Recombinant human tumour necrosis factor α

(rhTNFα) was purchased from Genzyme Corporation (Boston, MA). rhIL-6

was a gift from Dr. L.A. Aarden and had a specific activity of 10^9U/mg. A

cDNA probe for type I collagen was a gift from Dr. R. Poulsom, ICRF (London,

UK). cDNA probes for IL-1β, TGFβ and β actin were a gift from Glaxo Group

Research. Immunoradiometric assay kits for the detection of TNFα were

supplied by Medgenix diagnostics (Brussels, Belgium).

A monoclonal antibody directed against the common epitope of SV40

large T and small t antigens was obtained from Oncogene Science Inc.

(Manhasset, NY). Radioisotopes and multiprime DNA labelling system kits

were supplied by Amersham International (Amersham, UK). Other

chemicals were supplied by Sigma Chemical Company (Dorset, UK).

Multiwell plates and tissue culture dishes were obtained from Costar

(Cambridge, MA).

2.2   Patient details

Trabecular bone-derived cells were prepared from fragments of bone

obtained from a 17 year old male. The patient had undergone a

supercondylar osteotomy of the left femur for genus varum recurvatum. This

was necessary due to stapling of the medial aspects of the knees four years previously which had overcorrected growth on the left side. The patient had received no drugs and had no known underlying bone disorder.

2.3   Human bone cell culture.

Human osteoblast-like cells were isolated by maintaining explants of trabecular bone in culture. Cells isolated by this method have been well characterised and express markers of the osteoblast phenotype. The cells express alkaline phosphatase enzyme, produce osteocalcin in response to 1,25D, produce type I collagen and display a PTH-responsive adenylate cyclase (Beresford *et al*, 1984, MacDonald, Gallagher and Russell, 1986).

Trabecular bone was obtained from a 17 year old male (femoral osteotomy) and excised into 3-5mm pieces. Bone chips were washed several times in phosphate buffered saline (PBS) to remove any blood and loosely adherent marrow cells, and incubated overnight at 37°C in universal containers filled with complete MEM. The purpose of this overnight incubation is to allow fat globules from the bone to rise to the surface of the medium. Removal of fat in this way improves the yield of cells obtained from the chips as fat deposited on the tissue culture plastic decreases the area available for osteoblasts to settle.

Following this incubation, the medium was decanted and the bone explants washed again in PBS. Bone chips were then incubated in 100mm tissue culture dishes in 10ml per dish of complete MEM. The medium was changed every week until confluent monolayers of cells were obtained.

2.4   Maintenance of cell lines

Immortalised osteoblast-like cell lines (Bath Osteoblast Prototype (BOP)), and human osteosarcoma cells (MG-63) were maintained in Minimum Essential Medium supplemented with 10% foetal calf serum +2mM L-glutamine, 100IU/ml penicillin and 100μg/ml streptomycin (complete MEM). COS cells were maintained in DMEM with the same additions. The cells

were cultured in 100mm tissue culture dishes with 10ml of complete medium, which was replaced every 3-4 days. Primary cultures of osteoblasts and all cell lines were passaged using the following procedure: Culture medium was aspirated and the cell layer washed once with 10ml PBS, cell layers were incubated with 2.5ml per culture dish of trypsin (0.25%) + EDTA (0.2%) for 5-10 minutes at 37°C, the reaction was stopped by addition of complete medium and the cell suspension centrifuged for 5min at 230g. Cells were resuspended and counted using a haemocytometer.

2.5   Alkaline phosphatase and bone matrix proteins

BOP cells were passaged and seeded into 6 well plates at 4×10^4 per 30mm well and allowed to settle for 24h in complete medium. The medium was then changed to defined medium (MEM + 0.1% bovine serum albumin, $1\mu g/ml$ insulin, $10\mu g/ml$ transferrin and $3 \times 10^{-8}M$ $NaSeO_2$) supplemented with $50\mu g/ml$ L-ascorbate and $10^{-8}M$ vitamin K_1. After 24h the medium was changed again to defined medium + L-ascorbate and vitamin K_1 with either ethanol vehicle or $10^{-8}M$ 1,25D. Cultures were incubated for a further 48h and the conditioned media obtained stored at -20°C for the determination of bone matrix proteins. Cell layers were freed by addition of $500\mu l$ per well of 0.1% tween followed by scraping with a rubber policeman. Cell layers were sonicated for 5s (amplitude 15 microns). Alkaline phosphatase (ALP) activity and total protein content were determined on aliquots of sonicated cell samples.

a).   Alkaline phosphatase assay

ALP activity in cell sonicates was measured by monitoring the release of para-nitrophenol from disodium p-nitrophenyl phosphate at 37°C and pH 10.5. The reaction produces a coloured product and can be monitored spectrophotometrically at 410nm.

A standard curve was obtained by preparing serial dilutions of a 10μmol/ml stock of p-nitrophenol. The stock solution was diluted in 0.1% tween to obtain standards of 2, 1.8, 1.6, 1.4, 1.2, 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, and 0.05μmol/ml. Assay buffer was prepared as follows: 10.52g diethanolamine and 400mg magnesium chloride were dissolved in distilled water and the pH adjusted to 10.5 with 0.1M HCl. Immediately prior to the assay, 46.4mg of disodium p-nitrophenyl phosphate were added to 100ml of buffer.

50μl of each sample or standard were pipetted into a LP3 tube and the reaction started with 500μl of assay buffer. The tubes were incubated at 37°C for 30-60min until a yellow colour developed. The reaction was stopped with 1ml 0.2M NaOH when the colour of the samples matched that of the lower standards. The reaction time was noted and 200μl of each sample and standard were transferred to wells of a 96 well plate. Absorbance at 410nm was measured using a Dynatech micro-ELISA reader. A standard curve of p-nitrophenol concentration (abscissa) vs absorbance (ordinate) was plotted and sample results extrapolated. Results are expressed as μmoles per min per mg total protein.

b).   Total protein assay

The protein content of the cell sonicates was measured by the method of Lowry (Lowry *et al* 1951). This method is based on the reduction of phosphomolybdic-tungstic acid present in Folin and Ciocalteu reagent. Proteins effect a reduction by the loss of oxygen atoms from the mixed acid thereby producing a blue colour with maximum absorbance at 745-750nm. Copper is though to facilitate electron transfer to the mixed acid and so serves to increase the sensitivity to protein.

Bovine serum albumin (BSA) was used as standard. Serial dilutions of BSA in 0.1% tween were prepared to give standards of 100, 87.5, 75, 62.5, 50, 37.5, 25, 12.5, 7.5, and 5μg protein in 100μl.

The following reagents were prepared:

Copper stock solution:

   1% (w/v) tri-sodium citrate dihydrate in 0.5% (w/v) cupric sulphate

   $(CuSO_4.5H_2O)$.

Alkaline stock solution:

   10% (w/v) anhydrous Na_2CO_3 in 2% (w/v) NaOH.

Folin's reagent:

Stock diluted 1:2 parts with dH_2O, immediately prior to use.

Alkaline copper reagent was freshly prepared immediately prior to the assay by mixing 1ml copper stock solution, 20ml alkaline stock solution and 79ml dH_2O. 100μl of standard and sample were pipetted into LP3 tubes and 500μl of alkaline copper reagent added. Tubes were vortexed and left to stand for 10min at room temperature. 50μl of Folin's reagent were added, the tubes vortexed again and incubated at room temperature for 30min. 200μl of each standard and sample were transferred to wells of a 96-well plate and the absorbance at 750nm measured using a plate reader. A standard curve of protein concentration (abscissa) vs absorbance (ordinate) was plotted and sample results extrapolated.

c).   Osteocalcin assay

Osteocalcin, released into the experimental culture medium, was measured using a specific radioimmunoassay. Radiolabelled osteocalcin (^{125}I-osteocalcin) and cold osteocalcin, in standards or samples, compete for binding sites on the primary antibody (rabbit anti-bovine osteocalcin). A second antibody (goat anti-rabbit gammaglobulin) is used to precipitate the osteocalcin-primary antibody complex. Normal rabbit serum (2% v/v) was added to the tubes to produce a larger, more visible pellet. The amount of incorporated radioactivity in the precipitated pellet was monitored with a gamma counter.

55

The assay buffer used was Bent T (0.01M Tris pH 7.4, 0.14M NaCl, 0.025M EDTA, 0.1% tween, 0.25% BSA). A standard curve of purified bovine osteocalcin was prepared by serial dilution to give standards of 100, 50, 33, 20, 10, 5, 3.3, 2, and 1ng/ml.

$50\mu l$ of standard and sample were transferred to LP4 tubes, $50\mu l$ of 2% normal rabbit serum (NRS), $50\mu l$ of primary antibody and $100\mu l$ of Bent T were added and the tubes vortexed. Finally, $50\mu l$ of ^{125}I-osteocalcin were added. The tubes were vortexed again, covered, and incubated at 4°C overnight. In addition, the following control tubes were included: Non specific binding (NSB), included as a quality control to account for non specific binding of the radiolabel. NSB tubes contained $100\mu l$ of NRS, $150\mu l$ Bent T and $50\mu l$ of ^{125}I-osteocalcin. Maximum binding (MAX) was included to account for the binding of ^{125}I-osteocalcin to primary antibody in the absence of cold, competing osteocalcin. MAX tubes contained $50\mu l$ of primary antibody, $150\mu l$ Bent T and $50\mu l$ ^{125}I-osteocalcin. Total counts (TC) (^{125}I-osteocalcin alone) was included as an interassay quality control.

Following the first overnight incubation, $50\mu l$ of second antibody and $500\mu l$ of buffer were added to all tubes except TC. Tubes were vortexed and incubated again at 4°C overnight. Tubes were then centrifuged for 25min. at 2,000g and 10°C. Supernatant was aspirated and the amount of radioactivity in the tubes monitored. A graph of $\log_{10}$ concentration (abscissa) vs B/B_0 (ordinate) was plotted where B/B_0 is calculated from the following equation:

$$B/B_0 = \frac{\text{Counts per min. (standard/sample)-NSB}}{\text{MAX-NSB}}$$

A typical standard curve is shown in figure 2.1. Results were extrapolated from the curve and expressed as ng per ml of conditioned medium.
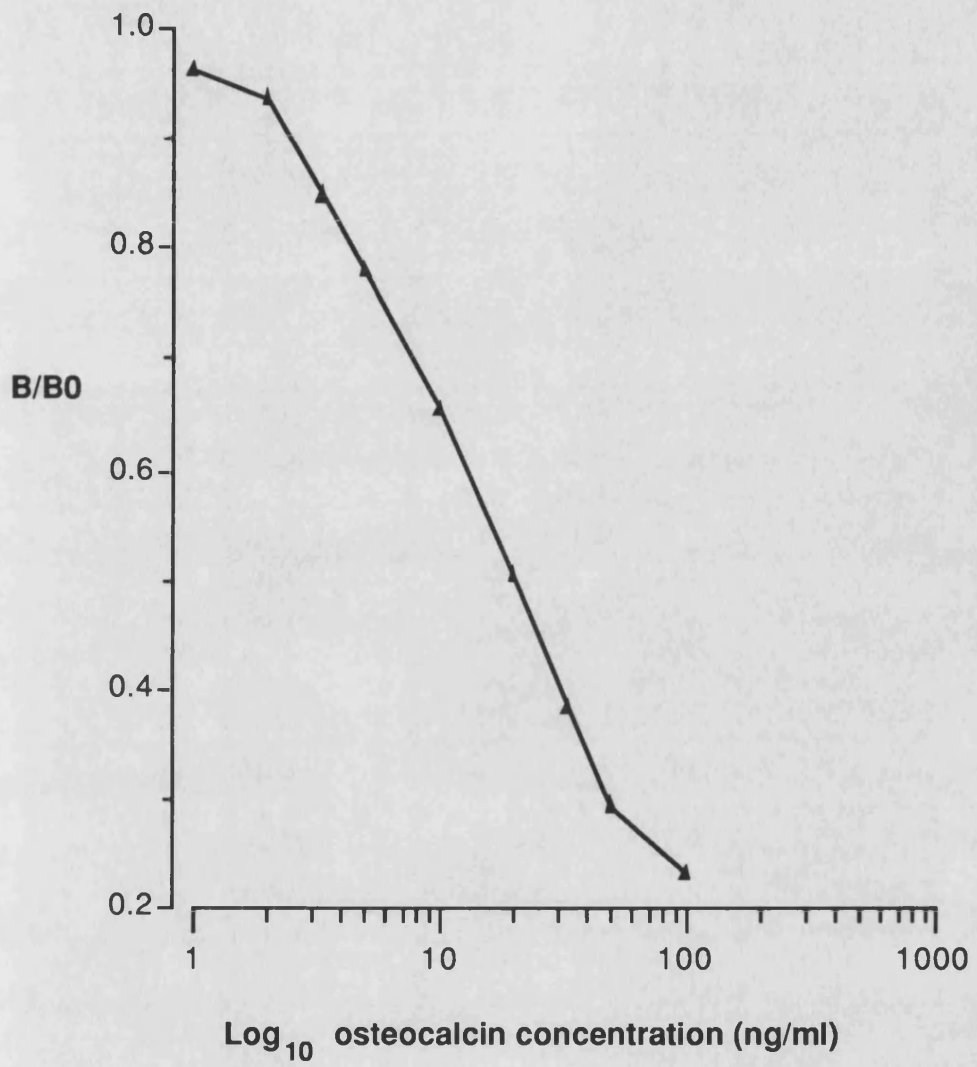
Fig. 2.1   A typical standard curve for the osteocalcin assay

2.6   Immunofluorescence

In order to assess the ability of transfected cell lines to express SV40 early gene products, indirect immunofluorescence was carried out using a monoclonal antibody directed against the common epitope of SV40 large T and small t antigens. Cells (at 10^3 per spot) were allowed to settle overnight onto poly-L-lysine coated multispot slides then slides were washed in PBS and fixed in acetone for 10min at room temperature. Slides were incubated for 30min in a humidified chamber at room temperature with optimally diluted primary antibody. Negative control spots incubated with mouse serum were included. Slides were washed in PBS+2% FCS over 30min and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (polyvalent IgG) optimally diluted in PBS+2% FCS+2% human AB serum. The AB serum is included to block non-specific binding of the FITC conjugate to human antigens on the cell surface. After 30min at room temperature slides were washed again and mounted in 90% (v/v) glycerol in PBS+2.5% (w/v) diazo-bicyclo-octane (DABCO). The DABCO prevents quenching of the fluorescence.

2.7   Molecular Biology Reagents

10X MOPS --	200mM 3-(N-morpholino) propanesulphonic acid, 50mM sodium acetate, 10mM ethylenediamine tetraacetic acid (EDTA)
20X SSC --	175.3g/l NaCl, 88.2g/l sodium citrate pH 7
20X SSPE --	175.3g/l NaCl, 27.6g/l NaH$_2$PO$_4$, 7.4g/l EDTA
50X Denhardt's --	5g Ficoll, 5g polyninylpyrrolidone (PVP), 5g BSA in 500ml of deionised water.
10X Tris acetate --	0.4M Tris, 0.05M sodium acetate, 0.01M EDTA pH 8.1.
10X Tris EDTA --	100mM Tris, pH 7.4, 10mM EDTA pH 8.

RNA loading dye --   20% (w/v) Ficoll, 0.2% (w/v) bromophenol blue.

DNA loading dye --   25% Ficoll, 0.25% bromophenol blue, 0.25%

                        xylene cyanol.

2.8   RNA isolation

RNA was isolated from cells maintained in 100mm culture dishes. Medium

was aspirated and 0.5ml guanidium thiocyanate + 0.1M 2-mercaptoethanol

(solution D) added to each petri dish. Cell layers were removed using a

sterile cell scraper and transferred to microfuge tubes. RNA was extracted

from the cell layers using the method of Chomczynski and Sacchi (1987).

To 0.5ml of cell lysate, 50μl of 2M sodium acetate (pH 4), 500μl phenol

(water saturated) and 100μl chloroform-isoamyl alcohol (49:1) were added

sequentially and tubes were mixed by inversion between each addition.

Tubes were shaken vigorously and allowed to cool on ice for 15min. Tubes

were microfuged at 11,600g for 5min and the aqueous layer decanted into a

fresh tube. An equal volume of chloroform-isoamyl alcohol was added,

tubes were centrifuged again and the aqueous layer decanted. An equal

volume of isopropanol was added to the final aqueous layer and the

microfuge tubes stored at -20°C overnight to precipitate the RNA.

Following the overnight step, RNA was microfuged at 11,600g for 10min.

The supernatant was discarded and the pellet redissolved in 150μl of

solution D. 150μl of isopropanol were added and the RNA precipitated

again at -20°C overnight. The RNA was then microfuged again for 10min

and the pellet washed twice in 70% ethanol and once in absolute ethanol.

The pellet was dried in a vacuum chamber and then redissolved in 50μl of

diethyl-pyrocarbonate (DEPC) treated water.

RNA was quantified by measuring the absorbance at 260nm and 280nm.

The ratio A_{260}/A_{280} should be 2.0 for RNA, a lower ratio indicates the

presence of contaminating phenol or protein. The concentration of RNA is calculated from the following equation: A_{260} X 40 X dilution = μg/ml RNA

2.9   Hybridisation of filters

Filters of RNA obtained by Slot and Northern blotting were prehybridised for 4h at 50°C in a pretreatment solution of 2X SSC, 10X Denhardt's, 33% (v/v) formamide, 10% (w/v) dextran sulphate, 0.5% (w/v) sodium dodecyl sulphate (SDS), 2mM EDTA and 10mg/ml denatured salmon sperm DNA (ssDNA). The ssDNA is included to prevent non-specific binding of the probe to the filter.

cDNA probes were labelled by the random hexanucleotide method using a Multiprime DNA labelling system (Amersham International U.K.). Linear double stranded DNA is denatured and added to Multiprime DNA labeling buffer which contains deoxy-adenine triphosphate (dATP), deoxy-guanine triphosphate (dGTP) and deoxy-thymidine triphosphate (dTTP) in Tris-HCl, pH7.8, magnesium chloride and 2-mercaptoethanol. Random sequence hexamers (primer solution) are added which recognise complementary sequences on the probe DNA strand and bind. The 'Klenow' fragment of DNA polymerase I initiates synthesis of DNA strands which are complementary to the probe and are radiolabelled by addition of ^{32}P deoxy-cytosine triphosphate ([32]PdCTP).

The labelling procedure is as follows:

10-20ng of probe DNA (1-10μl) are added to sufficient deionised water to make a final volume of 25μl in a small eppendorf tube. The probe is heated in a boiling water bath for 5min to denature the DNA and the tube quenched on ice. 5μl of buffer, 2.5μl of primer, 2.5μl of [32]PdCTP and 1μl of enzyme (Klenow) are added and the reaction allowed to proceed for 3-5h at room temperature. At the end of the incubation 75μl of water are added and the reaction stopped with 1μl of 500mM EDTA (final concentration 20mM EDTA). The enzyme requires Ca^{2+} and Mg^{2+} ions for the reaction to proceed, EDTA

binds these ions and thus stops the reaction. The probe is boiled again to release single stranded labelled DNA fragments and added to the hybridisation mixture.

Filters were hybridised at 50°C overnight and then subjected to high stringency washing. Filters were washed for 2h at 65°C in prewarmed buffer (2X SSC, 0.5% SDS), for 15min in 0.1X SSC and rinsed finally in 2X SSC. The filters were wrapped in cling film and exposed to X ray film in cassettes with intensifying screens. Films were developed after 24-72h.

To normalise the signal for equal loading of the RNA, filters were stripped by soaking in a boiling solution of 0.5% SDS which was allowed to cool to room temperature. Filters were reprobed for human β actin.

CHAPTER 3

Transfection of human osteoblast-like cells

ABSTRACT

With a view to obtaining a self-renewing population of cells expressing the osteoblast phenotype, primary cultures of trabecular bone-derived cells were transfected with SV40 DNA using the calcium phosphate/DNA co-precipitation technique. 1-2 weeks post-transfection numerous foci of mitotic cells appeared in the transfected population. The colonies were plucked and rapidly expanded to provide large numbers of cells. The cell lines expressed the enzyme alkaline phosphatase and certain cell lines responded to 1,25D by increased enzyme activity. In order to obtain clonal osteoblast-like cell lines, two lines were cloned by limiting dilution. Many of the clones entered crisis and senesced but two clonal cell lines survived and expressed very high levels of alkaline phospatase. Immortalisation of primary cells is a useful technique for obtaining large numbers of cells expressing the parent phenotype. The cell lines have been given the name BOP for Bath Osteoblast Prototype.

3.1   INTRODUCTION

Primary cultures of cells, obtained as outgrowths from tissues and organs, provide useful models for *in vitro* studies of cell function. Rodent cells form continuous cell lines in culture spontaneously whereas human cells survive only a limited number of generations *in vitro*. Clonal cell lines expressing the full phenotype of the mature osteoblast have been obtained by digestion of murine calvariae (Sudo *et al*, 1983). Clonal cells of human origin are more difficult to obtain, but are desirable as species differences occur with respect to the effects of hormones and cytokines on cells. Furthermore, studies on animal cells may not be directly relevant to the understanding of disorders of bone metabolism in humans.

Human osteoblast-like cells, derived from explants of trabecular bone obtained at surgery, have been well characterised and express all the

hallmarks of the mature osteoblast. The responses of these cells to calciotropic hormones, growth factors and immune-cell derived cytokines have been explored extensively and are more fully described in chapters 4 and 5. The disadvantages of these primary cultures are their limited capacity for growth *in vitro,* making them difficult to obtain in large numbers, and the heterogeneity of the trabecular bone cell population. Cells of different stages of the osteoblast differentiation pathway are likely to be present and contamination by other cell types, such as fibroblasts and marrow stromal cells, may also occur.

To overcome these problems osteosarcoma cell lines of human origin have been described and used for a variety of biochemical studies, large numbers of clonal cells are readily obtained and are convenient for *in vitro* use. The cells express some of the characteristics of mature osteoblasts but not all the phenotypic markers are present in a particular cell line. The transformed nature of the osteosarcoma cell lines may account for alterations in responsiveness to various hormones, and the loss of certain osteoblastic characteristics.

The technique of immortalising cells by transfection with oncogenic viruses and exogenous DNA has been widely used both to study the molecular and genetic events leading to transformation of cells, and to provide rapidly growing populations of cells derived from primary cultures. Rodent cells are more readily transformed by cellular oncogenes than are human cells, transfection with an oncogenic virus such as simian virus 40 (SV40) has proved more successful as an inducer of anchorage independent growth and loss of contact inhibition in human cells (Mayne *et al,* 1986, Oshima *et al,* 1977, Huschtscha and Holliday, 1983).

Land, Parada and Weinberg (1983) describe the transformation of rat embryo fibroblasts (REF) using the *ras* and *myc* oncogenes. When REF cells were transfected with either cloned *ras* or *myc* oncogenes the cells

failed to transform as assessed by their inability to form colonies in soft agar. When cells were cotransfected with both *ras* and *myc* oncogenes, anchorage independent growth was induced and the cells produced rapidly growing fibrosarcomas in nude mice. Thus for the tumorigenic conversion of primary fibroblasts, expression of a single oncogene was insufficient.

Simian virus 40 (SV40) is a member of the papova group of small, nonenveloped DNA viruses and causes lytic infection of permissive monkey cells. The genome of SV40 contains an early region encoding the large T and small t tumour antigens, the late region encodes the viral coat proteins. The origin of replication (ori) is found between the early and late regions. The SV40 T antigen is a large multifunctional protein of 90,000 daltons that binds specifically to the SV40 origin of replication and acts as an initiator of DNA replication (Myers *et al*, 1981).

SV40 has been used to transform a wide range of cell types such as human fibroblasts (Huschtscha and Holliday, 1983; Oshima *et al*, 1977), human juxtaglomerular cells (Pinet *et al*, 1985) bone marrow cultures (Singer *et al*, 1987) and rat calvarial osteoblasts (Heath *et al*, 1989). Table 3.1 shows a variety of vectors that were used in an attempt to transform pancreatic β islet cells and thyroid epithelial cells (Marini, personal communication). SV40 was the only vector capable of establishing a cell line from these primary cultures. SV40 would seem to be far more potent at inducing rapid cell growth and loss of contact inhibition in cells than cellular oncogenes.

Small, Gluzman and Ozer (1982) describe the transformation of human fibroblasts with both wild-type SV40 and an SV40 construct that lacked the origin of DNA replication (ori⁻). The vector SV40 pSV ori⁻ was demonstrably more efficient at inducing a loss of contact inhibition and inducing anchorage independent growth of transfected cells. When transfected cells were grown in soft agar, a greater number of larger colonies were obtained

VECTORS	Line established from:	Reference	Times tried and No. of lines established:			
			Pancreas		Thyroid	
SV40 (pX-8)	Rat F-11	Fromm and Berg, 1982	12	2	33	1
	Human fibroblasts	Mayne et al, 1986				
SV40 (PAS)	Human juxtaglomerular	Pinet et al, 1985	10	1	21	1
	cell tumour					
pSV Neo	Human thyrocytes	Whitley et al, 1987	8	-	13	-
Adenovirus Ad 12S/13S	Baby rat kidney cells	Haley et al, 1984	7	-	11	-
c-myc (SV40 promoter)	Rat embryo fibroblasts	Land, Parada and	5	-	4	-
and v-myc		Weinberg, 1983	5	-	4	-

Table 3.1   Vectors used to immortalise pancreatic β islet cells and thyroid epithelial cells. Cell lines were successfully established by transfection with SV40 DNA.

from cells transfected with ori⁻ than with wild type SV40. These authors suggest that the enhanced transformation efficiency of the origin defective vector was due to its inability to replicate in semipermissive cells.

Expression of the SV40 large T antigen is thought to be essential for inducing focus formation by transfected cells. Human fibroblasts transfected with an SV40 construct lacking the T antigen sequences displayed contact - inhibited growth and no morphological transformation occurred (Mayne *et al*, 1986). Further evidence of a role for T antigen in the initiation and maintenance of immortalisation was provided by Radna *et al* (1989). These workers isolated transformants after introduction of an origin-defective SV40 genome encoding a heat-labile large T antigen. The transformed human fibroblasts grew rapidly at 35°C but failed to form colonies at 39°C, whereas cells transfected with wild-type T antigen were able to grow rapidly at the higher temperature.

A characteristic of SV40 transformed cells is an initial limited growth potential. After a number of generations the cells enter a non-proliferating degenerative stage termed "crisis" and many cells senesce. A few cells are capable of rejuvenating from crisis and continue growing to form stable, post-crisis lines that can proliferate indefinitely. The frequency of such stable cell lines occurring is very low (Huschtscha and Holliday, 1982).

A number of techniques have been employed to facilitate the entry of exogenous DNA into cells in order to transform them. The efficiency of stable transfection varies widely depending on the cell type under study as different cells vary in their ability to take up and express exogenously added DNA. The most widely used method is transfection mediated by either calcium phosphate or DEAE-dextran. The mechanism of action of DEAE-dextran is not known but it is thought that binding of the polymer to DNA may promote endocytosis of the DNA. Transfection by DEAE- dextran is generally used only for transient expression of cloned genes rather than for

67

stable transfection. The technique is unsatisfactory for many cell types which may be due to the polymer's toxicity (Sambrook, Fritsch and Maniatis, 1989).

Uptake of DNA into cells may be markedly enhanced if calcium chloride is added to the DNA prior to addition to the cell monolayers (Graham and van der Eb, 1973). Formation of a calcium phosphate/DNA coprecipitate by mixing calcium chloride with sodium hydrogen phosphate is now a well-established method for transfecting cells. The precipitate is adsorbed onto the surface of the infected cells and may facilitate the entry of DNA into the cell by endocytosis, although the precise mechanism is unknown.

For cells resistant to transfection by the calcium phosphate technique, the use of pulsed electric fields (electroporation) to introduce DNA into cells has a high degree of efficiency of transformation. Brief, high voltage electric fields are passed through the cells to induce the uptake of DNA presumably through transient micropores in the cell membrane (Andreason and Evans, 1989). Disadvantages of this technique are that optimal conditions of field strength, length of pulse and temperature must be determined for every cell type studied and the cell viability of electroporated cells is very low (20-50% of cells survive) (Sambrook, Fritsch and Maniatis, 1989; Aizawa *et al*, 1990).

For the immortalisation of human trabecular bone-derived cells, the calcium phosphate/DNA coprecipitation technique was employed using a SV40 origin defective construct (called px-8). Following incubation of the cell monolayers with the precipitate, cells were subjected to a glycerol shock. This modification may further facilitate the uptake of DNA by the cells (Parker and Stark, 1979), thereby enhancing the efficiency of immortalisation.

3.2.   METHODS

Definition of the SV40 vector

Human osteoblast-like cells were transfected with a recombinant vector consisting of a 2.3 Kb portion of the plasmid pBR322, SV40 late region, and SV40 early region DNA which encodes large T and small t antigens. Ori, the sequence needed for initiation of SV40 DNA, was deleted by cleaᵛage of the Bgl I site followed by Bal 31 exonuclease digest of each end with insertion of the 8bp XhoI linker. The resultant pX-8 vector was shown to have a transforming efficiency of 100% of wild-type SV40 DNA. However, pX-8 fails to grow and replicate its DNA when transfected into COS cells (Fromm and Berg 1982).

Definition of the pX-8 vector was carried out by restriction endonuclease digests of vector DNA. The following restriction enzymes were used:-

1. XhoI   single digest
2. Eco RI   single digest
3. Bam HI and Xho I   double digest
4. Bam HI and Hpa II   double digest
5. Pst I   single digest

2 μg of plasmid DNA, in each of 5 microfuge tubes, were dissolved in water to make a final volume of 20μl. 2μl of the appropriate buffer for the enzyme used was added to each tube with 1μl of enzyme. All tubes were incubated in a 37°C water bath for 1h. 2μl of DNA loading buffer were then added to the tubes. DNA digests were electrophoresed on a 0.8% agarose gel with 0.01% ethidium bromide for 1h with a 50mV charge. Commercially obtained molecular size markers were included on the gel. These were EcoR I and Hind III digests of λDNA, and a Hind III digest of λ DNA.

Following electrophoresis the gel was photographed on a UV light

transilluminator, with a ruler placed next to the gel to allow measurement of

the distance travelled by the DNA fragments. A graph of $\log_{10}$ base pairs

(ordinate) versus distance moved in mm (abscissa) was plotted for the

molecular markers. The size of DNA fragments obtained by restriction

endonuclease digests of pX-8 could then be calculated by extrapolation

from the curve.

Transfection of osteoblast-like cells

Primary cultures of osteoblasts were seeded at 5 X 10^5 cells per 100mm

tissue culture dish and maintained in the presence of bone explants in

complete MEM for 18h prior to transfection. After 14h explants were removed

and the medium replaced with fresh complete tissue culture medium.

Plasmid DNA, sterilised in absolute ethanol, was dissolved in 10mM Tris

pH 8, 1mM EDTA. A $Ca_3 (PO_4)_2$/ DNA co-precipitate was prepared by

adding 500μl of 2X DNA precipitation buffer (50mM Hepes, pH 7.05, 1.5mM

Na_2HPO_4, 10mM KCl, 280mM NaCl, 12mM glucose) to 62μl of 2M $CaCl_2$

with 20μg plasmid DNA and sterile dH_2O to a final volume of 1ml. The

solutions were mixed in a microfuge tube in the presence of bubbling air and

the precipitate allowed to form for 20min at room temperature. 1ml of

precipitate was added to the 10ml of complete medium in each 100mm

tissue culture dish. Cells were incubated at 37°C for 4h. Cell layers were

then washed with serum free medium and subjected to a glycerol shock.

1ml of glycerol shocking buffer (3 parts glycerol, 7 parts dH_2O, 10 parts

2XDNA precipitation buffer) was added to each cell layer in the absence of

medium and the cells were incubated for 2min at room temperature. The

glycerol was then aspirated and the cell layers were washed again in serum

free MEM and maintained in complete medium. The culture medium was

changed every 3-4 days until colonies of mitotic cells appeared 1-2 weeks

post transfection. A total of 19 100mm dishes were treated in this way.

Culture dishes were placed under an inverted microscope stationed inside a laminar flow cabinet. Individual colonies were plucked using finely drawn-out Pasteur pipettes. Colonies were transferred into microfuge tubes, centrifuged for 5min. at low speed, washed once in PBS and dispersed with trypsin/EDTA. The reaction was stopped with 1ml complete medium, the tubes were centrifuged again and the colonies resuspended in 500μl complete medium. Individual colonies were seeded into separate 16mm wells of 48 well plates and colonies were numbered.

At confluence, cell lines were passaged as described in Chapter 2 and expanded into 24-well plates. Cell lines were then passaged again, counted, and seeded into 30mm wells with approximately 10^4 cells of each line seeded into microtitre wells. Cells in microtitre wells were allowed to settle for 24h then incubated for 72 or 96h with or without 10^{-8}M 1,25D in MEM+ 5% charcoal stripped FCS (CSFCS) supplemented with vitamin K_1, and ascorbate. Medium from the wells was discarded, cell layers washed with PBS, and ALP assays performed by addition of assay buffer directly to the cell layers. Cells in 6 well plates were expanded to 60mm tissue culture dishes and then to 100mm culture dishes. Cells were continuously maintained in 100mm culture dishes and cryopreserved.

Cloning by Limiting Dilution

Two cell lines, BOP 26 and 37 were cloned by limiting dilution. Cells were passaged as described in Chapter 2, counted, and resuspended to a cell density of 5 cells/ml in complete culture medium. 200μl of cell suspension were added to each well of 5 96-well microtitre plates, to give a cell density of one cell per well. Cell suspensions of 5 cells/2ml and 25 cells/ml were also prepared and used to seed 5 microtitre plates each, at 1 cell per two wells and 5 cells per well respectively.

Every 3-4 days the medium in the wells was replaced with fresh culture medium supplemented with medium conditioned by the parent cell line.

Growth of the cells was monitored daily by viewing plates under an inverted microscope. Confluent wells were identified and individual clones harvested as follows: medium was aspirated and the cell layers washed with 200μl of PBS. Wells were incubated for 5min at 37°C with 50μl of trypsin/EDTA per well. 200μl of complete medium were then added to each cell suspension. Individual clones were labelled alphabetically (e.g. 37A-Z, 37AA-WW) and expanded. Two reclones of each cell line (37WW, 26A) were subjected to a further round of limiting dilution cloning, in the manner described above. Clones of 37WW and 26A (labelled in numerical order) were expanded and cryopreserved.

Characterisation of reclones

Clones 26A4, 26A5, 26A12, 37WW3, 37WW5 and 37WW8 were revived and maintained in complete culture medium. Cells of two clones, 37WW3 and 37WW8, were seeded at 4×10^4/ml in 24-well dishes and allowed to settle for 24h. Cells were treated with 10^{-8}M 1,25D in fresh defined medium for 48h and cell layers were harvested for ALP assay and total protein as detailed in Chapter 2.

Cell division time

Cells of line BOP 26 were seeded at 4×10^4/ml in 24-well culture dishes and allowed to settle for 6h. Cells from 4 wells were harvested with trypsin/EDTA, resuspended in complete medium and counted with a Nebauer haemocytometer. 4 wells were counted every 24h and in all other wells half the medium was replaced daily. To provide a comparison a similar growth curve was obtained with cells of the human osteosarcoma line MG-63. Primary cultures of human osteoblast-like cells were seeded at 2×10^4/ml and counted daily (growth curve provided by J. Clover).

3.3   RESULTS

Definition of the SV40 vector

The linearised pX-8 vector was approximately 7.6 Kb in size. The fragments obtained by restriction endonuclease digest are shown in table 3.2, following extrapolation of distance moved through the gel from the graph (Figure 3.1). The SV40 early region was found to be 3Kb in size, 1.5Kb smaller than was reported by Fromm and Berg (1982). The 3Kb fragment containing early region genes was subsequently used for hybridisation of southern blots (see chapter 4). The SV40 late region was 1.44Kb in size.

Focus formation by transfected cells

1-2 weeks post-transfection, numerous foci of rounded, mitotic cells were observed in every plate of transfected cultures. Such colonies do not appear in primary cultures of normal trabecular bone cells. Initially, a total of 82 foci were plucked and dispersed as described in the methods sections of this chapter. Certain colonies were lost in the dispersion procedure, some colonies failed to grow and one was lost through bacterial contamination. Of 24 colonies that survived further expansion, 12 were able to support continuous, rapid growth *in vitro* and could be maintained for several passages. These cell lines were cryopreserved and quickly and readily revived after thawing.

The original parent cultures of transfected cells were returned to the incubator and 3 weeks later many more colonies were plucked. These were expanded and provided a further 62 cell lines all of which were cryospreserved after 4-5 passages.

The appearance of the colonies of mitotic cells, observed under phase contrast microscopy, is shown in figure 3.3(a). The phenotypic alteration of the cells, indicative of a loss of contact inhibition of growth, was also observed in post confluent cultures after a number of passages. Fig. 3.3b

shows a typical focus of mitotic cells observed in cell line number 4 at passage 4. After the first 12 cell lines had been maintained in culture for up to seven or eight passages, foci with the appearance of fig. 3.3a appeared in post confluent cultures of cells in 100mm dishes. Foci were often visible to the naked eye.

Morphology of the cell lines

The appearance of the original parent cultures of trabecular bone cells viewed under phase contrast microscopy is shown in figure 3.3c, displaying the characteristic fibroblastic morphology and heterogeneity of these cells. The morphology of cell line Bath Osteoblast Prototype (BOP) 26 is shown in figure 3.3d.

Expansion of immortalised cell lines

The capacity of SV40 transfected cells for rapid growth is illustrated in figure 3.4. These graphs demonstrate the large number of cells that could be obtained assuming that all harvested cells were replated at each passage. For example, BOP 15 seeded at 2×10^4 cells in one well of a 6 well plate could be expanded to 2.7×10^{10} cells in 40 days.

The growth curve (Fig. 3.5) shows that BOP 26 has a cell division time of 30h compared with 26h for MG-63 and 120h for primary cultures of trabecular bone cells.

Alkaline phosphatase activity

The expression of the enzyme alkaline phosphatase by SV40 immortalised osteoblasts is shown in table 3.3. A range of activities was observed, cell lines 26, 31 and 33 for example having much lower activity than cell lines 19, 71 and 72. Treatment with 10^{-8}M 1,25D enhanced ALP activity in cell lines 16, 31, and 62, but had no effect on cell lines 7, 33 and 72.

<u>Growth of clonal cell lines</u>

Two cell lines (BOP 26 and 37) were chosen for cloning by limiting dilution as they closely resembled the parent cultures with respect to the osteoblast phenotype (described in chapter 4). After two rounds of limiting dilution cloning many cell lines entered crisis and senesced. 3 cell lines from BOP 37 and five from BOP 26 grew sufficiently to be cryopreserved. After thawing of cell lines 37WW3, 37WW5 and 37WW8, 26A4, 26A5 and 26A12 only the two clonal cell lines 37WW3 and 37WW8 provided sufficient numbers of cells for characterisation of ALP activity. As displayed in table 3.4 the clonal cell lines expressed very high levels of ALP activity and were also capable of responding to 1,25D by enhanced levels of the enzyme.
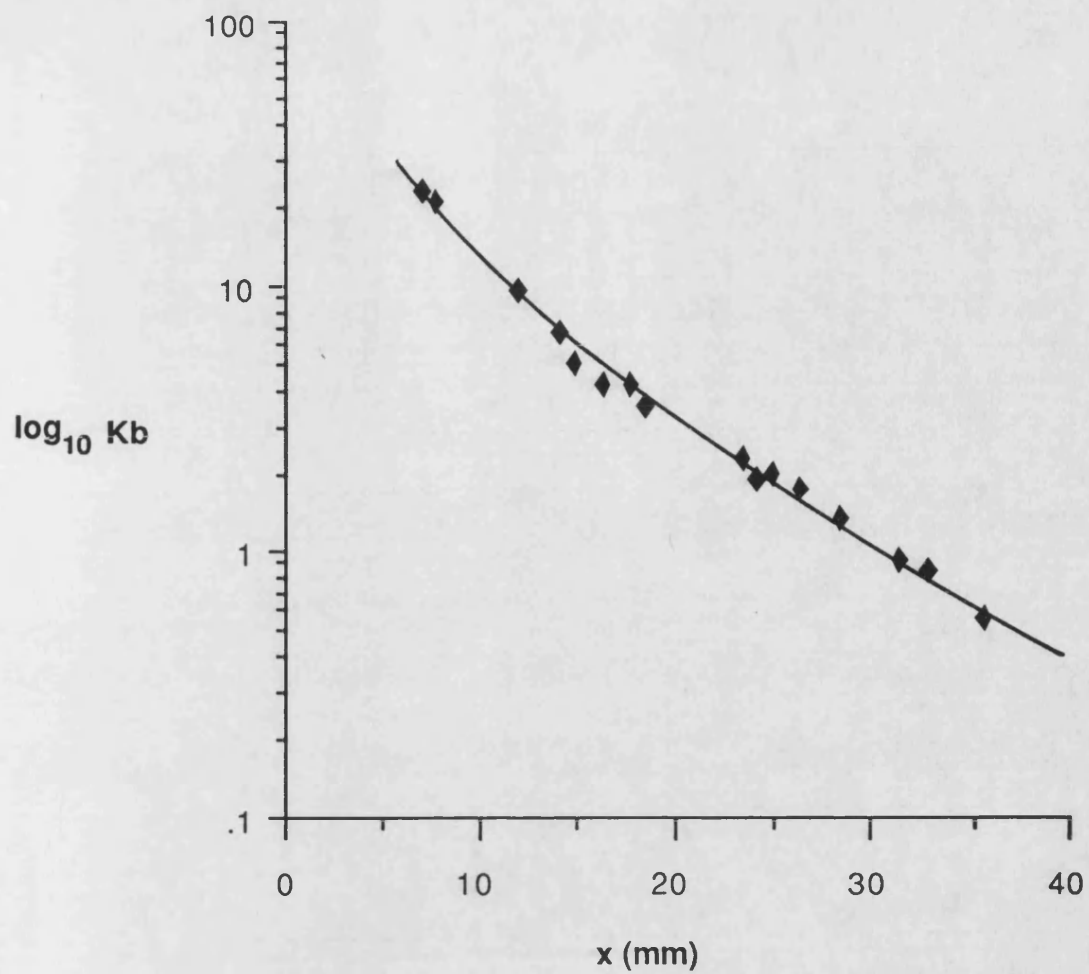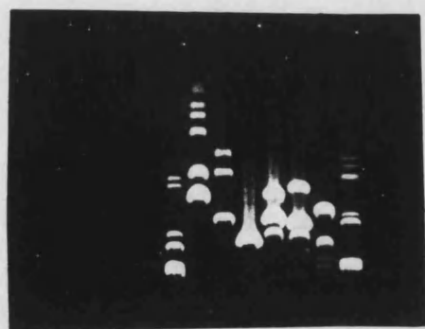
Fig. 3.1   (a) Log$_{10}$ Kb versus distance moved through the gel for Eco RI

and Hind III digests of λ DNA subjected to electrophoresis.   (b) Agarose gel

of the DNA digests.   Lane A, Hind III digest of λ DNA; lanes B-F, digests of

pX-8, B: Hpa II and Bam HI, C: Pst I, D: Xho I, E: Bam HI and Xho I, F: EcoRI.

Lane G, uncut plasmid.   Lane H, Hind III and EcoRi digests of λ DNA.

Restriction enzyme	Fragment size (Kb)
Xho I	7.6 (linearised vector)
Eco RI	7.4 5.4 2.6
Bam HI + Xho I	6.2 5 2.9
Pst I	5 1.9 1.2
Bam HI + Hap II	3 1.9 0.76 0.56 0.47 0.35

Table 3.2   Restriction digest fragments of the pX-8 vector, obtained by incubation of vector DNA with the appropriate enzyme, followed by gel electrophoresis as described in methods. Fragment size was calculated by extrapolation of distance moved from the graph (Fig. 3.1)
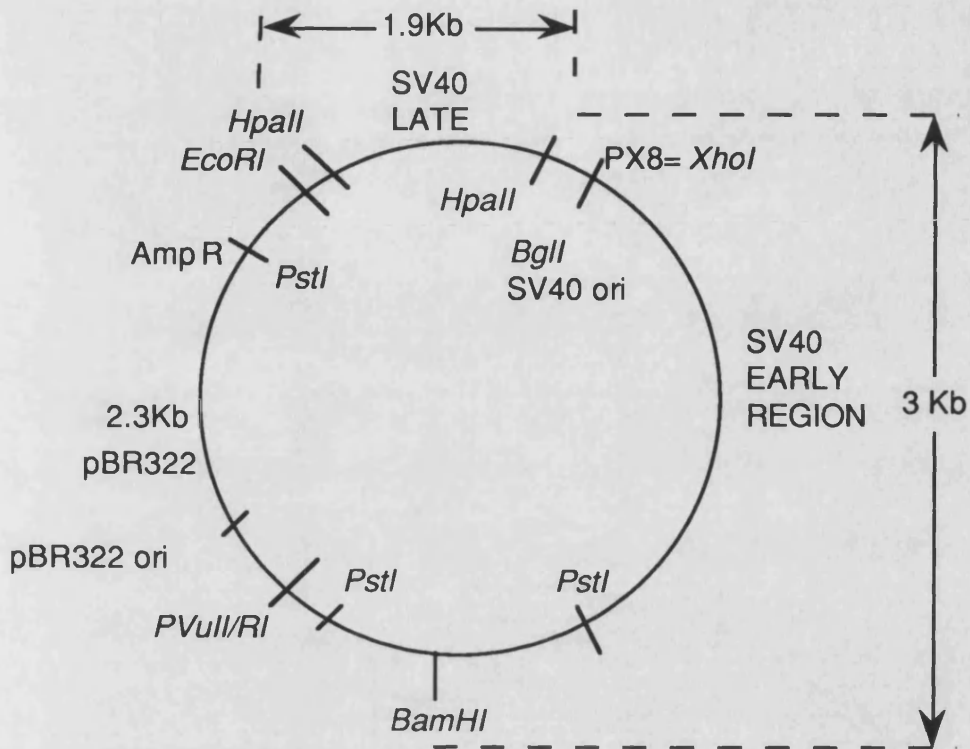
Fig. 3.2 Restriction map of the pX-8 vector with sizes of the SV-40 early and late regions indicated. (Modified from Fromm and Berg, 1982).

Fig. 3.3. Morphology of parent and SV40-transfected cells. Primary cultures of human osteoblast-like cells were transfected with pX-8 and maintained in complete medium. (a) Focus formation by transfected cells. Discrete foci of rapidly dividing cells were observed in all transfected cultures (arrowed). (b) A focus of mitotic cells in a culture of cell line BOP 4 at passage 4 (arrowed). (c) The characteristic heterogeneous morphology of parent cultures of ostoblasts; (d) The morphology of cell line BOP 26, the cells were polygonal and presented a more homogeneous appearance than primary cultures. Magnification X 100.

(d)

(c)

(b)
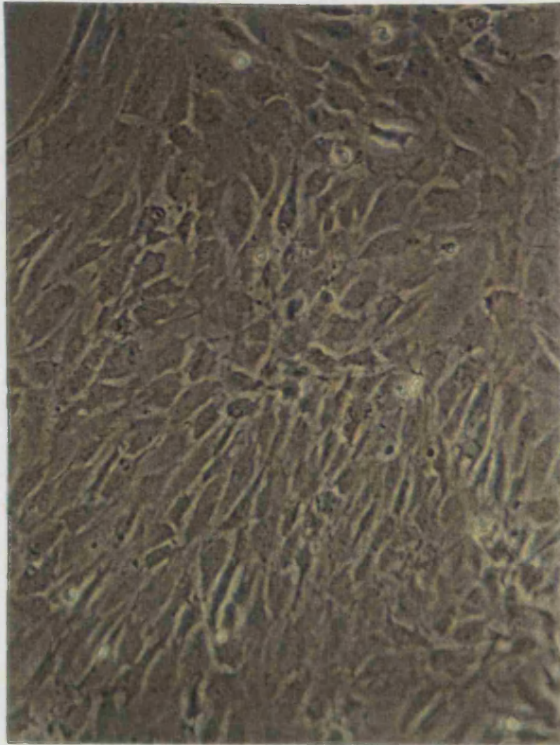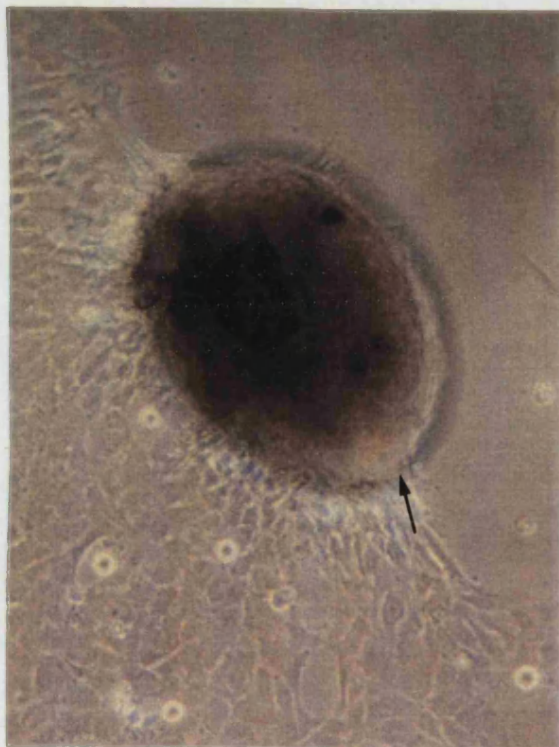
(e)
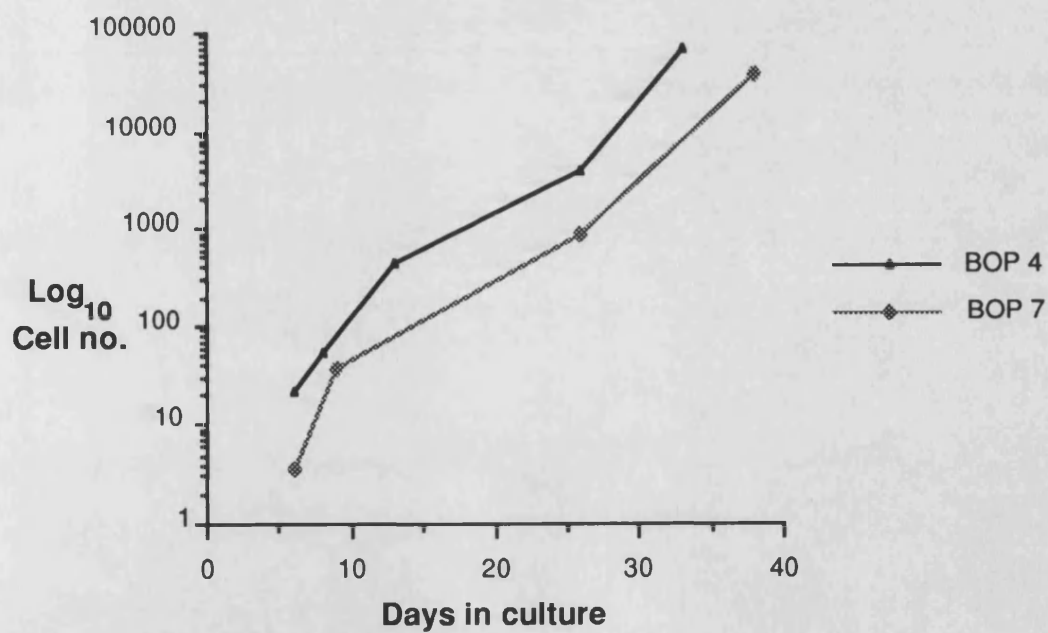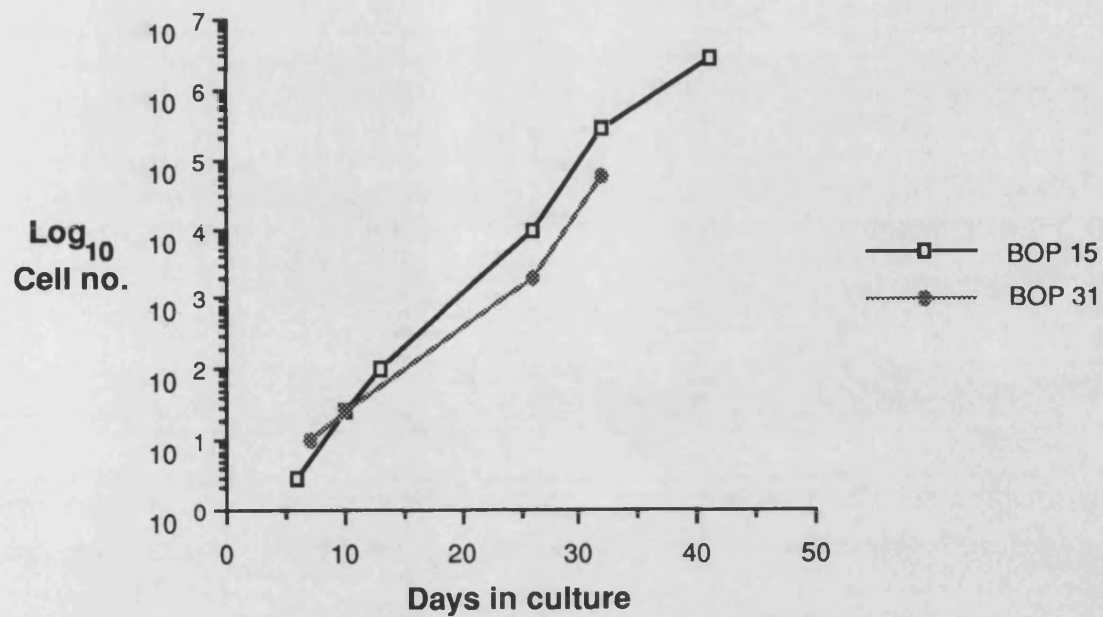
Fig. 3.4   The rapid expansion of four cell lines maintained in continuous
culture. Each point on the graph represents one passage. Cell number was
calculated assuming all harvested cells had been replated at each passage.
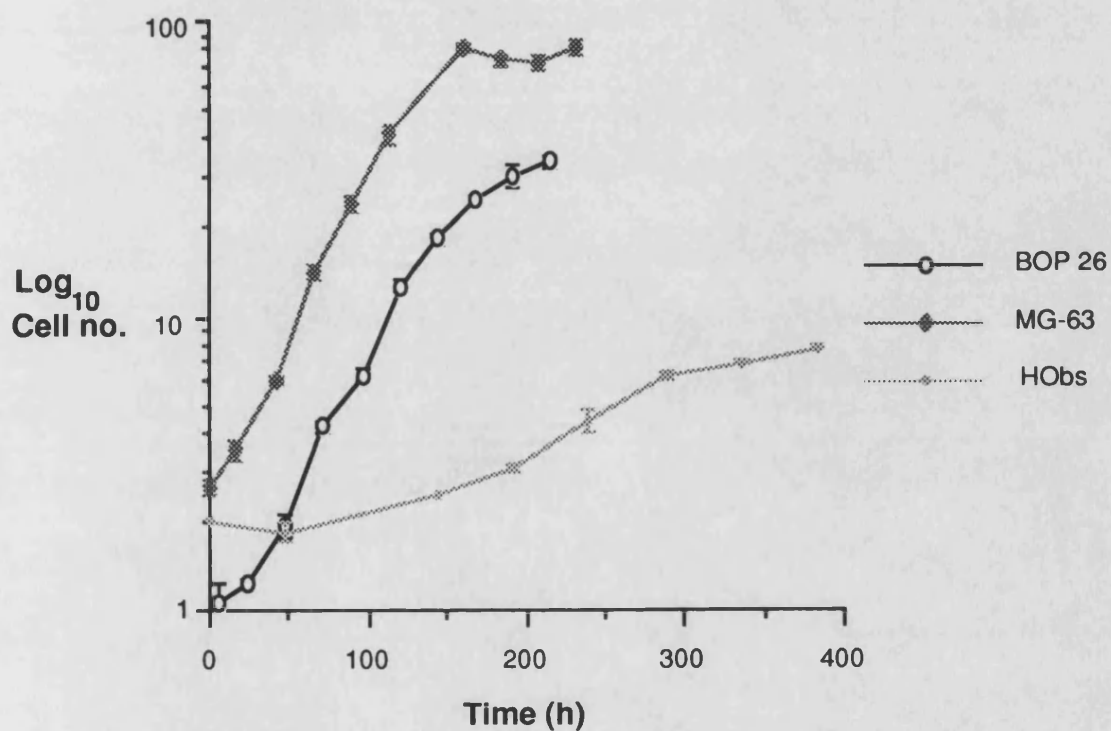
Fig. 3.5   Growth curves for the immortalised osteoblast-like cell line BOP 26, cells of the human osteosarcoma line MG-63 and primary cultures of human osteoblasts (HObs). Cell counts were performed on trypsinised cell layers after 6h and then every 24h. Error bars show standard error of the mean (SEM), n=4.

Alkaline phosphatase

(μmoles/min)

BOP	Control	1,25D	T/C	Time with 1,25D (h).
4	0.081	>0.093	-	96
7	0.013	0.0012	1.1	96
12	0.052	>0.093	-	96
16	0.004	0.014	3.5	96
18	0.015	0.023	1.5	96
19	>0.02	>0.02	-	72
26	0.008	0.005	0.6	72
31	0.005	0.023	4.6	72
33	0.002	0.002	1.0	72
37	0.065	0.057	0.9	72
62	0.002	0.032	16	72
70	0.027	0.040	1.5	72
71	>0.08	0.050	-	72
72	0.042	0.039	0.9	72
74	0.040	>0.067	-	72

Table 3.3   Alkaline phosphatase activity in immortalised cell lines. Cells were seeded into single microtitre wells and allowed to settle for 24h. Cells were then treated with 10^{-8}M 1,25D for the length of time indicated and cell layers were assayed for ALP activity in situ as described in methods. T/C is the ratio of 1,25D treated to control values.

Alkaline phosphatase umoles/min/mg			
Clone	Control	1,25D	T/C
37WW3	4.81 ± 0.92	7.68 ± 0.55	1.6
37WW8	9.07 ± 0.52	16.49 ± 0.92	1.8

Table 3.4   Alkaline phosphatase activity in clonal cell lines. Cells were treated with 1,25D for 48h as described in methods. ALP and total protein assays were performed on cell layers. Data are shown ± standard error of the mean (SEM), n=4.

3.4 DISCUSSION

The calcium phosphate/DNA coprecipitation technique has been used to transfect a variety of cell types such as human juxtaglomerular cells (Pinet *et al*, 1985), neonatal rat Schwann cells (Tennekoon *et al*, 1987) and human fibroblasts (Mayne *et al*, 1986). Primary cultures of human trabecular bone-derived cells were readily transformed by origin defective SV40 DNA using the calcium phosphate method. The vector induced a loss of contact inhibition and proved to be potent at inducing the cells to undergo repeated mitotic divisions. The foci of rapidly dividing cells observed in transfected cultures also appeared in confluent cell lines that had been maintained *in vitro* for a number of passages (Fig. 3.1) displaying that the initial loss of contact inhibition of growth was sustained for many generations.

Comparison of the growth curves for human osteoblasts and immortalised BOP cells demonstrates the rapid proliferation rate of the latter (Fig. 3.5). Primary cultures divided in 125h whereas BOP cells took only 30h to divide, slightly more than the cell cycle time for human osteosarcoma cells (MG-63) which completed division in 26h. This is in contrast with the observations of Huschtscha and Holliday (1983). These authors transfected human foetal lung fibroblasts (MRC-5) with wild-type SV40 virus strains and obtained two immortalised cell lines. The parent cultures, at an early passage, divided in 18-21h whereas the immortalised cells took 38h to divide. Human bone marrow stromal cells transfected with a recombinant SV40-adenovirus vector divided in 30h (Aizawa *et al*, 1991). Cells of different origin behave differently following transfection and these differences may be observed irrespective of the vector used, for example human juxtaglomerular cells transfected with origin defective SV40 were slow to divide and were subcultured once a month (Pinet *et al*, 1985).

Immortalisation of cells with SV40 is clearly a useful procedure for obtaining large numbers of cells derived from parent cultures. From an

initial population of 9.5×10^6 trabecular bone cells a total of 74 cell lines were obtained each capable of providing over 10^{10} cells. The mechanism by which SV40 effects this dramatic transformation is unknown but expression of the large tumour antigen has been shown to be essential for the induction of anchorage independent growth of transfected cells (Mayne *et al*, 1986; Radna *et al*, 1989). Expression of the large T antigen by BOP cells is demonstrated in chapter 4.

Cell lines transfected with SV40 typically enter a period termed "crisis" after a limited number of divisions; cells are shed into the medium and remaining cells senesce. Some cells may survive and emerge as stable, post-crisis immortalised lines but the frequency of such lines is low (Oshima *et al*, 1977; Huschtscha and Holliday, 1983). When BOP cells were subjected to two rounds of limiting dilution cloning, in order to obtain clonal cell lines, many clones failed to divide and large, irregular, senescent cells were observed. Of six clonal lines that were cryopreserved and revived only two survived and grew in sufficient numbers for the assay of alkaline phosphatase activity. These may represent stable post-crisis cell lines.

Use of a dominant selectable marker has been reported to improve the frequency of establishing a stable, immortalised cell-line. Mayne *et al*, (1986) employed an origin-defective SV40 construct encoding T antigen and the bacterial gene xanthine-guanine phosphoribosyl transferase (gpt). Human fibroblasts transfected with this plasmid were able to grow in medium containing the metabolic inhibitors mycophenolic acid and aminopterin. Other workers have used the neomycin resistance gene as a selectable marker (Heath *et al*, 1989; Kellerman *et al*, 1990). Use of a selectable marker, however, does not prevent the crisis stage of pre-immortalised cells from occurring (Mayne *et al*, 1986; Keeting *et al*, 1992).

Primary cultures of trabecular bone cells express both constitutive and 1,25D-inducible alkaline phosphatase activity (Beresford, Gallagher and

Russell, 1986). This enzyme is not osteoblast-specific but is a characteristic of the cell type and is widely used as a marker of the osteoblast phenotype. Human osteoblast-like cells transfected with SV40 DNA produced the enzyme alkaline phosphatase and activity of the enzyme was enhanced by 1,25D. The two clonal cell lines 37WW3 and 37WW8 also expressed very high levels of the enzyme and responded to 1,25D by an enhancement of enzyme activity.

Large numbers of rapidly dividing cells were obtained following transfection of primary cultures of human osteoblast-like cells with SV40 DNA. The cells retained the capacity to express an enzyme characteristic of the parent cultures and to respond to the osteotropic hormone 1,25D. The cell lines had a limited growth potential and are likely to represent the "pre-immortalised" state described by Huschtscha and Holliday (1983). Two cell lines that emerged following limiting dilution cloning may represent stable post-crisis lines.

CHAPTER 4

Characterisation of immortalised cell lines.

ABSTRACT

Rapidly dividing BOP cells were characterised for established markers of the osteoblast phenotype. Thirteen cell lines expressed alkaline phosphatase activity which was enhanced by 1,25D and the cells responded to 1,25D by production of the mineralised tissue-specific protein osteocalcin. BOP cells displayed the ability to produce osteonectin and bone sialoprotein constitutively and production of these proteins was unaffected by 1,25D. The cells secreted thrombospondin and 1,25D elicited variable responses with respect to production of this protein. The cells displayed the ability to mineralise *in vitro* as visualised by both von Kossa and Alizerin Red staining. mRNA for Type I Collagen was detected by Northern blotting in seven cell lines. Two cell lines produced PGE_2 in response to IL-1. Incorporation of SV40 DNA was confirmed by Southern blotting and expression of large T antigen was demonstrated by immunofluorescence. The data demonstrate that immortalised cell lines derived by transfection of primary cultures of osteoblasts with SV40 DNA are capable of retaining the full phenotype of the parent cells.

4.1   INTRODUCTION

An essential requirement of any model used for the study of osteoblast function *in vitro* is the expression of the phenotypic characteristics of osteoblasts *in vivo*. A variety of current models are described in chapter 1 (Table 1.1). Primary cultures of human osteoblasts derived as outgrowths from trabecular bone have been extensively characterised with respect to extracellular matrix production and responsiveness to calciotropic hormones. The cells express high levels of the bone-liver-kidney isoenzyme alkaline phosphatase and enzyme activity is enhanced by 1,25D (Beresford, Gallagher and Russell, 1986). The cells produce predominantly type I collagen but a small percentage of the total collagen produced is type III

which may indicate contamination of the cultures by fibroblasts (Aufmkolk, Hauschka and Schwartz, 1985). Cells maintained in medium supplemented with β glycerophosphate, which serves as a substrate for alkaline phosphatase, may produce a thick, extracellular, mineralised matrix *in vitro* (Gehron Robey and Termine, 1985). Production of bony nodules by human bone cells transplanted intramuscularly into mice has also been reported although some strains of cells did not display *in vivo* osteogenic activity (Yamamoto, Ecarot and Glorieux, 1991).

A characteristic of many osteoblast model systems from different sources is a PTH responsive adenylate cyclase. PTH also stimulates the proliferation of human bone-derived cells and enhances secretion of PGE_2 (MacDonald, Gallagher and Russell, 1986; MacDonald *et al*, 1984). Osteoblasts also produce PGE_2 in response to IL-1 (Gowen, 1988).

The most specific marker available for the identification of osteoblast-like cells is the noncollagenous matrix protein osteocalcin. Primary cultures of trabecular bone cells produce low or undetectable levels of osteocalcin which are enhanced by 1,25D (Aufmkolk, Hauschka and Schwartz, 1985; Yamamoto, Ecarot and Glorieux, 1991). Human osteoblast-like cells also secrete the matrix proteins osteonectin, bone proteoglycan and thrombospondin (Gehron Robey and Termine, 1985; Gehron Robey *et al*, 1989).

The ability of cell lines obtained by transfection of primary cultures with SV40 DNA to retain the full osteoblastic phenotype was assessed. BOP cells were characterised for the production of noncollagenous matrix proteins, expression of type I collagen mRNA, synthesis of PGE_2 and production of a mineralised matrix.

4.2   METHODS

Cell lines were incubated in the presence and absence of 1,25D and
characterised for alkaline phosphatase expression and production of
osteocalcin as described in chapter 2 (general methods). Production of
SV40 large T antigens by transfected cells was assessed by
immunofluorescence (chapter 2).

Production of a mineralised matrix

Cells were seeded at 10^4 per well of multichamber slides and maintained in
complete medium for 3-4 days. Cells were then incubated for a further 7 days
in defined medium + L-ascorbate + vitamin K_1 in the presence and absence
of 5mM β glycerophosphate (βGP) ± 10^{-8}M 1,25D. The glycerophosphate
serves as an organic substrate for the enzyme alkaline phosphatase.
Following incubation cell layers were washed in phosphate buffered saline
(PBS), fixed in 80% ethanol, and stained for phosphate by the von Kossa
method and for calcium deposition using Alizerin Red S.

a).   von Kossa stain

The von Kossa stain is a well established method for visualising mineral
deposition *in vitro*. Fixed cells are covered with silver nitrate and exposed to
ultraviolet light (uv). The uv light causes the silver to cross-link with
hydroxyapatite in the extracellular matrix forming a black deposit of silver
phosphate. The staining procedure is as follows:

(i).   Wash fixed sections twice for 10min in distilled water.

(ii).   Add freshly made up 1% (w/v) silver nitrate in distilled water and
expose to uv light for 30min.

(iii).   Rinse in distilled water.

(iv).   Rinse in 5% (w/v) sodium thiosulphite for 30s.

(v).   Wash in tap water for 10min.

(vi).   Wash in distilled water for 10min.

(vii).   Wash in 70% ethanol for 10min.

(viii). Wash in absolute ethanol for 10min.

(ix). Clear in cedar wood oil.

b). **Alizerin Red-S stain**

Alizerin sodium sulphonate is a salt that binds metal ions, but preferentially binds Ca^{2+} ions.

(i). Wash fixed slides in 50% ethanol.

(ii). Wash in distilled water.

(iii). Cover with 2% (w/v) alizerin red in distilled water pH 4.1 to 4.3 (adjust pH with dilute $NH_4(OH)_2$) for 30s-5min.

(iv). Rinse with distilled water twice.

(v). Mount in cedar wood oil.

Prostaglandin E_2 production

Cells, in 16mm wells, were incubated in the presence and absence of 10U/ml rhIL-1α for 48h in MEM+3% FCS. Conditioned media from these cultures were stored at -20°C for the subsequent determination of PGE_2 production. PGE_2 production was measured by specific radioimmunoassay, using rabbit antisera. The specificity of the antisera is shown in table 4.1.

Standards of 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, 0.02 and 0.01ng/100μl PGE_2 were prepared in assay buffer (0.1% w/v gelatin in 0.01M Trizma base, 0.14M NaCl, pH 7.4). 100μl of sample or standard were transferred to LP3 tubes and 100μl of both antiserum and tritiated PGE_2 added. A medium blank was included and also total counts, maximum binding (100μl each of buffer, antibody and tracer) and non-specific binding (100μl tracer, 150μl buffer) as with the osteocalcin assay (described in chapter 2). Tubes were vortexed and incubated at 4°C overnight.

600mg of Norit A charcoal + 125mg Dextran T70 were mixed with 100ml assay buffer and left on a magnetic stirrer at 4°C until the charcoal was

Prostaglandin E_2	100.0 %	
Prostaglandin E_1	10.0 %	
Prostaglandin $F_{1\alpha}$	3.3 %	
Prostaglandin B_1	3.0 %	
Prostaglandin D_2	0.01	%
Prostaglandin A_1	0.01	%
Prostaglandin A_2	0.01	%
Prostaglandin $F_{2\alpha}$	0.01	%
13,14-Dihydro-6-keto-prostaglandin $F_{1\alpha}$	0.01	%
6-Keto-prostaglandin $F_{1\alpha}$	0.01	%
Thromboxane B_2	0.01	%
13,14-Dihydro-15-keto-prostaglandin $F_{2\alpha}$	0.01	%

Table 4.1   Comparative cross-reactions of the antiserum raised in rabbits against prostaglandin E_2.

completely in suspension. The charcoal is used to adsorb any unbound

tracer. On day 2 of the assay 200μl of charcoal were added to each tube,

tubes were vortexed and then centrifuged at 2,000g for 15min at 4°C. 200μl

of the clear supernatant were decanted into scintillation vials, 500μl of

scintillant added and the amount of incorporated radioactivity monitored

using a Rackbeta scintillation counter. A graph of concentration vs B/B_0 was

plotted as for the osteocalcin assay and results extrapolated. A typical

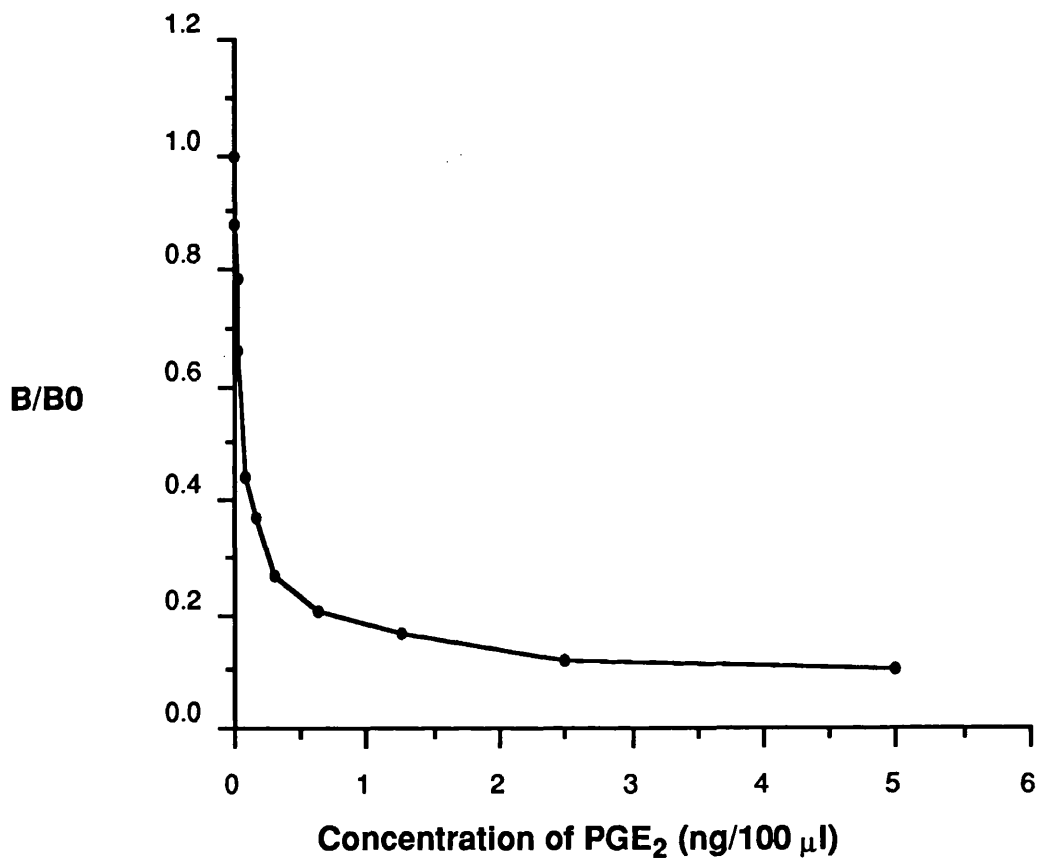standard curve is shown in figure 4.1. Results are expressed as ng/ml.

Fig. 4.1 A typical standard curve for the prostaglandin E_2 assay

Noncollagenous matrix proteins

Osteocalcin was measured as described in Chapter 2. Thrombospondin, osteonectin and bone sialoprotein in the same conditioned media were detected by specific radioimmunoassay. These assays were performed by Chantal Chenu and co-workers at INSERM, Lyon, France.

Expression of type I collagen mRNA

RNA was isolated from confluent, unstimulated cells as described in chapter 2. Expression of mRNA for type I collagen was assessed by Northern blotting.

For Northern blot analysis 10μg per sample of RNA were dissolved in 10μl of loading buffer prepared as follows:

60μl 10XMOPS, 300μl formamide, 100μl formaldehyde, 140μl DEPC water. 1μl of RNA gel loading dye was added to each sample.

RNA was electrophoresed in a denaturing gel of 1% (w/v) agarose with 14% (v/v) formaldehyde in 1X MOPS buffer, at 30mAmps for at least 7h. RNA was transferred to a Hybond-N+ filter by overnight blotting with 20X SSC as the transfer buffer. Filters were fixed for five minutes in 50mM sodium hydroxide and stored wrapped in cling film at <4°C. Filters were hybridised as described in chapter 2, with a cDNA probe for α_1 pro-collagen (type I).

Incorporation of pX-8

The incorporation of the SV40 vector into host cell DNA was confirmed by Southern blotting.

a). DNA isolation

Chromosomal DNA was isolated from cells by a salting-out procedure (Miller, Dykes and Polesky, 1988). Cell pellets were resuspended in 3ml nuclei lysis buffer (10mM Tris, 400mM NaCl, 2mM Na$_2$EDTA, pH 8.2) and protease K and 100μl of 20% SDS added. After a 3h incubation at 55°C 1ml of 6M NaCl was added and the suspension shaken vigorously. Tubes were

centrifuged for 25min at 2,000g. The supernatant was transferred to a clean

tube, 8ml of absolute ethanol added, and the contents mixed by inversion.

Precipitated DNA was removed using a sealed pasteur pipette and

dissolved in Tris-EDTA.

b)   Southern blot analysis

Restriction endonuclease digests of BOP cell DNA were carried out with

the enzymes Xba I and Bst XI. There are no restriction sites for Xba I on the

pX-8 vector but there is one Bst XI site in the SV40 early region at base pair

4770. COS cell DNA was included as a positive control as these cells

constitutively express SV40 early region genes. 10μg of DNA were diluted

in water to make a final volume of 25μl, 1μl of enzyme and 2μl of appropriate

buffer were added. Xba I digests were incubated at 37°C and Bst XI digests

were incubated at 45°C overnight.

Southern blots of DNA digests were performed by overnight electrophoresis

in 0.8% agarose gels with 1X Tris acetate as the running buffer. λ Hind

digests of λ DNA were used as molecular size markers. The gel was then

placed in 0.25M HCl until the dyes changed colour (bromophenol blue to

green, xylene cyanol to yellow) and left for a further 10min. DNA was

transferred to Hybond-N+ filters by alkali blotting, with 50mM NaOH as the

transfer buffer. Filters were prehybridised in a solution of 6X SSPE, 5X

Denhardt's, 5% dextran sulphate, 1% SDS for 4h at 65°C and hybridised with

a 3Kb fragment of SV-40 early DNA excised from the pX-8 vector. Filters

were washed twice in 2X SSPE, 0.5% SDS for 15min at 65°C and washed

once in 0.2% SSPE, 0.5% SDS for 10min at 65°C. Hybridised, washed filters

were subjected to autoradiography.

4.3   RESULTS

Alkaline phosphatase expression

The constitutive and 1,25D-inducible alkaline phosphatase activity of SV40 immortalised osteoblasts is shown in table 4.2. The cell lines exhibited a range of enzyme activities and differential responses to 1,25D. For example, in experiment 1 cell lines 7, 12, 62 and 72 had almost undetectable ALP activity and were unaffected by treatment with 1,25D. Cell lines 15 and 26 had higher levels of ALP which were enhanced by 1,25D. In experiment 2 enzyme activity for all cell lines was very low compared with the other 3 experiments and again great variation between cell lines with respect to both constitutive ALP and 1,25D responsiveness was observed. The ALP activity of the parent, nontransfected trabecular bone cell cultures (HObs) was enhanced almost twofold by 1,25D (Table 4.2b). Cell line BOP 37 maintained high levels of ALP and responsiveness to 1,25D in all 4 experiments. BOP 26 also exhibited high ALP activity. By comparison, BOP 7 displayed lower levels of ALP expression.

Table 4.2 The following two pages show data for alkaline phosphatase expression by BOP cells assayed in four experiments. Cells were incubated in defined medium ± 1,25D (10^{-8}M) for 48h. Cell layers were freed by scraping with a rubber policeman and assayed for ALP activity as described in Chapter 2. (a) and (b), alkaline phosphatase activity in 13 BOP cell lines and parent cultures of osteoblasts (HObs) (μmoles/min/mg total protein). (c) and (d), ALP activity in seven cell lines. SEMs are shown in (d). T/C is the ratio of 1,25D treated to control values.

Alkaline phosphatase
(μmoles/min/mg)

BOP	Control	1.25D	T/C
4	0.066	0.362	5.5
7	<0.033	<0.033	-
12	<0.033	<0.033	-
15	0.117	0.200	1.7
19	0.083	0.117	1.4
26	0.050	0.133	2.7
31	0.167	0.333	2
37	0.225	0.300	1.3
62	<0.033	<0.033	-
71	0.083	0.133	1.6
72	<0.033	0.033	-
74	0.050	0.205	4.1

a).   Experiment 1

BOP	Control	1.25D	T/C
4	0.0150	0.0147	1.0
7	0.0230	0.1230	5.4
12	0.0210	0.0095	0.4
15	0.0002	0.0080	40.0
19	0.0100	0.0045	0.5
26	0.2780	0.0340	1.2
31	0.0068	0.0109	0.6
33	0.0023	0.0843	36.6
37	0.0295	0.0541	1.8
62	0.0322	0.0081	0.3
71	0.0278	0.0822	3.0
72	0.0446	0.0350	0.8
74	0.0000	0.0000	-
HObs	0.0784	0.1460	1.9

b).   Experiment 2

	Alkaline phosphatase (µmoles/min/mg)		
BOP	Control	1.25D	T/C
7	0.570	0.863	1.5
12	0.660	>1.5	-
26	>1.5	>1.5	-
31	>1.5	>1.5	-
33	0.11	0.175	1.6
37	0.715	1.5	2.1
70	>1.5	>1.5	-

c).   Experiment 3

BOP	Control	1.25D	T/C
7	0.19 ± 0.02	0.23 ± 0.01	1.2
12	1.70 ± 0.10	2.94 ± 0.06	1.7
26	1.85 ± 0.17	3.50 ± 0.44	1.9
31	3.71 ± 0.18	4.30 ± 0.61	1.1
33	0.13 ± 0.01	0.24 ± 0.02	1.9
37	0.85 ± 0.14	2.51 ± 0.48	2.9

d).   Experiment 4, n=3.

Production of noncollagenous matrix proteins

The production of osteocalcin by BOP cells is shown in figure 4.2 and a separate experiment is shown in table 4.3. With the exception of BOP 31 which produced a very low level of osteocalcin (0.1ng/ml) constitutively, the cell lines produced osteocalcin only in response to 1,25D. The cell lines produced variable amounts of osteocalcin; for example lower levels were produced by BOP 7 than by BOP 26 and 37.

The cell lines all produced similar levels of bone sialoprotein (BSP) which were not affected by treatment with 1,25D (Fig. 4.3). All the cell lines produced osteonectin again with very little variation between cell lines or response to 1,25D (Fig 4.4). With respect to thrombospondin production (Fig. 4.5) a much greater variation was observed between different cell lines; for example, BOP 7 produced very low levels of thrombospondin (11ng/ml) compared with BOP 37 (210ng/ml) and BOP 19 (120ng/ml). 1,25D enhanced thrombospondin secretion in cell lines 26, 61, 72 and 74, but reduced production of the protein in lines 19, 37, 71 and 31. The effects of 1,25D on thrombospondin release were not consistent with BOP number but varied between experiments in cell lines 12, 26 and 37.
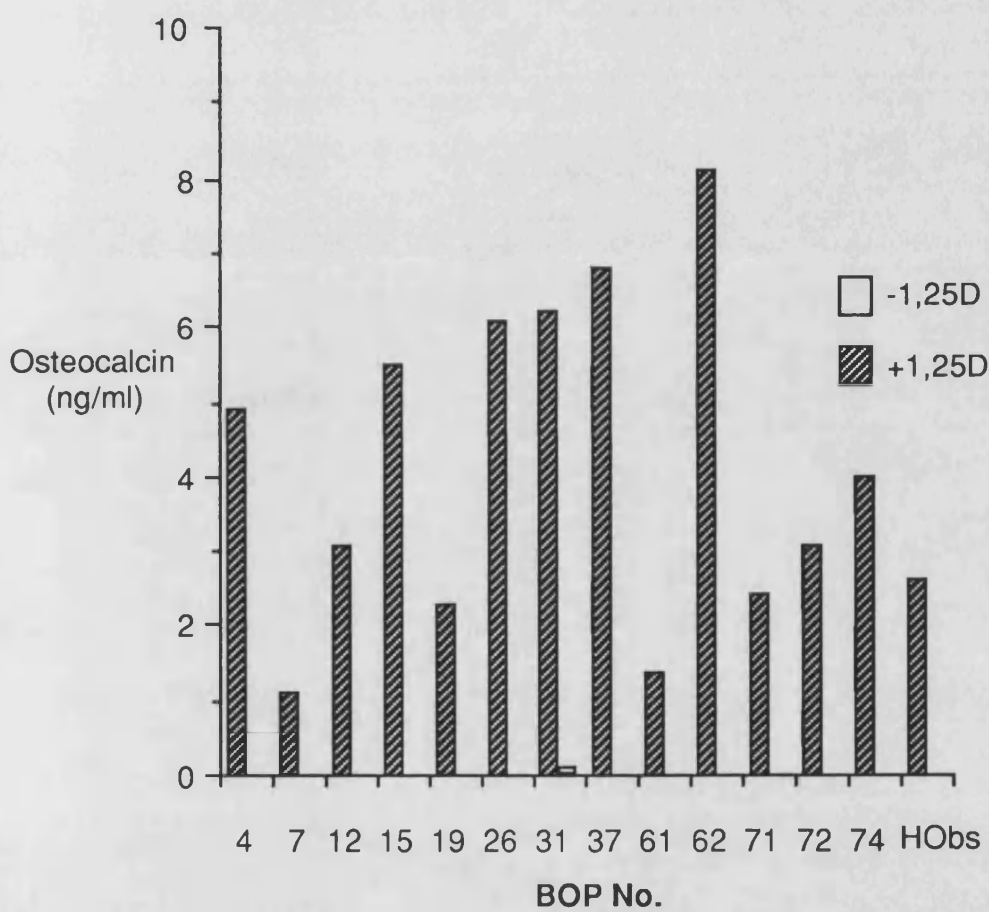
Fig. 4.2 Osteocalcin production by BOP cells, mean value for 1,25D treated BOP cells is 4.2 ± 0.6 ng/ml. Cells were seeded into 30mm wells, allowed to settle for 24h, and treated with ethanol vehicle or 10^{-8}M 1,25D for 48h. Conditioned media were assayed for osteocalcin using a specific radioimmunoassay as described in chapter 2.

BOP	Control	1.25D
4	0	17.8
7	0	3.4
12	0	15.8
15	0	1.1
19	0	1.1
26	0	9.8
31	0	7.5
33	0	16.5
37	0	9.1
62	0	13.0
72	0	6.2
74	0	0.7

Table 4.3 Osteocalcin production (ng/ml) by BOP cells. Cells were treated for 48h with 1,25D (10^{-8}M) and osteocalcin in conditioned media measured using a specific radioimmunoassay.

Fig. 4.3 Bone sialoprotein production by BOP cells. Conditioned media were prepared as described in the legend to Fig. 4.2 and assayed for bone sialoprotein by radioimmunoassay. Mean values for control and 1,25D treated BOP cells are 2.2 ± 0.18 and 2.4 ± 0.13ng/ml respectively.

105

Fig. 4.4 Osteonectin production by BOP cells. Conditioned media were prepared as described in the legend to Fig. 4.2 and assayed for osteonectin by radioimmunoassay. Mean values for control and 1,25D treated BOP cells are 32.9 ± 3.1 and 30.6 ± 2.6ng/ml respectively.
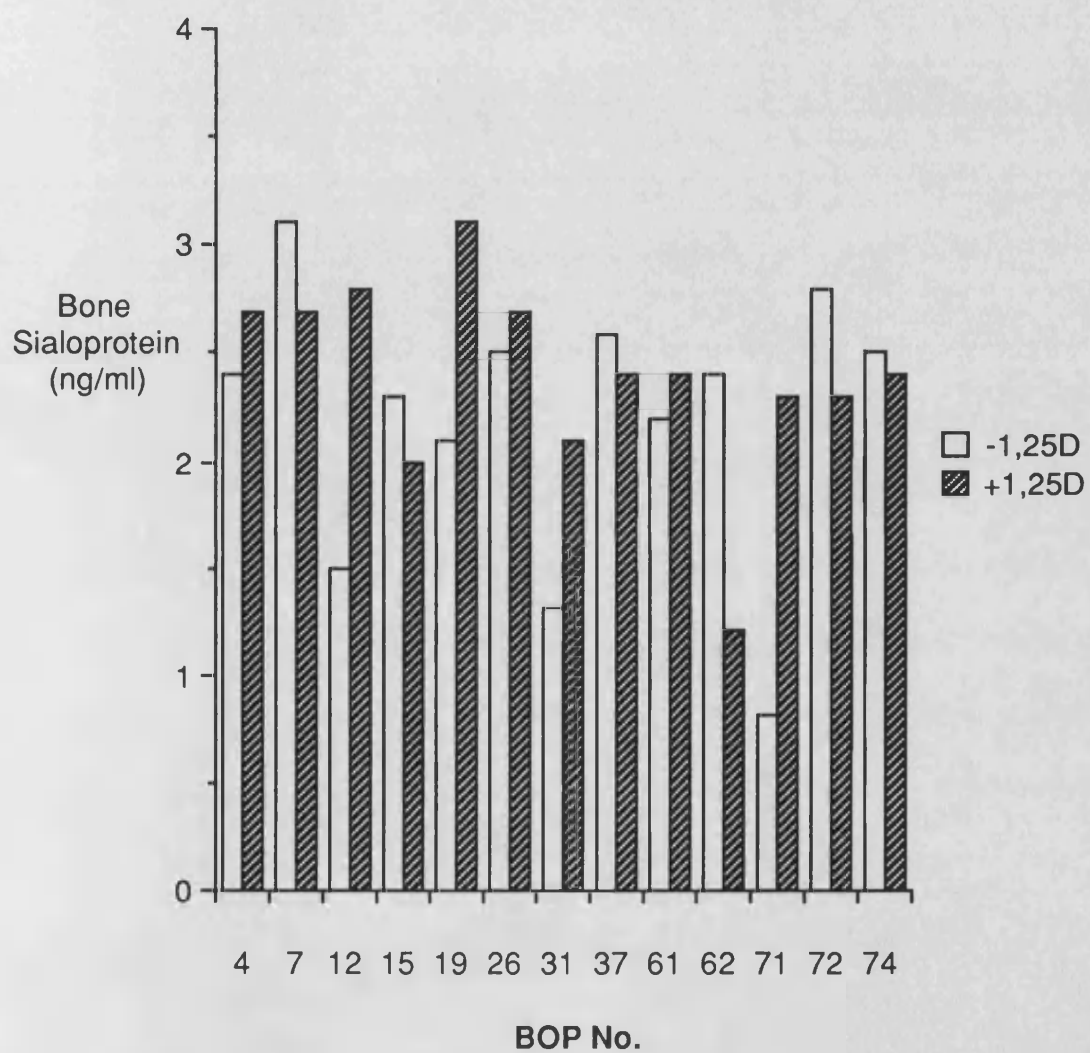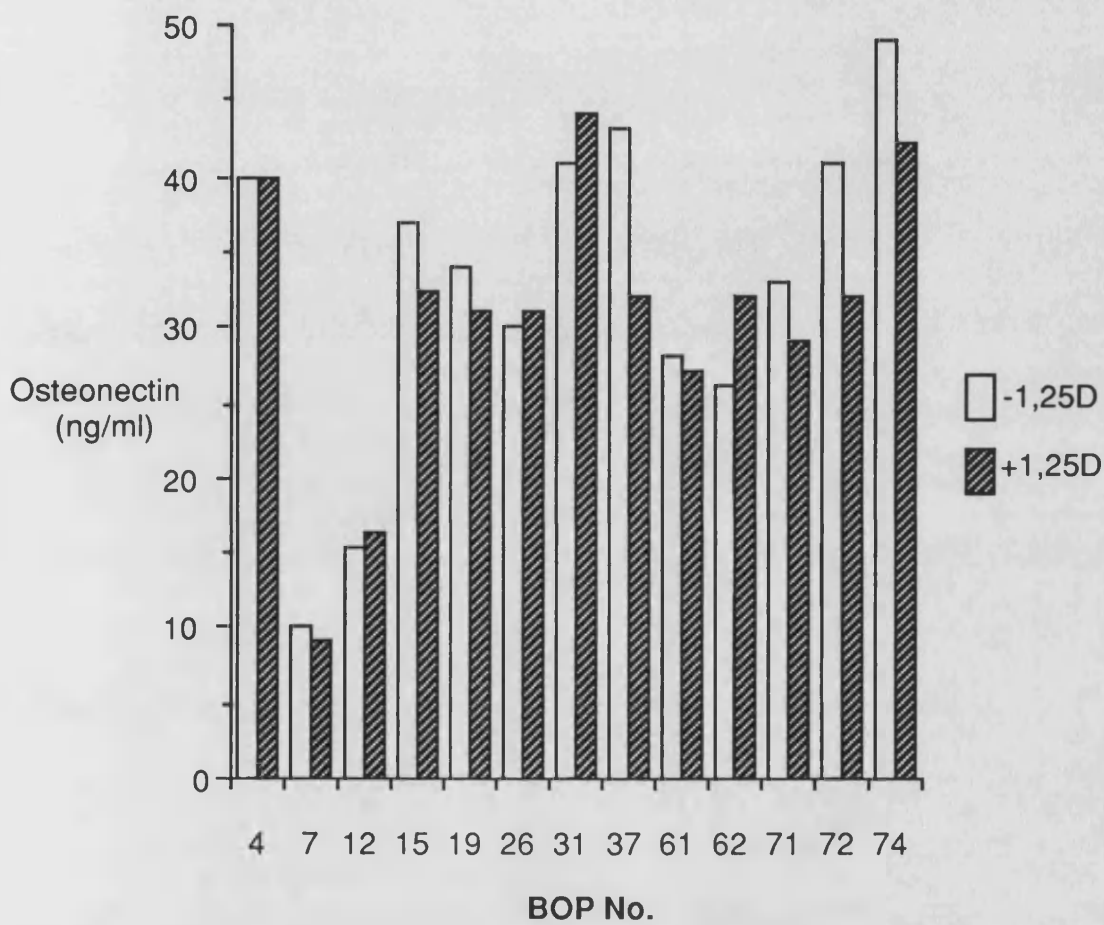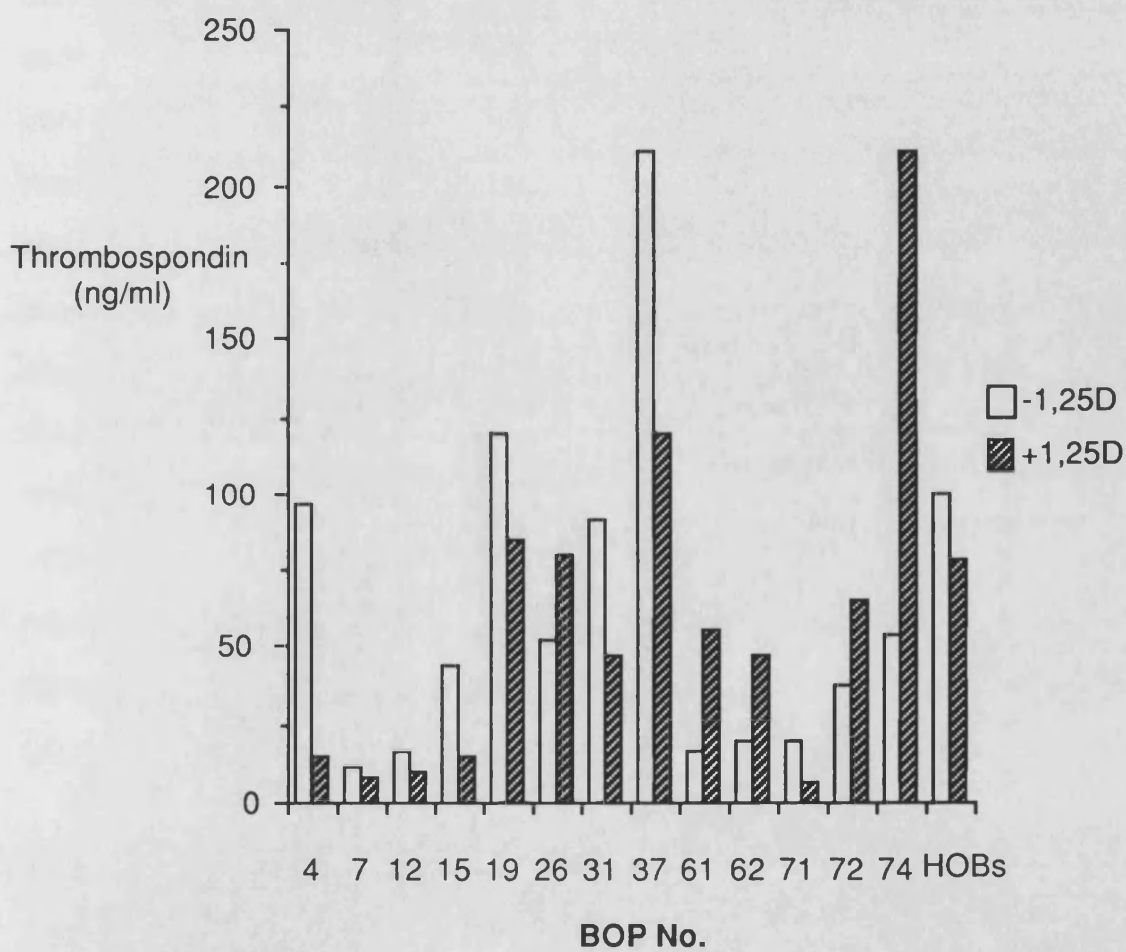
Fig. 4.5 Thrombospondin production by BOP cells. Conditioned media were prepared as described in the legend to Fig. 4.2 and assayed for thrombospondin by radioimmunoassay. Mean values for control and 1,25D treated BOP cells are 60.8 ± 15.7 and 58.9 ± 4.42ng/ml respectively.

Mineralisation *in vitro*

The ability of the immortalised cell lines to mineralise *in vitro* was assessd by the von Kossa stain which demonstrates phosphate deposition, and the Alizerin Red S stain which binds Ca^{2+} ions. The amount of staining present in fixed cells is shown in table 4.4, the degree of staining observed was recorded as absent (-), weakly present (+/-), or present to varying intensities (+). In experiment 1(Table 4.4a) no von Kossa staining was observed in control cells but varying amounts of phosphate deposits were present in cells that had been incubated with β glycerophosphate. In experiment 2 no von Kossa staining was observed in control or 1,25D treated cells; weak reactivity occurred in β glycerophosphate treated cells of BOP 26 and 37 and staining was enhanced by 1,25D in these cell lines. Other cell lines produced a positive von Kossa reaction only when both β GP and 1,25D were present. Spearman rank tests demonstrated no correlation between the intensity of the von Kossa stain and the levels of ALP, osteocalcin, bone sialoprotein, osteonectin or thrombospondin. The staining pattern of BOP 37 cells is shown in figure 4.6. Cell lines displayed varying Alizerin Red reactivities (Table 4.4b), calcium deposition was enhanced by addition of β glycerophosphate to the medium but not dramatically affected by 1,25D.

Prostaglandin production

BOP cells failed to produce PGE_2 constitutively and only two cell lines, BOP 26 and 62 secreted PGE_2 in response to interleukin 1 (Table 4.5).

Expression of type I collagen mRNA

Cell lines 7, 12, 26, 31, 33, 37 (at passage (p) 9 and 19) and 70 all expressed mRNA for type I collagen as shown in figure 4.7.

Incorporation of vector DNA and expression of SV40 T antigens

The seven cell lines listed above expressed SV40 T antigens, immunolocalisation of the antigens in the nuclei of cell lines 31 and 37 is shown in figure 4.8. Southern blot analysis demonstrated the presence of

SV40 DNA in six cell lines tested. There are no sites recognised by the restriction endonuclease Xba I in the pX-8 vector and digestion of BOP cell DNA with this enzyme resulted in one band appearing on the Southern blot for each of six cell lines. The exception was BOP 31 which gave two bands, suggesting that this cell line was derived from cells which had incorporated the vector into two distinct sites of host DNA. The enzyme Bst XI cuts the pX-8 vector approximately mid-way along the 3Kb segment used to hybridise the Southern blot. Digestion of BOP cell DNA with Bst XI yielded at least two bands (Fig. 4.9).

Experiment 1

BOP	βGP
4	weak+
7	+++
12	+++
15	++
19	+
26	++
31	weak+
37	++
62	+++
71	weak+

Experiment 2

βGP	βGP + 1.25D
-	weak+
-	-
-	+++
-	-
-	-
weak+	++
-	-
weak+	++++
-	+
-	+

a). von Kossa staining reaction of BOP cells

BOP	Control	1.25D	βGP	1.25D+βGP
7	-	-	weak +	++
12	-	-	++	+++
26	+/-	+/-	+/-	++
31	+	+	++++	++
33	-	-	-	-
37	-	-	+++	+++
70	+/-	+	+++	+++

b). Alizerin red S.

Table 4.4 Mineralisation *in vitro* by BOP cell lines. Cells were incubated in wells of multichamber slides and treated with 1,25D (10^{-8}M) + 5mM β glycerophosphate (βGP) for seven days. Fixed preparations were stained by the von Kossa method (a) or with Alizerin Red S (b). Staining was absent (-) or present to varying intensity (+).
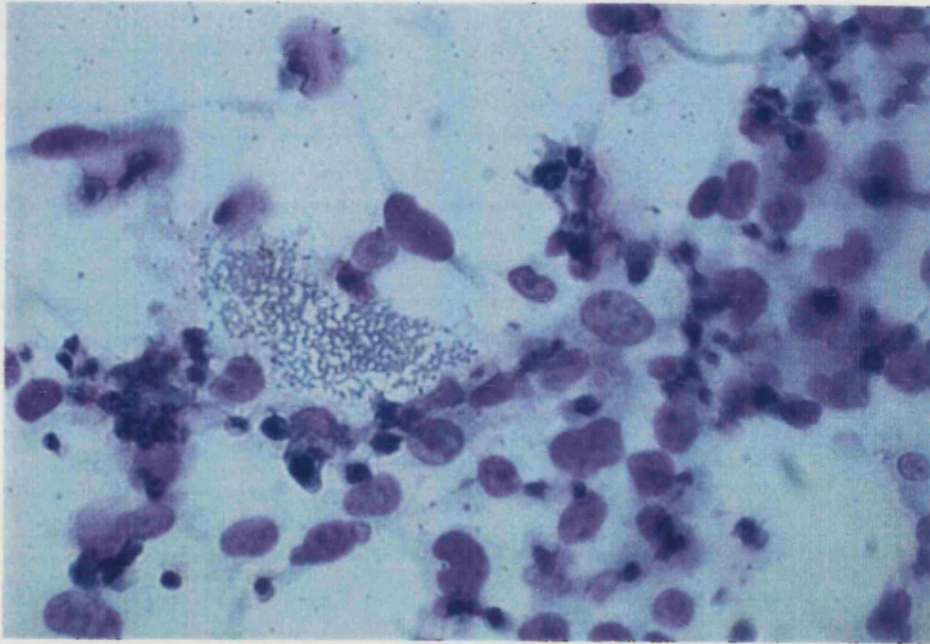
(a)
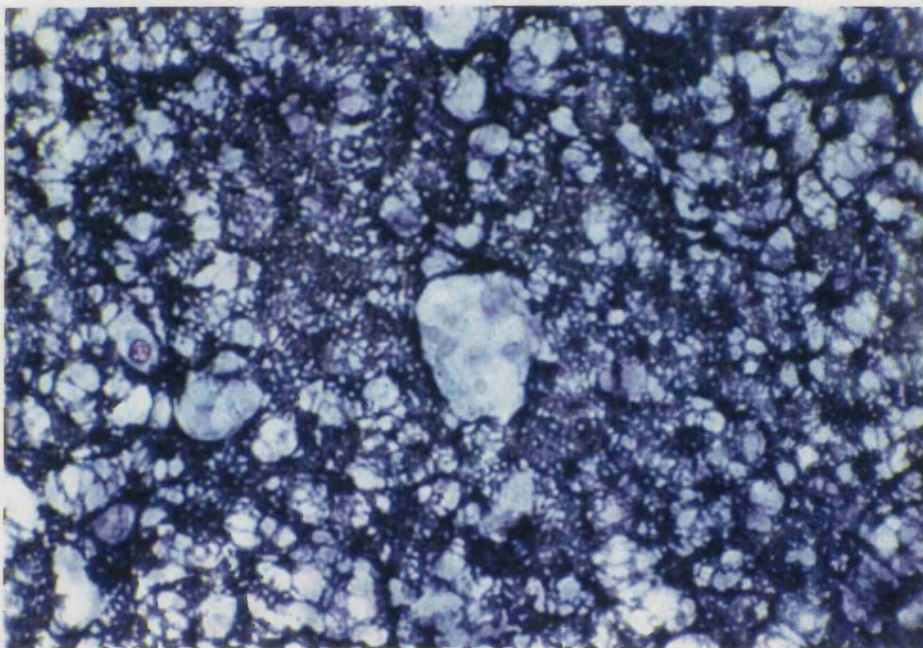
(b)

Fig 4.6   von Kossa staining reaction in BOP 37. (a) Cells incubated with β
glycerophosphate and (b) cells treated with β glycerophosphate + 1,25D.
X400.

BOP	Control	IL-1α
4	0	0
7	0	0
12	0	0
15	0	0
19	0	0
26	0	11.65
37	0	0
62	0	2.15
71	0	0
72	0	0
74	0	0

Table 4.5   Production of PGE_2 (ng/ml) by BOP cells. Cells were incubated in the presence and absence of 10 U/ml rh IL-1α for 48h in MEM + 3% FCS. PGE_2 in conditioned media was measured by radioimmunoassay.
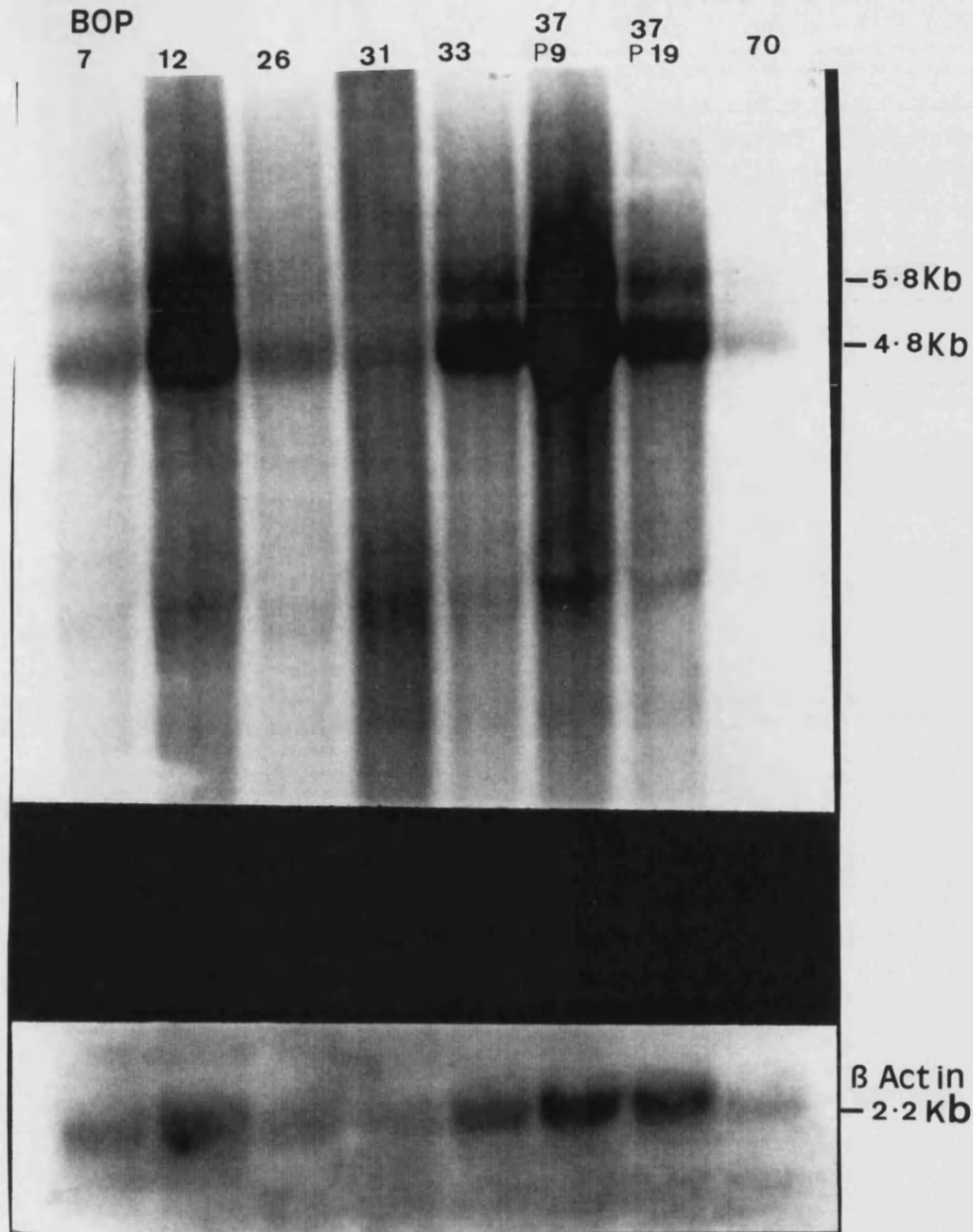
Fig. 4.7   Type I collagen mRNA expression in BOP cells. RNA was prepared from confluent monolayers of cells and subjected to gel electrophoresis and Northern blotting Filters were hybridised with a cDNA probe for α_1 pro-collagen then stripped and reprobed for human β actin as described in methods.
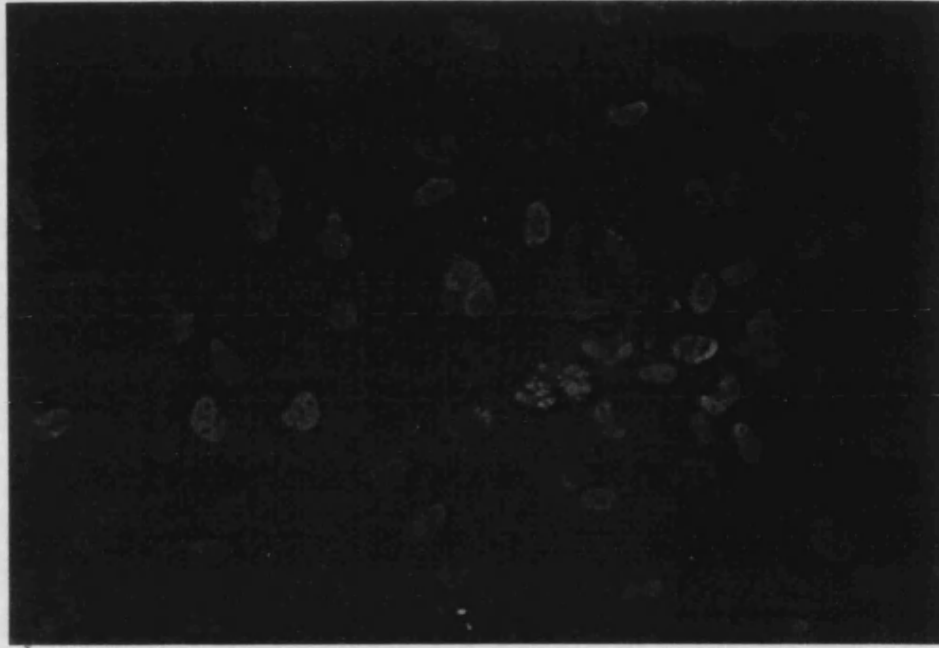
(a)
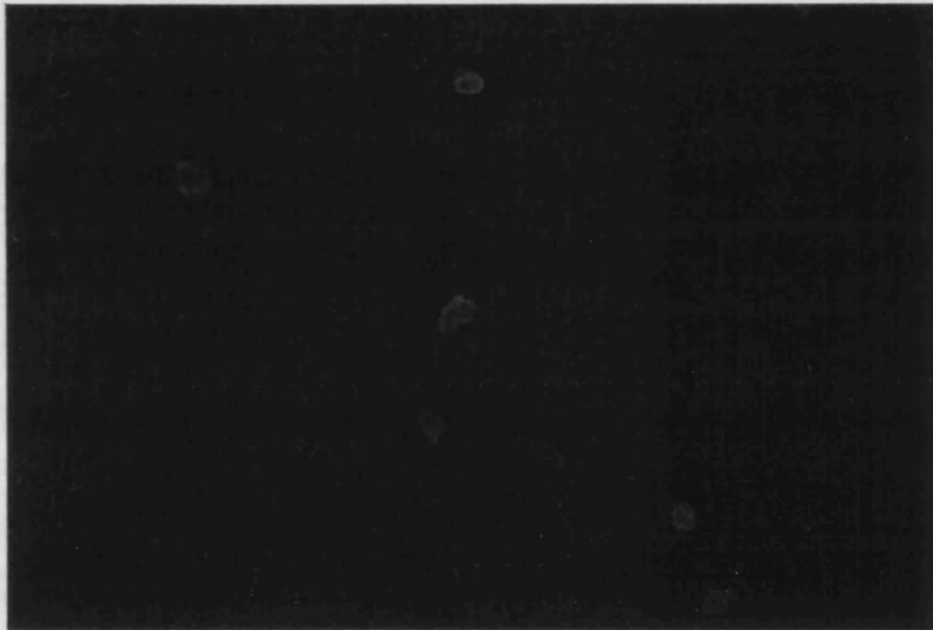
(b)

Fig. 4.8   Expression of large and small SV40 T antigens in transfected BOP
cells. Cells were seeded onto poly-L-lysine treated glass multispot slides
and allowed to settle overnight. Preparations were fixed in acetone and
stained by indirect immunofluorescence as described in chapter 2. (a) BOP
31, (b) BOP 37. X100.

114
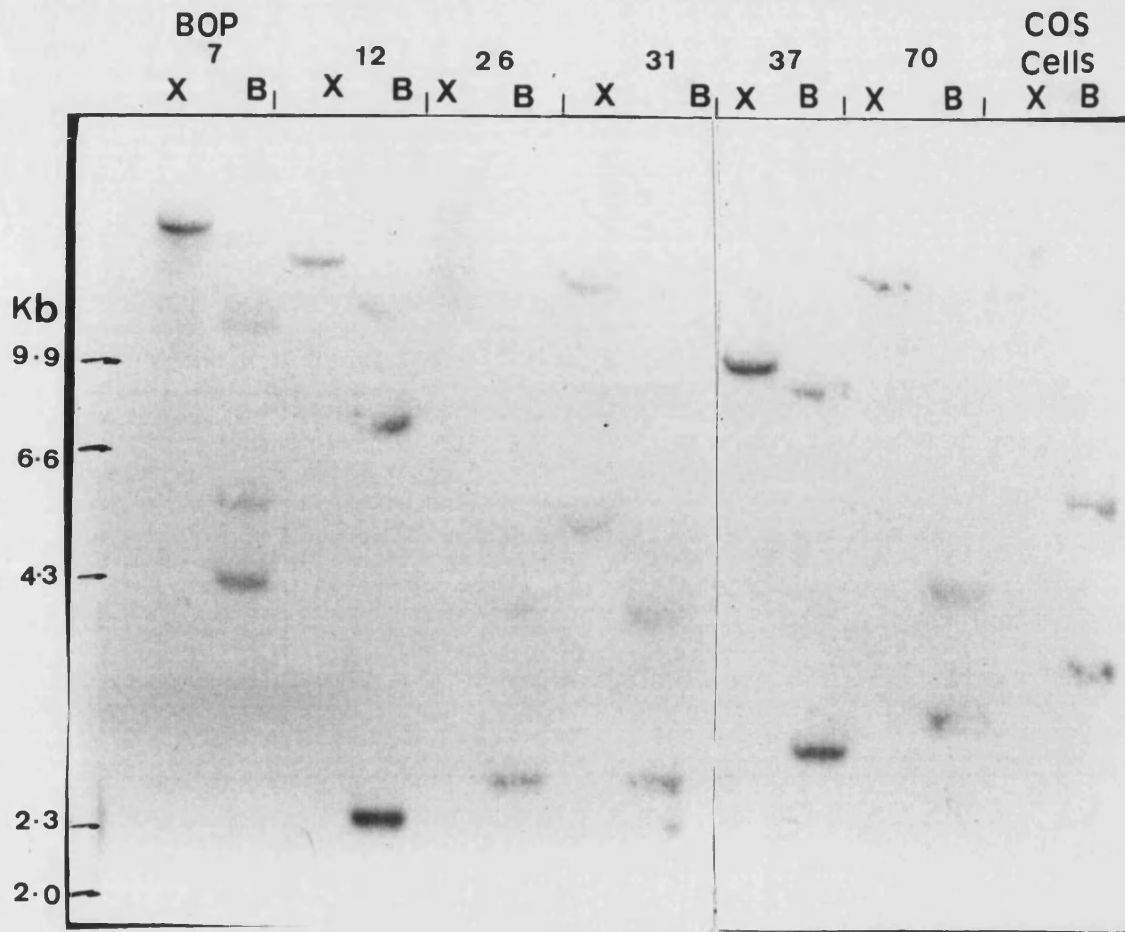
Fig. 4.9   Incorporation of the pX-8 plasmid into transfected cells. DNA was

prepared from BOP cells as described in methods and subjected to

restriction endonuclease digestion, gel electrophoresis and Southern

blotting. Molecular size is indicated on the left hand side. X=Xba I, B=Bst XI.

4.4   DISCUSSION

Characterisation of immortalised BOP cells demonstrated that the cell lines were capable of expressing the phenotype of mature osteoblasts. The cell lines expressed mRNA for type I collagen, the major constituent of bone extracellular matrix, and secreted bone-associated noncollagenous proteins. In addition, cells were responsive to the major calciotropic hormone 1,25D. Only two cell lines produced prostaglandin E_2 in response to IL-1 which may reflect the heterogeneity of the parent cultures.

Expression of the bone isoenzyme alkaline phosphatase was extremely variable when assayed at different time points in both control and 1,25D treated cells. A similar variation has been reported for the murine clonal osteoblastic cell line MC3T3-E1, these cells display low alkaline phosphatase activity when in the logarithmic phase of growth and 100 fold higher levels at confluence (Sudo *et al,* 1983). Primary cultures of human osteoblasts contain subpopulations of alkaline phosphatase positive cells with strong, weak or undetectable activity (Matsuyama, Lau and Wergedal, 1990). This variation has been attributed to the asynchronous nature of the cultures and to cell cycle changes. Fedarko *et al* (1990) obtained synchronous populations of human osteoblasts by a thymidine-aphidicolin double blockade method and observed a drop in the levels of cell-associated ALP activity during mitosis. ALP activity returned slowly during G_1 and reached a peak during S phase. When the constitutive ALP activity of BOP 26 was assayed every two days over a period of nine days a small increase in activity was observed as cells attained confluence (Bell, Williams and Gowen, unpublished observations). The levels of ALP activity in BOP cells were higher than are commonly observed with cells of the human osteosarcoma cell line MG-63 which express very little ALP activity when treated under the same conditions (Clover, personal communication).

BOP cells produced osteocalcin in response to 1,25D displaying their ability to differentiate when incubated in the presence of the hormone, in common with primary cultures of osteoblasts. The pattern of noncollagenous protein synthesis in cell lines 37 and 26 was considered to most closely resemble the behaviour of normal trabecular bone cells (Delmas, personal communication). BOP 7 produced low levels of both ALP and osteocalcin but high levels of bone sialoprotein. This cell line provides an interesting negative comparison and may represent less well differentiated osteoblasts that were present in the parent cultures. The cell lines displayed differential responses to 1,25D with respect to thrombospondin production. The precise role of this protein is unknown but it has been demonstrated to promote the attachment, but not spreading, of osteoblasts *in vitro* (Gehron Robey *et al*, 1989). Similarly, bone sialoprotein has cell attachment activity whereas osteonectin lacks an RGD sequence but may be important in regulating mineralisation.

The variation observed in von Kossa staining reactivities is likely to relate to changes in the activity of alkaline phosphatase. The von Kossa stain is not a specific monitor of bone matrix production. β glycerophosphate added to the culture medium provides an organic substrate for alkaline phosphatase resulting in the extracellular deposition of phosphate which is visualised by conversion to silver phosphate in the von Kossa reaction. Cells expressing high levels of alkaline phosphatase would be expected to demonstrate a positive reaction. Alizerin Red S is a salt that binds calcium and demonstrates that BOP cells produce a calcium phase *in vitro* that is likely to be calcium phosphate.

The rapid growth of BOP cells described in chapter 3 is attributable to the production of SV40 T antigen. When early region cRNA encoding T antigen is injected into epithelioid cells production of the viral protein induces chromatin replication and mitosis (Graessmann and Graessmann, 1976). T

antigen is able to catalyse the hydrolysis of ATP to ADP+P$_i$ and binds DNA. The protein may cause the unwinding of portions of duplex DNA, allowing polymerases to bind and initiate replication (Tjian and Robbins, 1979).

Immortalised cell lines derived by transfection of primary cultures with SV40 DNA provide a self-renewing population of cells that retain the characteristics of parent cultures. BOP cells expressed all the hallmarks of mature osteoblasts. Variation between cell lines with respect to production of noncollagenous proteins, prostaglandins and expression of alkaline phosphatase may reflect the heterogeneity of the parent cells. Two cell lines, BOP 26 and 37, were considered to most closely resemble primary trabecular bone cells; in contrast BOP 7 was less well differentiated and may represent an osteoblast precursor.

CHAPTER 5

Cytokine production and responsiveness in immortalised cells

ABSTRACT

Locally-produced cytokines may be important regulators of bone remodelling; IL-1 and TNF for example stimulate both osteoclastic resorption and osteoblast proliferation. Human osteoblast-like cells derived from trabecular bone are capable of producing the cytokines IL-1, IL-6, TNF and TGFβ. Seven BOP cell lines were characterised for their ability to produce the cytokines IL-1, TNF and IL-6. The effects of oestradiol (E_2) on cytokine release and on the expression of mRNA for TGFβ were also assessed.

All seven cell lines produced IL-1 in response to TNF; constitutive levels of IL-1 were very low and were not affected by treatment with 17β-oestradiol. Treatment with IL-1α enhanced expression of mRNA for IL-1β in six cell lines tested. Seven cell lines released bioactive IL-6 and production of this cytokine was enhanced by IL-1 and TNF but unaffected by E_2. The cell lines released high levels of immunoreactive TNF constitutively, production of TNF was not affected by IL-1 and was slightly enhanced by treatment with E_2 in one cell line only. Constitutive expression of TGFβ mRNA was low in six cell lines tested and was not affected by treatment with E_2.

The production of IL-1 and responsiveness of the cells to IL-1 and TNF were similar to the results previously observed with primary cultures. High, aberrant production of TNF may be a result of the SV40-induced transformation process.

5.1 INTRODUCTION

Immune-cell derived cytokines such as IL-1 and TNF are potent stimulators of osteoclastic resorption and may be important physiologic regulators of bone turnover. Together with a variety of other growth factors they are produced locally by cells in the bone microenvironment and may act in a paracrine or autocrine fashion to affect bone cell function. IL-1 and TNF have anabolic effects on bone by stimulating the proliferation of human

osteoblasts (Gowen, 1992). The proliferative effect of TNF was not seen in the absence of serum, suggesting that additional growth factors are required. This enhanced proliferative activity is accompanied by a reduction of differentiated function; IL-1 inhibits alkaline phosphatase activity and antagonises the production of osteocalcin induced by 1,25D (Evans, Bunning and Russell, 1990). Similarly TNF inhibits basal and 1,25D enhanced alkaline phosphatase activity and osteocalcin release and, like IL-1, stimulates the release of prostaglandins (Gowen, MacDonald and Russell, 1988). These two cytokines, in conjunction with other growth factors, may be important regulators of osteoblast proliferation prior to a new round of bone formation at the Howship's lacuna.

Interleukin 6 is a cytokine with similar activities to IL-1, most notably induction of the acute phase response and induction of fever. IL-6 has been reported to either enhance osteoclastic resorption or have no effect and this variation may result from the different models used (chapter 1, 1.6.2(a)). Reports of the effects of IL-6 on osteoblast function also vary depending on the model system studied. Fang and Hahn (1991) report a dose-dependent increase of tritiated thymidine incorporation induced by IL-6 in cells of the rat osteosarcoma cell line UMR-106-01. IL-6 also increased prostaglandin release, decreased the percentage of collagen synthesised but had no effect on alkaline phosphatase activity in this model. In human osteoblast-like cells IL-6 had no effect on DNA synthesis or on the expression of mRNA for type I collagen (Linkhart et al, 1991). IL-6 failed to affect IL-1 induced proliferation of either human osteoblasts or the clonal rat osteosarcoma cells ROS 17/2.8, and did not affect basal or 1,25D induced alkaline phosphatase activity in these cell types. Neither human osteoblasts nor ROS 17/2.8 cells produced PGE_2 in response to IL-6 and IL-6 did not affect IL-1-induced PGE_2 release (Littlewood et al, 1991a). The osteotropic effects of IL-6 may

be restricted to the stimulation of osteoclast formation from precursors in the bone marrow (Roodman, 1992).

Human osteoblast-like cells derived from trabecular bone produce bioactive TNF in response to stimulation by IL-1, bacterial lipopolysaccharide (LPS) and granulocyte/macrophage colony-stimulating factor (GM-CSF). IL-1, at an optimal concentration of 10U/ml, was as potent as LPS in inducing TNF production. The osteotropic hormones 1,25D, PTH and calcitonin did not affect TNF release in this model (Gowen et al, 1990). The constitutive production of both TNF and IL-1 by human osteoblasts is minimal but the cells are stimulated to produce IL-1 by both TNF and LPS. The hormones PTH and oestradiol were shown to have no effect on either constitutive or TNF induced IL-1 release (Keeting et al, 1991a). Human trabecular bone derived cells produce IL-6 in response to LPS, TNF and IL-1 (Linkhart et al, 1991; Littlewood et al, 1991b). PTH enhances IL-6 production in ROS 17/2.8 cells but has no effect on the production of IL-6 by primary cultures of human osteoblasts. These reports would suggest that locally produced cytokines in the bone microenvironment may be regulated by other cytokines and local factors independently of the hormone status of the individual. One possible exception to this is TGFβ, a component of bone matrix with potent effects on the growth and differentiation of many cell types (chapter 1, 1.6.2(d)). Oestradiol and PTH enhance TGFβ mRNA in human osteoblast-like cells (Merry et al, 1991) and oestradiol enhances TGFβ production (Oursler et al, 1991). Oestradiol has also been reported to inhibit the release of IL-1-induced TNF from adult human osteoblasts (Rickard, Russell and Gowen, 1992).

BOP cells were assessed for their ability to produce cytokines characteristic of primary cultures of trabecular bone cells. Seven BOP cell lines were treated with TNF, IL-1 and oestradiol and their production of IL-6, IL-1 and TNF assayed. The effect of IL-1α treatment on IL-1β mRNA

expression, and the effect of 17β-oestradiol on TGFβ mRNA were also measured.

5.2 METHODS

Cytokine production

Cells were seeded at $4X10^4$ per well of 24 well plates in phenol red-free defined medium and treated with10U/ml recombinant human (rh) IL-1α, 1,000U/ml rhTNFα, or $10^{-9}M$ 17β oestradiol (E_2) for 72h. Triplicate wells were set up and results are expressed ± SEM. Fresh conditioned media were assessed for IL-6 content using the B9 assay and for TNF production utilising the WEHI bioassay. Aliquots of conditioned media were stored at - 20°C and subsequently assayed for interleukin 1 content using $D10N_4M$ cells, and for TNF by immunoradiometric assay (IRMA).

IL-6 bioassay

IL-6 was measured by specific bioassay utilising an IL-6 dependent hybridoma cell line B9 (Helle *et al*, 1988). B9 cells proliferate in response to IL-6 but do not respond to other cytokines except for a small response to murine IL-4. Proliferation of the cells is quantified by addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Uptake of this dye measures the overall metabolic activity of the cells.

B9 cells were maintained in RPMI-1640 medium supplemented with 5% FCS, 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine, $5X10^{-5}M$ 2-mercaptoethanol and 0.1% (v/v) monocyte conditioned medium (a source of IL-6).

For the assay, B9 cells were washed twice in complete RPMI lacking the IL-6 and seeded at 5000 cells per microtitre well. Cells were cultured for 72h with 100μl medium and 100μl samples or known amounts of rhIL-6. Serial dilutions of 100, 30, 10, 1, 0.1 and 0 U/ml of hrIL-6 were prepared for the standard curve. Cells were incubated for 72h, 50mg/ml of MTT in PBS (20μl

per well) was added 4h before the end of the culture period. Medium was then aspirated and replaced with 100μl of 0.04M HCl in propan-2-ol to dissolve the metabolised dye. Samples were measured spectrophotometrically using a Dynatech micro-ELISA reader with 570nm and 630nm as test and standard wavelengths respectively. $OD_{570-630}$ was plotted against standard concentrations to generate a standard curve. Sample $OD_{570-630}$ was used to quantify the amount of IL-6 in the conditioned medium.

D10N₄ M assay for IL-1

IL-1 production was assayed using the D10N4M murine T cell line. D10N4M cells proliferate in response to IL-1 and are responsive, but to a lesser extent, to IL-2 and murine IL-4. Cells were maintained in RPMI + 10% FCS, rhIL-2 (20U/ml), concanavalin A (5μg/ml) and 2-mercaptoethanol $(5X10^{-5}M)$. For the assay, cells were seeded at 10^4 per well of 96-well plates in medium with concanavalin A and saturating levels of IL-2 (20U/ml). This last masks the response to any IL-2 present in the samples and also increases sensitivity to IL-1. A standard curve was prepared by serial dilution to give standards of 10, 1, 0.1, 0.03, 0.01, 0.003, 0.001U/ml rhIL-1α. For the last 18h of a 72h culture cells were pulsed with 1μCi per well of tritiated thymidine (^{3}H-Tdr). At the end of the culture period cells were harvested onto filter paper using a cell harvester (Skatron instruments, Norway). The amount of incorporated radioactivity was measured using a Rackbeta scintillation counter (LKB-Pharmacia, Uppsala, Sweden). Results were extrapolated from a plot of log_{10} concentration of standard against counts per minute (CPM). Results are expressed as ng/ml.

WEHI assay

Bioactive TNF in the conditioned media of BOP cells treated with IL-1 and oestradiol was assayed using the murine fibrosarcoma cell line WEHI 164 clone 13. These cells are lysed by TNFα and β. WEHI cells were maintained

in RPMI 1640 + 10% FCS. For the assay cells were dispersed with trypsin, centrifuged and resuspended in 10ml of complete RPMI. Cells were seeded at 2×10^4 per microtitre well in a volume of 100μl and allowed to settle for 4h at 37°C. 100μl of rhTNFα standards prepared to give final concentrations of 0. 0.001, 0.01, 0.1, 1, 10, 100 and 1,000U/ml were added to wells in triplicate. Samples were added in quadruplicate and plates were incubated for 24h at 37°C. 10μl of MTT solution (5mg/ml in PBS) were added to each well and plates incubated for a further 4h. The medium was then aspirated with a syringe and 50μl of isopropanol containing 0.04M HCl added to dissolve the MTT. Absorbance was read at reference and test wavelengths of 630nm and 570nm respectively. A standard curve of percentage cytotoxicity (1-[OD sample/OD zero standard] X100) vs TNF concentration was plotted, the percentage cytotoxicity was calculated for each sample and the corresponding TNF concentration extrapolated from the standard curve.

TNF IRMA

TNFα in BOP cell conditioned media was measured using a specific immunoradiometric assay. In this assay several monoclonal antibodies directed against distinct epitopes of TNFα are coated on the lower and inner surface of plastic tubes. Standards or samples are added to the tubes and will show affinity for the antibodies. The signal antibody is labelled with ^{125}I and triggers the immunological reaction. After washing, the remaining radioactivity bound to the tube reflects the antigen concentration.
200μl of sample or standard were dispensed into the precoated tubes and 50μl of tracer added. Tubes were incubated for 18h at room temperature, then the contents aspirated. Tubes were washed and counted using a gamma counter. A graph of $\log_{10}$ concentration (abscissa) vs CPM (ordinate) was plotted and results extrapolated.

Expression of cytokine mRNA

Cells in 100mm culture dishes were incubated for 24h with phenol-red free defined medium then treated for 6h with fresh medium with or without rhIL-1α (10U/ml) or for 9h with 10^{-9}M 17β oestradiol. RNA was extracted and subjected to slot blot analysis.

Slot blot analysis

For slot blot analysis 10μg per sample of RNA were diluted to 50μl with DEPC water and 50μl of formamide added. RNA samples were denatured by incubation at 65°C for 10min followed by quenching on ice. 8μl 3M sodium acetate were added and samples were diluted to 5μg/100μl with a solution of 50% formamide and 0.25M sodium acetate (solution A). 2-fold serial dilutions of the samples were made in solution A in microtitre wells and kept on ice.

The manifold slot blot apparatus was soaked in DEPC water for 30min prior to use. The Hybond-N+ filter was presoaked first with water then with 2X SSC and the manifold assembled. 100μl of each sample were blotted onto the filter by applying a vacuum. 100μl of solution A was then added to each slot. Filters were fixed with 50mM NaOH, rinsed in 2X SSC, and stored wrapped in saran wrap at <4°C.

Filters of RNA from control and IL-1α treated cells were hybridised with a cDNA probe for IL-1β, and filters of RNA from 17β oestradiol treated cells were probed for TGFβ. Hybridisation of filters and autoradiography were carried out as described in chapter 2. The intensity of each slot on the autoradiographs was assessed using a Digit-X densitometer.

5.3   RESULTS

Interleukin 6 production by BOP cells

Seven BOP cell lines were assessed for their ability to produce bioactive IL-6 constitutively and in response to IL-1, TNF and oestradiol. IL-1 induced

IL-6 production is shown in table 5.1, all seven cell lines were capable of secreting IL-6 but levels varied between experiments and between different cell lines. Constitutive and inducible IL-6 levels were lower in experiment 2. IL-6 production was enhanced to varying degrees by IL-1 in all seven cell lines, for example in experiment 1 BOP 7 responded to IL-1 with a 15-fold increase in IL-6 production, a 72 fold increase was observed in BOP 37. Certain cell lines, for example BOP 31, 37 and 70, also responded to TNF with enhanced IL-6 activity as shown in table 5.2. Treatment of the cells with 17β-oestradiol for 72h had no effect on the IL-6 production by the cells (Table 5.3).

Interleukin 1 production

The production of bioactive IL-1 by the cells is shown in table 5.4. Constitutive production of IL-1 by all seven cell lines was very low but was considerably increased by treatment of the cells with TNFα. BOP 7 and 31, for example, displayed a greater response to TNF than cell lines 37 and 12. Oestradiol had no effect on the constitutive IL-1 production of any of the BOP cell lines tested.

TNF production

The production of TNF by BOP cells was assayed in two separate experiments. In one experiment conditioned media were stored at -20°C for the subsequent analysis of TNFα content by IRMA. In a second experiment fresh BOP cell conditioned media were assayed directly for bioactive TNF using the sensitive WEHI cell line. All seven cell lines produced high levels of immunoreactive TNFα (Table 5.5) and this production was not affected by treatment of the cells with IL-1. Oestradiol had no effect on the TNFα secretion in six cell lines but caused a small increase in the amount of TNFα produced by cell line BOP 7. TNF produced by the cells was not bioactive, no cytotoxic effect was observed when conditioned media were assayed using the WEHI cell line.

Expression of IL-1β and TGFβ mRNA

The expression of mRNA for IL-1β in control and IL-1α treated cells is shown in Fig 5.1. Treatment with rhIL-1α for 6h enhanced mRNA for IL-1β in six cell lines, the greatest responses were seen in BOP 31 and 37. Constitutive expression of TGFβ mRNA was very low (Fig. 5.2). E_2 had no effect on the expression of TGFβ mRNA by BOP cells. The relative slot blot intensities for cytokine/β-actin mRNA are shown in table 5.6.

BOP	Control	IL-1α (10U/ml)	T/C
7	13 ± 1	150 ± 30.5	15
12	673 ± 175	1800 ± 0	2.7
26	14 ± 2.4	103 ± 3	7.4
31	11 ± 0.3	>100 <1,000	-
33	12.3 ± 2.3	<100	-
37	130 ± 17.3	9333 ± 1667	72
70	120 ± 15.3	560 ± 23	4.7

(a).   Experiment 1

BOP	Control	IL-1α (10U/ml)
7	<10	11.7 ± 1.7
12	<10	205 ± 58
26	<10	198 ± 58
31	<10	115 ± 15
33	<10	89 ± 41
37	<10	15 ± 1
70	<10	10.3 ± 0.3

(b).   Experiment 2

Table 5.1   IL-6 production (U/ml) in BOP cells. Cells were treated with IL-1α for 72h and IL-6 in conditioned media assayed using the B9 hybridoma cell line. T/C is the ratio of treated to control values. Values for the same conditioned media are shown in Tables 5.2 and 5.3.

BOP	Control	TNFα	T/C
		1,000 U/ml	
7	13 ± 1	20 ± 2.6	1.54
12	673 ± 175	1117 ± 101	1.66
26	14 ± 2.4	18 ± 4.3	1.3
31	11 ± 0.3	127 ± 14.5	11.5
33	12.3 ± 2.3	20 ± 2.3	1.6
37	130 ± 17.3	1117 ± 242	8.6
70	120 ± 15.3	583 ± 136	4.7

(a).   Experiment 1

BOP	Control	TNFα
		1,000 U/ml
7	<10	11.7 ± 1.7
12	<10	205 ± 84
26	<10	137 ± 22
31	<10	235 ± 35
33	<10	33 ± 12
37	<10	17 ± 3
70	<10	10.3 ± 0.3

(b).   Experiment 2

Table 5.2   IL-6 production in BOP cells treated with 1,000U/ml TNFα for 72h.

	Experiment 1		Experiment 2	
BOP	Control	E_2	Control	E_2
		$(10^{-9}m)$		
7	13 ± 1	13 ± 1.5	<10	<10
12	673 ± 175	357 ± 64.4	<10	<10
26	14 ± 2.4	12 ± 2	<10	<10
31	11 ± 0.3	19 ± 2.5	<10	<10
33	12.3 ± 2.3	<10	<10	<10
37	130 ± 17.3	147 ± 37	<10	<10
70	120 ± 15.3	247 ± 62	<10	<10

Table 5.3   IL-6 production (U/ml) in cells treated with $10^{-9}M$ 17β oestradiol for 72h. These are the same conditioned media as in Tables 5.1 and 5.2.

BOP	Control	TNFα	T/C	E2
7	0.01± 0.004	0.26 ± 0.08	26	0.00
12	0.07 ± 0.01	0.11 ± 0.02	1.6	0.07 ± 0.004
26	0.00	0.1 ± 0.007	-	0.01 ± 0.006
31	0.02 ± 0.003	0.3 ± 0.15	15	0.00
33	0.02 ± 0.006	0.14 ± 0.02	7	0.02 ± 0.01
37	0.06 ± 0.01	0.154 ± 0.04	2.6	0.11 ± 0.06
70	0.1 ± 0.02	0.466 ± 0.03	4.7	0.08 ± 0.01

Table 5.4   IL-1 prodution (U/ml) by BOP cells treated with 1,000U/ml rhTNFα or 10^{-9}M 17- β oestradiol (E2) for 72h. Conditioned media were assayed for IL-1 using the D10N4 M assay.

BOP	Control	IL-1α	E2
7	7.48 ± 0.08	6.94 ± 0.32	9.16 ± 0.09
12	4.27 ± 0.46	4.95 ± 0.44	5.24 ± 0.4
26	6.47 ± 0.34	6.53 ± 0.07	5.71 ± 0.18
31	6.45 ± 0.18	7.26 ± 013	7.38 ± 0.55
33	7.6 ± 0.38	9.35 ± 0.37	7.62 ± 0.3
37	8.44 ± 0.5	9.15 ± 0.17	7.77 ± 0.7
70	5.36 ± 0.2	5.9 ± 0.3	5.41 ± 0.15

Table 5.5   TNFα production (U/ml) by BOP cells treated in defined medium with 10U/ml rhIL-1α or 10^{-9}M 17- β oestradiol (E2) for 72h. Conditioned media were assayed for TNFα using an immunoradiometric assay.
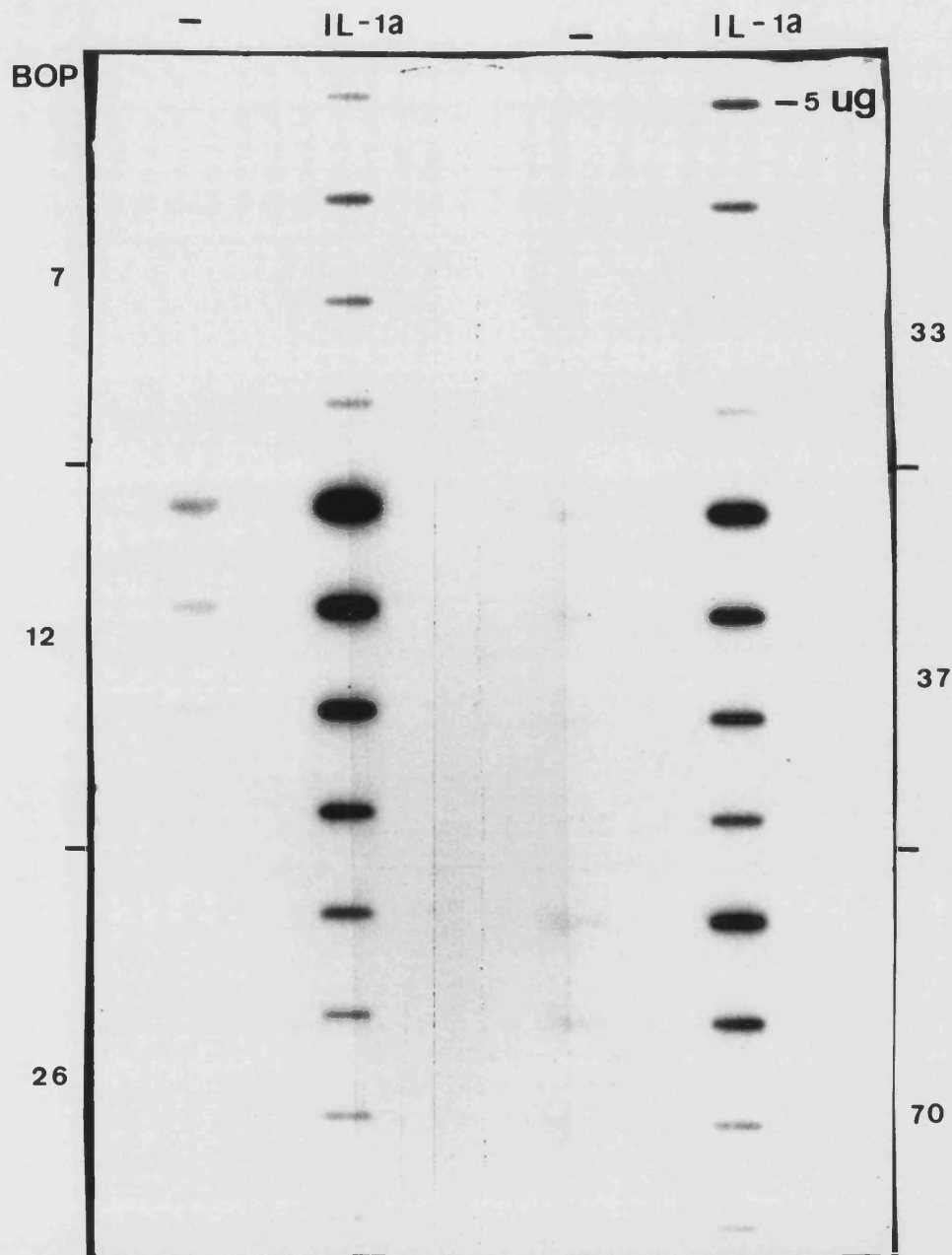
Fig. 5.1 (a). Interleukin 1β mRNA expression in control and IL-1α treated

BOP cells. Cells were incubated with 10U/ml IL-1α for 6h and RNA extracted

as described in chapter 2. 5μg of RNA were loaded in the first slots as

indicated and 3 serial dilutions of each sample were loaded. Slot blots were

hybridised with a cDNA probe recognising IL-1β. Equal loading of the RNA

was confirmed by probing stripped filters for human β actin (b) (next page).
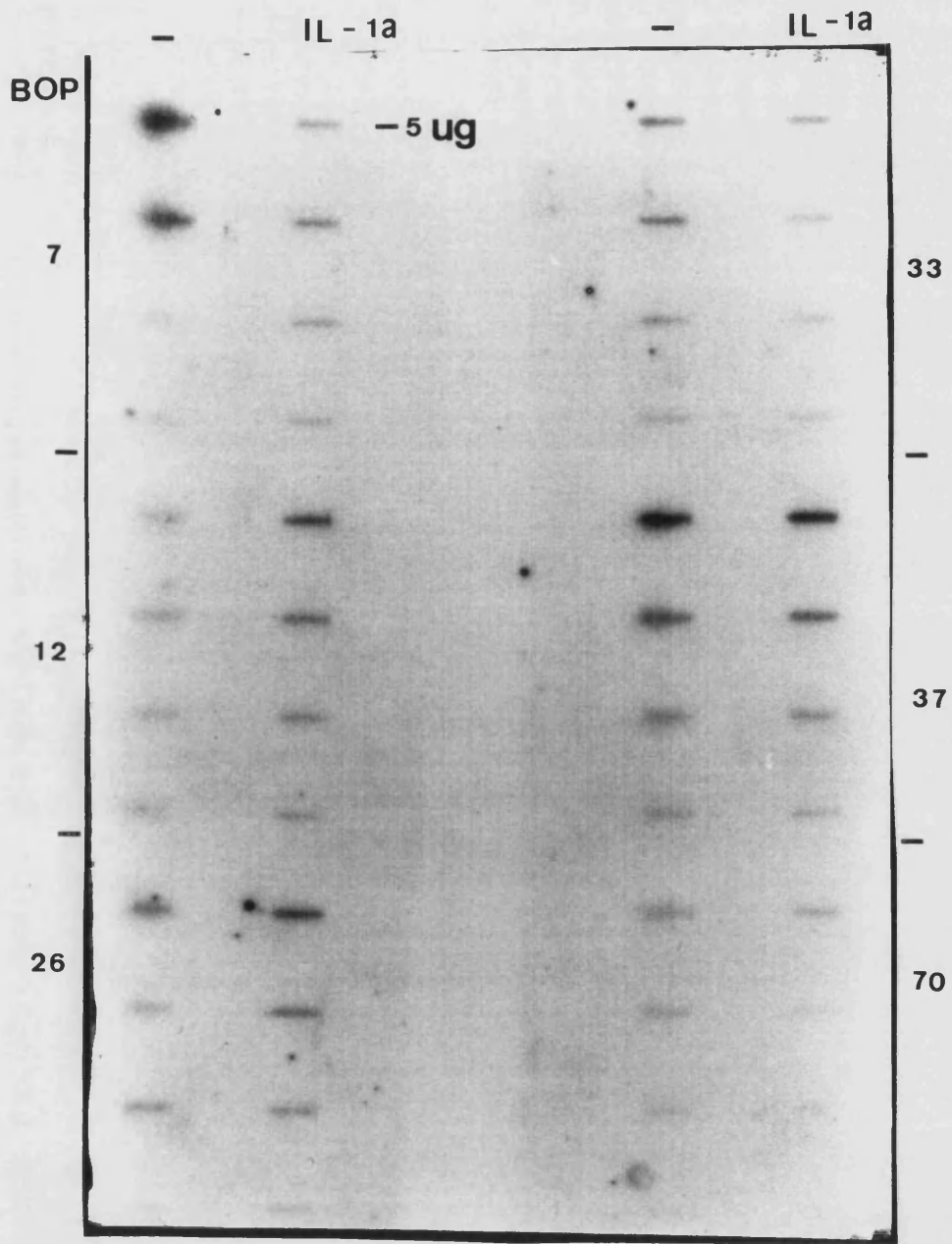
Fig. 5.1(b). Actin control.
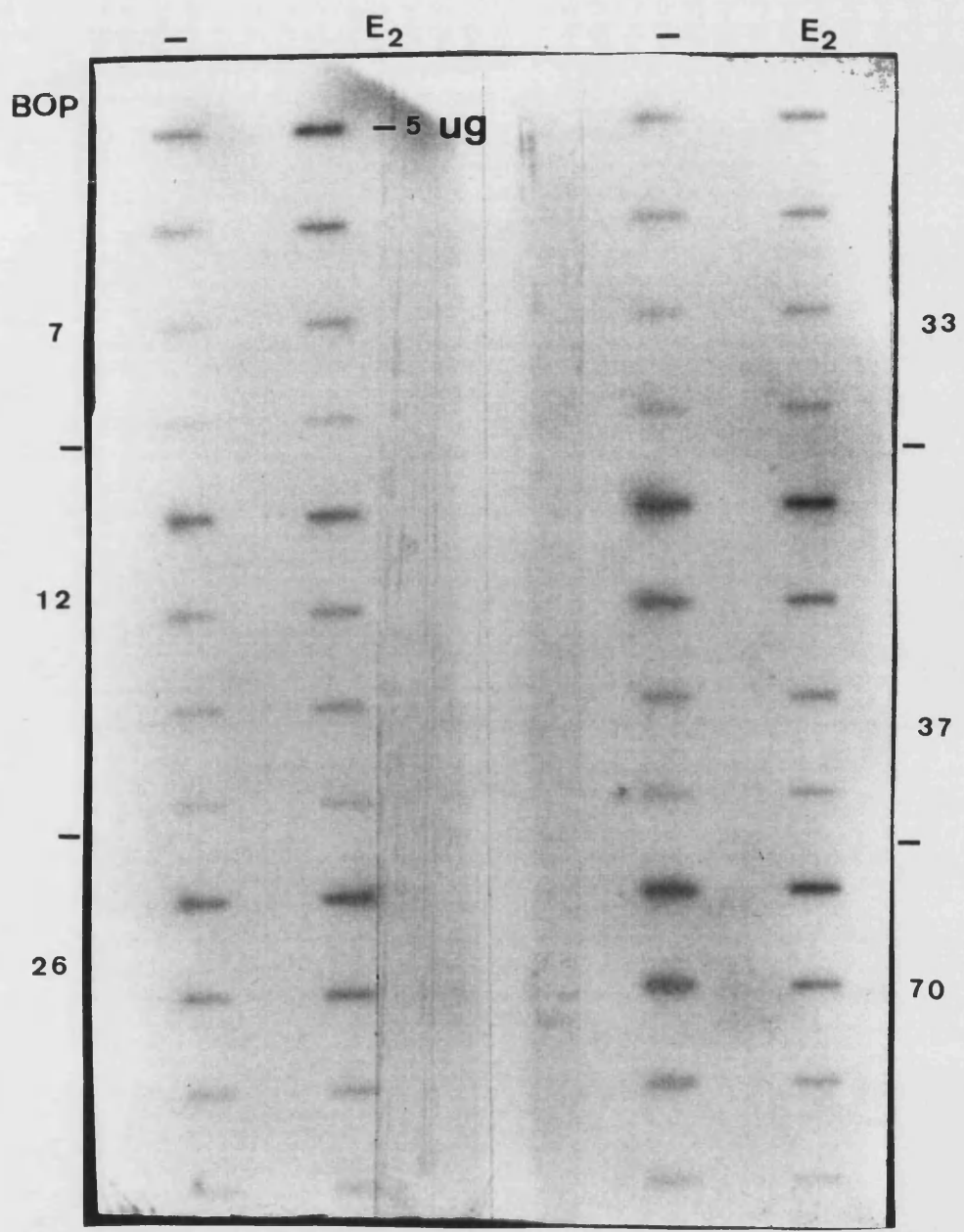
Fig. 5.2   (a).   TGFβ mRNA expression in six BOP cell lines treated with

10⁻⁹M 17β-oestradiol for 9h. The actin control is shown overleaf (b).
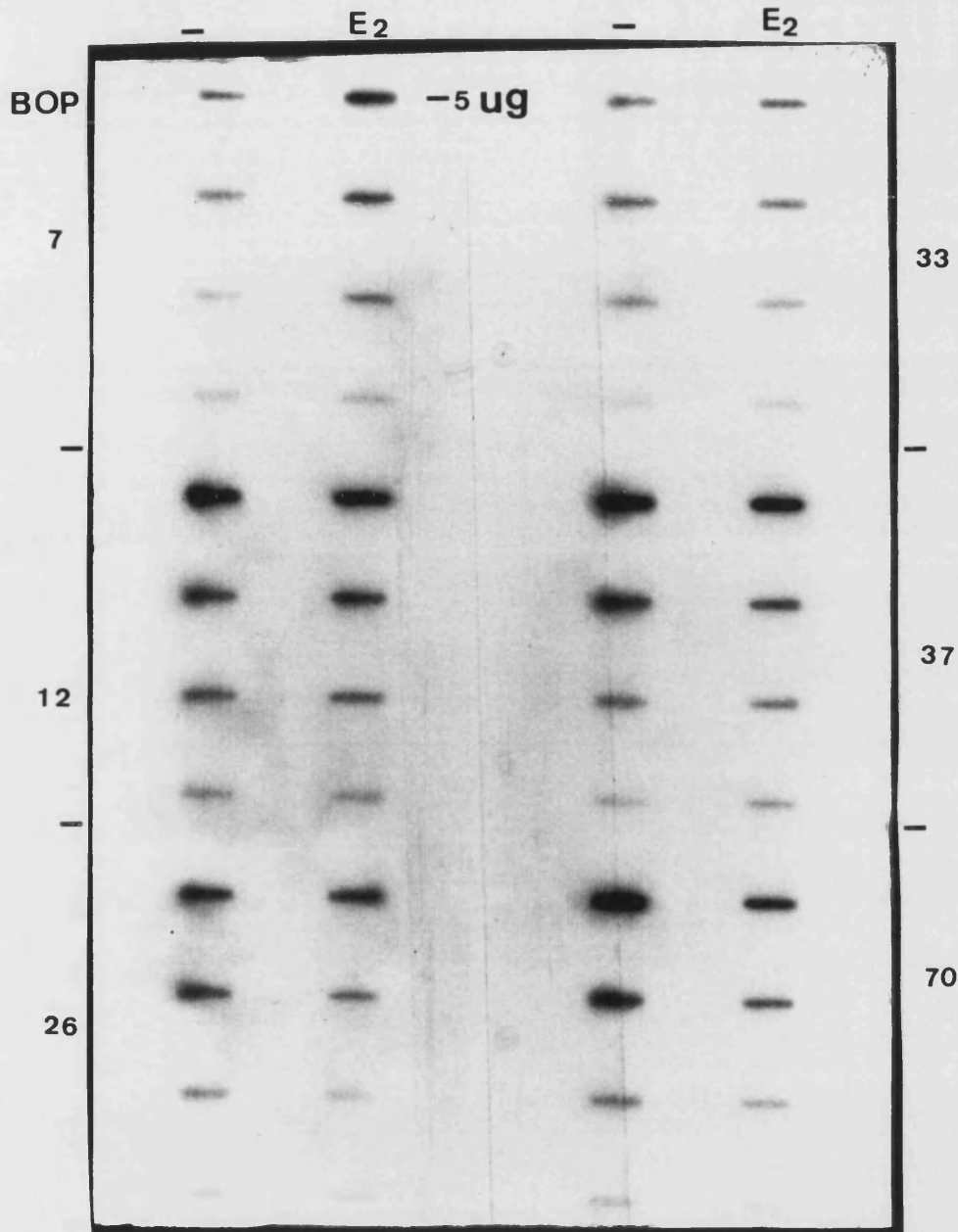
Fig. 5.2 (b).   Actin control.

BOP	Control	IL-1α
7	1.0	5.8
12	1.0	2.1
26	1.0	6.4
33	1.0	16.7
37	1.0	23
70	1.0	17

(a)

BOP	Control	E$_2$
7	1.0	1.7
12	1.0	0.8
26	1.0	0.6
33	1.0	0.8
37	1.0	0.9
70	1.0	0.8

(b)

Table 5.6   Relative slot blot densities for IL-1β/β-actin (a) and TGFβ/β-actin (b) mRNA normalised as 1.0 for the ratio of densities obtained with mRNA from unstimulated cells.

5.4   DISCUSSION

Cytokines such as IL-1, IL-6 and TNF may be important osteotropic agents and are produced by osteoblast-like cells *in vitro*. The ability of SV40-immortalised osteoblasts to produce cytokines and to display the cytokine responsiveness characteristic of parent cultures was studied. Oestrogen deficiency is strongly linked to the development of postmenopausal osteoporosis but the mechanism of oestrogen action remains undefined. A potential role for oestrogen as a regulator of cytokine production has been suggested by Pacifici *et al* (1989) and oestradiol may regulate cytokine release by osteoblasts (Rickard, Russell and Gowen, 1992). The effects of oestradiol on the basal production of IL-1, IL-6 and TNF by BOP cells and on the expression of TGFβ mRNA in the cell lines was assessed.

Littlewood *et al* (1991b) report that primary cultures of human osteoblasts produce high levels (1000-2050 U/ml) of IL-6 constitutively and the release of IL-6 is dramatically increased by treatment with IL-1 and TNF. The amount of IL-6 released by unstimulated BOP cells was much lower but the cells were capable of responding to IL-1 and TNF with enhanced IL-6 production. The low levels of constitutive IL-6 in BOP cells are similar to those produced by the human osteosarcoma cell lines MG-63 and HOS TE85, two cell lines which also respond to IL-1 and TNF with enhanced IL-6 production (van Damme *et al*, 1987; Rickard, Clover and Gowen manuscript in preparation). Linkhart *et al* (1991) describe the secretion of IL-6 by cells isolated from normal human bone and report no detectable constitutive IL-6 activity but secretion was induced by IL-1. The differences in basal IL-6 release observed for primary cultures may reflect the heterogeneity and variable growth rate of cells derived from different sources. Treatment of BOP cells with 17β-oestradiol did not affect the basal release of IL-6 in any of the cell lines tested. Similarly, Rickard, Russell and Gowen (1992) report no

consistent effect of oestradiol on either basal or stimulated IL-6 release in primary cultures of human osteoblasts.

BOP cells released high levels of TNF constitutively, much higher than the reported values of 0-0.25U/ml for primary cultures (Gowen *et al*, 1990). IL-1 had no effect on TNF release by BOP cells. Oestradiol inhibits the IL-1-induced TNF production of trabecular bone-derived cells (Rickard, Russell and Gowen, 1992) but the hormone had no effect on the constitutive release of TNF in six of the BOP cell lines tested. In cell line BOP 7, oestradiol caused a slight enhancement of TNF production. This cell line may represent a subpopulation of oestrogen responsive cells that were present in the parent cultures. The TNF produced by the cells was found to be immunoreactive but not bioactive. One possible explanation for this is the production of natural inhibitors of TNF. A human inhibitor of TNFα has been purified from the urine of febrile patients and the inhibitor can be measured in an assay of cytotoxicity using a TNF-susceptible cell line (Seckinger, Isaaz and Dayer, 1988). A soluble form of the 55kDa TNF receptor exists which binds free TNF and inhibits its interaction with cell-surface receptors (Schall *et al*, 1990). The enhanced constitutive production of TNF by BOP cells may be caused by upregulation following SV40-induced transformation and this excessive production of TNF may give rise to the secretion of natural inhibitors.

BOP cells produced low levels of IL-1 constitutively and responded to TNF by enhanced release of IL-1. Treatment with IL-1α also enhanced expression of mRNA for IL-1β suggesting autocrine regulation in the cell lines as has been observed for primary cultures of trabecular bone cells (Merry *et al*, 1991). The immortalised cell lines displayed low constitutive expression of TGFβ mRNA and oestradiol did not enhance this specific mRNA unlike the effect of oestradiol that has been reported for primary cultures (Oursler *et al*, 1991).

BOP cell lines display the characteristics of mature osteoblasts with respect to alkaline phosphatase activity and production of bone-associated proteins. The immortalised cell lines were also capable of responding to both IL-1 and TNF with enhanced IL-6 production, and responded to TNF with enhanced IL-1 production as has been reported for primary cultures of osteoblast-like cells. The high levels of TNF produced by the cell lines may be a result of the SV40-induced transformation process. The cell lines failed to respond to oestradiol with enhanced TGFβ mRNA expression but one cell line, BOP 7, displayed enhanced TNF release when treated with oestradiol and may represent a subpopulation of oestrogen-responsive cells present in the original parent cultures.

CHAPTER 6

Stability of the immortalised-cell phenotype.

ABSTRACT

Cell lines obtained by transfection of primary cultures with SV40 commonly display an extended lifespan *in vitro* but eventually the cells senesce. BOP cells express many features of the parent cultures of osteoblast-like cells. The stability of this phenotypic expression was explored by assessing alkaline phosphatase activity and osteocalcin production as a function of time in culture. BOP 12 showed high ALP activity and a functional response to 1,25D for up to 15 passages but reduced ALP activity at passages 18 and 19; osteocalcin production was maintained to passage 18 but lost at passage 19. Similarly, BOP 26 showed reduced ALP activity at passage18 but osteocalcin production was maintained. BOP 7 was stable for 13 passages. Expression of SV40 T antigens was persistant in BOP 12 to passage 18. The loss of phenotypic expression by the cells may be a reflection of the crisis stage that is a characteristic of SV40 transformed cells.

6.1   INTRODUCTION

An important consideration when using a cell line for *in vitro* studies is the stability of the phenotype as cells are continuously maintained and subcultured. Human trabecular bone-derived cells have only a limited lifespan *in vitro*. Primary cultures of a variety of cell types, when transfected with SV40, commonly yield cell lines which display an extended lifespan and express the phenotype of the parent cultures. However, little information is available concerning the long-term stability of phenotypic expression by the transformed cell type.

Pinet *et al* (1985) transfected human juxtaglomerular cells with SV40 DNA and obtained three cell lines that retained the ability to secrete the hormone renin. The quantity of renin produced declined after the first and second subcultures but then remained stable for the following five subcultures. Singer *et al* (1987) used SV40 to transform human bone marrow stromal

cells and obtained clonal cell lines that persistantly contained both round (haemopoietic) cells and adherent (stromal) cells. The stromal cells synthesised proteins characteristic of the parent cells, such as interstitial and basal lamina collagens, muscle-type actin, vimentin and thrombospondin. The cell lines survived up to 36 passages (11 months in culture) but no data is presented detailing the persistance of the characteristic proteins with time in culture. In a subsequent paper, Nemunaitis *et al* (1989) described the production of colony stimulating factors by round cells of the same series of cell lines. If cells were stimulated with IL-1α or β, or TNFα they produced high levels of colony stimulating factors which were detectable for up to 16 passages.

Other authors describing the transfection of rabbit kidney cells with SV40 early region genes demonstrate the ability of transformed cells to express the characteristics of parent cells (Scott *et al*, 1986). The cell lines obtained were capable of an extended life with continued expression of the phenotype. Similarly, transformation of neonatal rat Schwann cells resulted in a cell line with established Schwann cell properties (the ability to synthesise sulphogalactosyl ceramide and express mRNA for for the myelin protein P_0) which could be maintained for 180 doublings (Tennekoon *et al*, 1987). Two bone cell lines were established from rat calvarial osteoblasts by immortalisation with SV40 large T antigen and the neomycin resistance gene. One cell line, RCT-3, constitutively expressed markers of the osteoblast phenotype. The cells possessed alkaline phosphatase activity, expressed mRNA for type I collagen and osteopontin and displayed a PTH responsive adenylate cyclase. A second cell line, RCT-1, could be induced to express the above characteristics by treatment with retinoic acid (Heath *et al*, 1989). These cells have a limited lifespan *in vitro* (Baron, personal communication).

Keeting *et al* (1992) transformed primary cultures of trabecular bone cells with a vector incorporating SV40 early region genes and resistance to the antibiotics neomycin and G418. The cell line obtained grew rapidly but slowed after eight passages, cells entered crisis and became senescent between passages 12 and 14. The pX-8 SV40 vector used to produce BOP cells had been used to transfect pancreatic β islet cells, the cell line obtained secreted insulin for eight passages and then stopped releasing the hormone (Marini and Soldevila, personal communication).

In order to assess the stability of the phenotype of immortalised BOP cells, and to define the useful lifespan of the cell lines, three lines were subcultured continuously and characterised for two markers of the osteoblast phenotype at regular intervals. The ability of the cells to continue producing SV40 T antigens with time in culture was also monitored.

6.2   METHODS

Cell lines BOP 7, 12 and 26 were maintained in continuous culture and assayed for control and 1,25D responsive alkaline phosphatase activity and osteocalcin production at every second or third passage until the cells entered crisis and failed to grow. Cells were seeded at 4×10^4/ml in 24-well plates, allowed to settle for 24h and treated with ethanol vehicle or 10^{-8}M 1,25D for 48h. Conditioned media were assayed for osteocalcin production and cell layers for ALP activity as detailed in chapter 2. Triplicate wells were assayed and results expressed ± SEM.

At passages 10,13, 15 and 18, cells of BOP 12 were seeded onto poly-L-lysine treated glass slides, allowed to settle for 24h, then fixed and stained for T antigen expression as described in chapter 2.

6.3   RESULTS

Cell growth

After approximately 15 passages *in vitro,* BOP cells failed to attain confluence and many cells were shed into the medium. The remaining cells continued dividing when passaged and seeded into fresh medium but after approximately 19/20 passages the cells failed to grow and sparse cells with the morphological appearance shown in figure 6.1 remained in the culture dishes. The remaining cells were large, irregular in shape and granular in appearance.

Alkaline phosphatase expression

The alkaline phosphatase activity of cell line BOP 12 maintained in continuous culture and assayed every few passages is shown in table 6.1. Constitutive ALP activity was stable for 17 passages but was reduced at passage 18 and 19. The cells responded to 1,25D with enhanced ALP activity with the greatest response seen at passages 10 and 12 and a reduced response at passage 19. BOP 26 (Table 6.2a) showed a decline in ALP activity with time in culture but responsiveness to 1,25D was maintained. BOP 7 was stable for 13 passages (Table 6.2b).
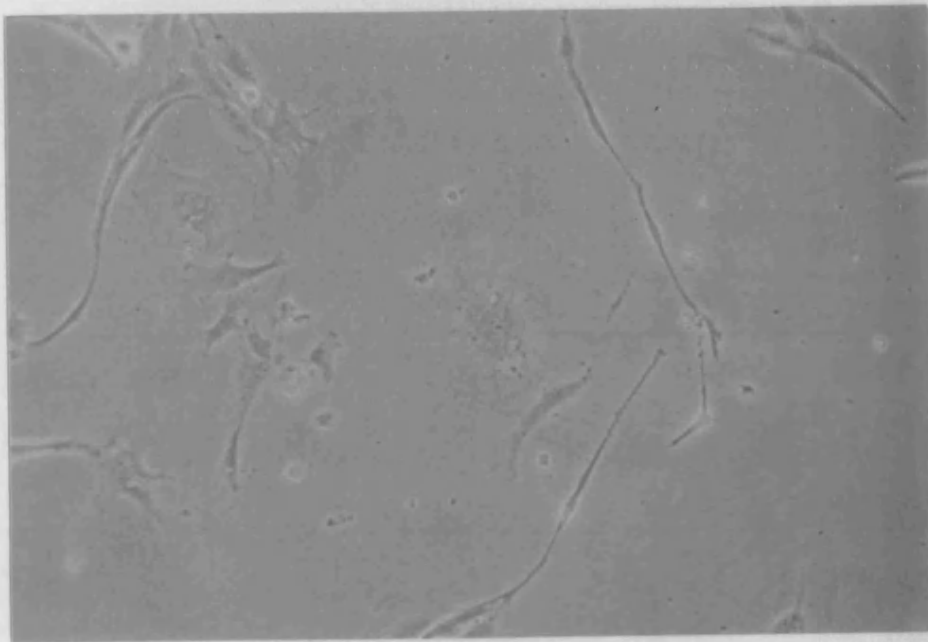
Fig. 6.1   Phase contrast micrograph of senescent cells of BOP 26 at passage 20. X100.

Alkaline phosphatase
(μmoles/min/mg)

	BOP	Control	1,25D	T/C
(a)	12p10	1.48 ± 0.18	5.20 ± 0.25	3.5
	12p13	1.68 ± 0.21	3.62 ± 0.25	2.15
	12p15	2.62 ± 0.21	5.08 ± 0.50	1.94
	12p17	1.09 ± 0.04	1.92 ± 0.08	1.76
	12p18	0.12 ± 0.02	0.29 ± 0.02	2.42
(b)	12p12	1.02 ± 0.02	3.3 ± 0.2	3.24
	12p17	1.24 ± 0.15	1.92 ± 0.11	1.55
	12p19	<0.50	0.7 ± 0.05	1.4

Table 6.1   Alkaline phosphatase activity in control and 1,25D treated BOP
12 assayed at different passages throughout continuous culture. (a) and (b)
are the results of two separate experiments. T/C is the ratio of treated to
control values.

		Alkaline phosphatase (μmoles/min/mg)		
	BOP	Control	1.25D	T/C
(a)	26p12	2.46 ± 0.3	5.55 ± 0.37	2.26
	26p15	1.05 ± 0.04	2.6 ± 0.16	2.48
	26p18	0.12 ± 0.02	0.24 ± 0.02	2
(b)	7p8	<0.50	0.68 ± 0.05	1.36
	7p12	0.58 ± 0.02	1.42 ± 0.05	2.45
	7p13	<0.50	0.75 ± 0.05	1.5

Table 6.2   Alkaline phosphatse activity in (a) BOP 26 and (b) BOP 7 assayed at different passages.

Osteocalcin production

None of the cell lines produced constitutive osteocalcin but production was induced by 1,25D. Osteocalcin production by BOP 12 was greatest at passage 10 and then declined, cells at passage 19 failed to synthesise osteocalcin (Table 6.3). BOP 26 cells produced similar levels of osteocalcin at the three time points assayed. Osteocalcin production was low in BOP 7 and the low levels were maintained to passage 13 (Table 6.4).

T antigen production

The production of SV40 T antigens by cells of BOP 12 at different passages is shown in figure 6.2. Cells displayed positive nuclear staining with the antibody at early passage (p10) and cells continued to produce T antigens at passage 18.

	BOP	Osteocalcin (ng/ml) ±1,25D
(a)	12p10	8.34 ± 0.15
	12p13	1.52 ± 0.23
	12p15	3.89 ± 0.11
	12p17	2.75 ± 0.12
	12p18	2.4 ± 0.18
(b)	12p12	2.69 ± 0.30
	12p17	1.16 ± 0.30
	12p19	0

Table 6.3   Osteocalcin production in cells of BOP 12 treated with 1,25D at various time points throughout continuous culture. (a) and (b) represent two separate experiments.

	BOP	Osteocalcin (ng/ml) ±1,25D
(a)	26p12	3.47 ± 0.09
	26p15	2.4 ± 0.11
	26p18	2.45 ± 0.32
(b)	7p8	1.52 ± 0.1
	7p12	0.6 ± 0.3
	7p13	1.85 ± 0.03

Table 6.4   Osteocalcin production in (a) BOP 26 and (b) BOP 7 cells treated with 1,25D at different passages.

Fig. 6.2 (overleaf)   Expression of SV40 T antigen in cells of BOP 12.

(a) Cells at passage 10, (b) passage 13, (c) passage 15 and (d) passage

18. X100.
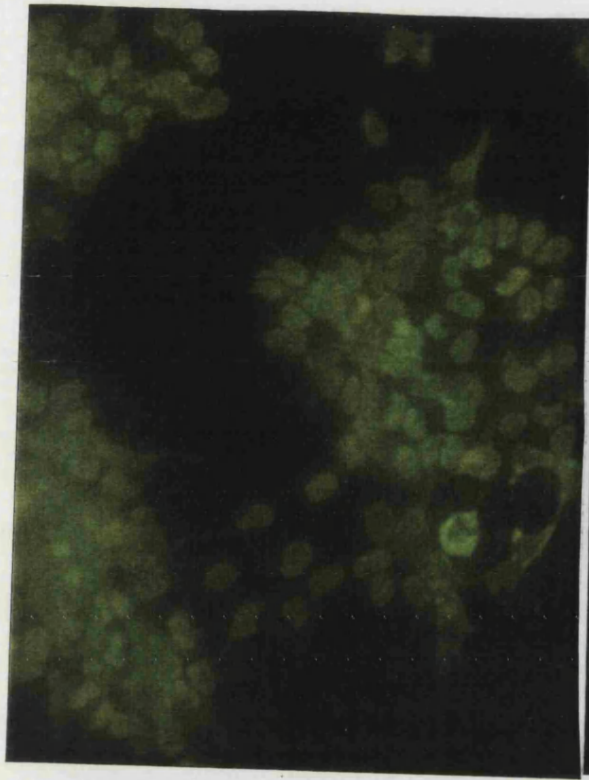
(a)

(b)

(c)

(d)

6.4   DISCUSSION

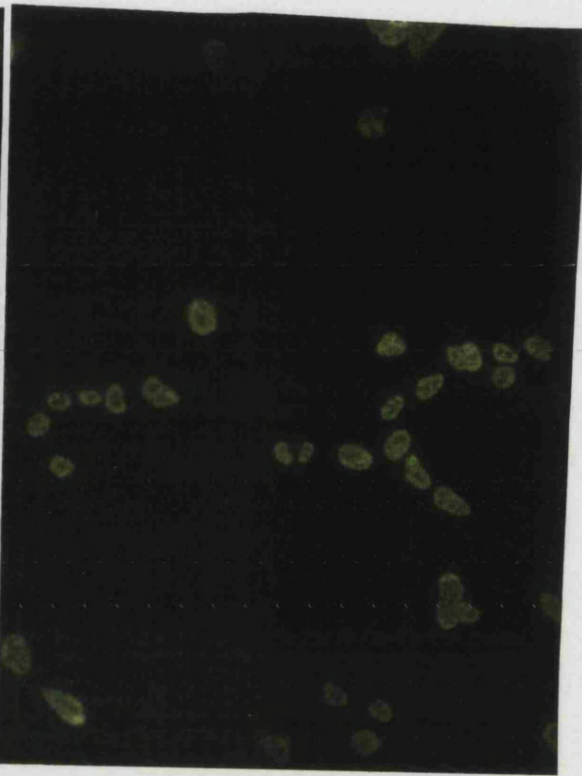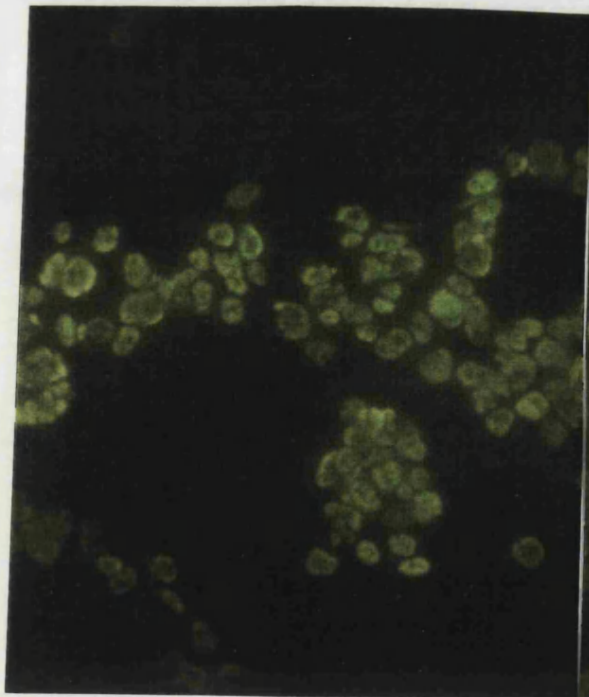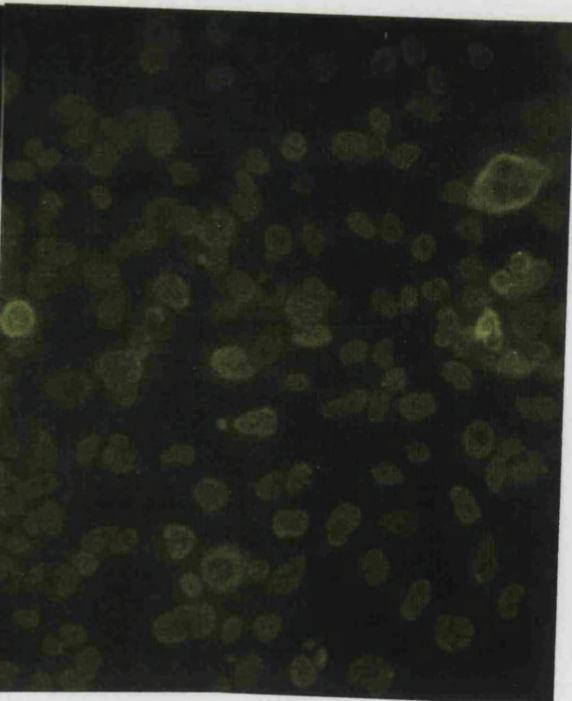The phenotype of the osteoblast-like cell lines was stable for a limited number of passages, after passage 15/16 expression of the osteoblast characteristics alkaline phosphatase activity and osteocalcin production was reduced or lost. A fall in the responsiveness of cells to 1,25D may be a result of a loss of functional 1,25D receptors in cells undergoing or about to enter the crisis state. Cells transfected with SV40 DNA typically divide for many generations beyond the expected lifespan of the parent primary cultures. The cell lines (described as "pre-immortalised" by Huschtscha and Holliday, 1983) then undergo a period of crisis, cells are shed into the medium and the remaining adherent cells fail to divide and ultimately senesce. The BOP cell lines, when initially isolated, were capable of post-confluent growth and formed multilayered foci (Chapter 3, Fig. 3.3) which persisted for a number of passages in culture. After 12-15 passages confluent cells in 100mm dishes did not form foci but were shed into the medium and by passage 17 cells failed to grow to confluence. Remaining cells were able to grow sufficiently for a further two subcultures but with smaller yields of cells. The cells appeared to be senescent by passage 19. Production of T antigen persisted in the cells to passage 18, suggesting that the decline of phenotypic expression by the cells, or the crisis state, was not due to a loss of the transforming vector or a failure of early gene transcription.

The pX-8 vector used to transfect primary cultures of osteoblasts contained an origin-defective SV40 early region, 8 regions at the Bgl I site were removed and replaced by a Xho I linker (Fromm and Berg, 1982; see Fig 3.2). The portion of DNA lost from the pX-8 vector contains binding regions for the large T antigen. In wild-type vectors, T antigen is able to bind to the origin of replication and autoregulates early region gene expression by inhibition of transcription initiation (Myers *et al*, 1981). T antigen is

overproduced in cells which have stably incorporated an origin defective SV40 vector and this may account for the enhanced transformation efficiency of ori⁻ observed by Small, Gluzman and Ozer (1982).

The precise mechanism of T antigen-induced mitosis of transfected cells is not known but the protein has been shown to stimulate chromatin replication in epithelioid cells (Graessman and Graessmann, 1976). T antigen, when injected into the cytoplasm of recipient cells, migrates across the nuclear membrane and stimulates cellular DNA synthesis (Tjian, Fey and Graessmann, 1978). In addition, large T antigen, purified from SV40-transformed human fibroblasts, exhibits ATPase activity which is enhanced severalfold by the DNA homopolymer poly (dT) (Giacherio and Hager, 1979). The ability of T antigen to bind both double stranded and single stranded DNA and its nucleic acid-stimulated ATPase suggests a role for T antigen in initiation of DNA synthesis by separating double stranded DNA and binding to the single strands to prevent reforming of the double helix. T antigen is able to block differentiation in preadipocytes by suppressing the expression of adipocyte differentiation-dependent genes (Cherington *et al*, 1988). BOP cells were capable of differentiation in response to 1,25D but gradually lost this ability, prolonged overexpression of T antigen may have resulted in prevention of the cells from differentiating. However, the clonal cell lines 37WW3 and 37WW8 described in chapter 3, which may represent stable post-crisis lines, displayed high alkaline phosphatase activity and responsiveness to 1,25D. The loss of phenotype of the pre-immortalised cell lines may be a function of the crisis stage which is so characteristic of SV40-transfected cells. Oshima *et al* (1977) observed no differences between precrisis and postcrisis cell lines with respect to growth rate, ability to form colonies on confluent monolayers or production of T antigen. The majority of cells do not survive crisis but the small number that are able to form post-

crisis lines may do so by continually producing stimulatory factors making them capable of unlimited growth.

It is interesting to note that BOP cells have a longer lifespan in culture of 19/20 passages (approximately 120 generations) than the immortalised osteoblastic cell line HOBIT which survives for more than 80 generations (Keeting *et al*, 1992). This may reflect the age of the donors used, BOP cells are derived from a 17 year old male, HOBIT cells from a 68 year old female. Primary cultures of osteoblasts obtained from young donors grow more rapidly *in vitro* than those from elderly patients and attain confluence more quickly. Oshima *et al* (1977) have suggested that the crisis stage of immortalised cells is a reflection of the limited lifespan of normal human diploid fibroblasts as the onset of crisis is shorter for cells infected near the end of their normal lifespan in culture. BOP cells may thus survive longer because the original primary cultures were transfected early in their normal life.

BOP cell lines obtained by transfection of primary cultures with SV40 DNA displayed a stable phenotype for 15 passages *in vitro*. A reduction of alkaline phosphatase activity and osteocalcin production, and a reduced response to 1,25D, were observed in cells maintained for more than 15 passages. This loss of phenotype coincided with shedding of cells into the medium and their ultimate senescence. The crisis stage of BOP cells did not appear to be a result of a loss of T antigen expression as the cells continued to support SV40 early gene expression.

CHAPTER 7

GENERAL DISCUSSION

7. GENERAL DISCUSSION

7.1   Immortalisation of primary cultures

Transfection of primary cultures of human osteoblasts with SV40 DNA yielded populations of cells with a capacity for rapid growth, large numbers of cells could be obtained which expressed hallmarks of the osteoblast phenotype. The phenotype of the cells was stable for 15 passages and then declined, cells were shed into the medium and the remaining cells ultimately senesced. Two cell lines which survived limiting dilution cloning may represent stable, post-crisis cell lines. Oshima *et al* (1977) have demonstrated that SV40 transformed human fibroblasts have a different DNA content per cell than precrisis cells and Huschtscha and Holliday (1983) observed that post-crisis cell lines contained a very high percentage of hyper-diploid cells. Oshima *et al* have suggested that the recovery of a post-crisis cell line depends upon the formation of a small number of variant cells. Picking single transformed foci and subsequent recloning before crisis, as has been done for this thesis, would reduce the chance of obtaining a long-term immortalised cell line if recovery from crisis was acheived by a small number of cells with an altered genetic complement. Growing bulk cultures of cells and maintaining them until post-crisis cells emerge may be a more efficient method of obtaining a long-term cell line.

Mayne *et al* (1986) transfected human fibroblasts with a plasmid containing both SV40 early regions and the bacterial gene gpt and reported that use of the selectable marker increased the probability of establishing an immortal cell line. However, these authors report that all transfected cells were maintained in bulk cultures and one cell line was obtained from each culture as would be expected. Other workers using antibiotic resistance genes as selectable markers have obtained cell lines with a limited growth potential but do not report the successful maintenance of long-term cell lines derived from senescent cultures (Heath *et al*, 1989; Keeting *et al*, 1992).

Scott *et al* (1989) have demonstrated that the neoplastic phenotype of
SV40 transformed cells can be suppressed by inducing nonterminal
differentiation. These workers obtained an adipocytic cell line by
transfection of murine mesenchymal stem cells with SV40 DNA. Culturing
the cells in heparinised DMEM containing 25% human plasma induced the
cells to revert to a nontransformed state and repressed expression of the
large T antigen. Cells that had been induced to nonterminally differentiate
failed to form colonies in semi-solid medium. This may be a potentially
useful mechanism for controlling the growth of immortalised cell lines and
allowing the normal processes of differentiation to go ahead. Use of a vector
coding for a mutant temperature-labile T antigen to transfect cells is another
potential mechanism for controlling the transformed phenotype of the
immortalised cell lines. Cells transfected with this mutant T antigen when
maintained at 39°C failed to form colonies and the transformed phenotype
was repressed (Radna *et al*, 1989). The disadvantage of using this
technique is the abnormal temperature required to inhibit T antigen
production which may also affect other cell functions.

The senescence of cells transfected with SV40 DNA may be related to the
limited growth potential of the parent primary cultures. BOP cells that had
been preserved in liquid nitrogen and subsequently revived had similar
growth properties and phenotypic characteristics to cells prior to
cryopreservation, but cell lines persistantly senesced after around 20
passages irrespective of the stage at which they were frozen. It is possible
that the programmed cell death of the cell lines was due to a loss of
stimulation of the cells, perhaps due to a cessation of production of certain
growth factors. Apoptosis in human monocytes is reported to be prevented
by addition of stimulatory factors such as lipopolysaccharide, TNFα and IL-1β
(Mangan, Welch and Wahl, 1991). Pre-crisis human fibroblastic cells were
able to grow well in the medium from senescent cells (Oshima *et al*, 1977)

but the ability of medium conditioned by pre-crisis cells to affect the growth of cells undergoing senescence has not been tested.

7.2   Phenotypic expression of immortalised cells.

Primary cultures of trabecular bone-derived cells are thought to contain osteoblasts at various stages of differentiation. It was initially proposed that the action of the transforming SV40 vector would be to "freeze" the cells at their particular stage of differentiation and produce a panel of cell lines representing different stages of the osteogenic pathway. Owen *et al* (1990) have described the progressive development of the rat osteoblast phenotype *in vitro*. Three principal stages occur, proliferation, extracellular matrix maturation and mineralisation. During the proliferative period there is a peak of DNA synthesis and enhanced expression of the H4 histone. Type I collagen, fibronectin and TGFβ are actively expressed. Enhanced expression of alkaline phosphatase is observed immediately following the proliferative period, later on enhanced expression of osteocalcin and osteopontin occurs at the onset of mineralisation. Bianco *et al* (1989) have shown that bone sialoprotein is produced late in the differentiation pathway. Developmental studies of the expression of bone matrix proteins suggest the sequence of events shown in figure 7.1 (Gehron Robey, personal communication). For BOP cells to represent each specific stage different cell lines would be expected to produce only those proteins occurring at each stage. In fact, with the possible exception of BOP 7 which produces very small amounts of osteocalcin, all BOP cells produce all the noncollagenous proteins assayed and all express mRNA for type I collagen. The developmental sequence of the osteoblast may be a gradient of differentiation necessary for the ordered production of a mineralised extracellular matrix rather than a series of specific steps. The ability of BOP cells to mimic *in vitro* the ordered developmental sequence of bone matrix

Fig. 7.1   Production of matrix proteins in osteoblast differentiation

Early

Intermediate

Late

Biglycan

Decorin

Alkaline phosphatase

Osteonectin

Collagen

Osteopontin

Bone sialoprotein

Osteocalcin
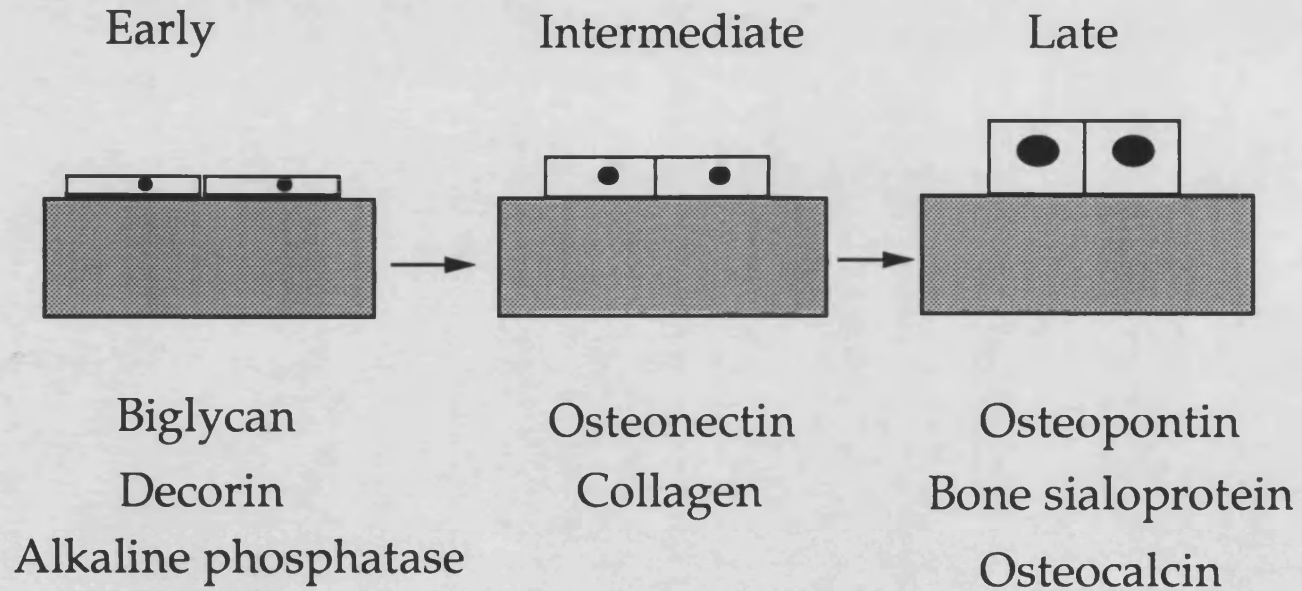
formation has not been studied. The onset and progression of osteoblast differentiation are closely related to cell growth (Stein, Lian and Owen, 1990) and as the BOP cell lines have growth characteristics resembling a transformed rather than a normal phenotype they may not reproduce the normal sequence of events.

With respect to the expression of markers associated with the osteoblastic phenotype BOP cells resembled primary cultures of human osteoblast-like cells more closely than do osteosarcoma cell lines currently in use. For example, cells of the human osteosarcoma line MG-63 respond to 1,25D by producing osteocalcin but express very low levels of alkaline phosphatase. The human osteosarcoma cell line HOS TE85 have higher levels of alkaline phosphatase but fail to produce osteocalcin in response to 1,25D (Clover, personal communication). Another osteoblastic cell line, FM30, produces osteocalcin constitutively, in the absence of 1,25D suggesting that in this cell line the osteocalcin gene is upregulated as a result of the transformation process. BOP cells display the responses of normal osteoblasts to the calciotropic hormone 1,25D, are more readily available in large numbers than primary cultures, and may prove useful in establishing the effects of other hormones, such as glucocorticoids, on matrix production. The responses of BOP cells to the cytokines IL-1 and TNF followed a similar pattern to that observed with primary cultures. However, the cells produced abnormally high levels of TNF which may be related to their rapid growth rate.

7.3   Future studies

Other workers in this laboratory have raised a series of monoclonal antibodies against 1,25D treated primary cultures of human osteoblasts. One antibody, OBL, reacts with osteoblasts lining the bone surface and also recognises plump osteoblasts which are actively forming bone in cryostat

sections of human osteophyte tissue. A second antibody, OBM, is reactive with osteocytes. Antibody OBL recognised epitopes on the surface of HOS TE85 cells and reacted positively with BOP cell lines 7, 12, 26, 31 and 37. OBM reacted with only select cells of cell lines BOP 12, 26 and 31, and select cells of MG-63 and HOS TE85. These data demonstrate that BOP cells have similar antigenic components to osteoblasts *in situ*. As a source of a large number of cells expressing the osteoblast phenotype more closely than osteosarcoma cell lines BOP cells may be used to purify potential antigens recognised by the monoclonal antibodies.

Human osteoblasts are amenable to transfection by the calcium phosphate/DNA coprecipitation technique. It may be possible to retransfect cells with cDNA coding for certain matrix proteins and assess the effects of overproduction of the proteins on other aspects of cell function, such as cell adhesion and matrix mineralisation. Conversely, production of a particular matrix protein may be blocked by allowing the cells to internalise a specific antisense oligonucleotide, the effect of deprivation of certain matrix proteins could then be studied.

Turksen and Aubin (1991) have described the immunoselection of subpopulations of osteoprogenitor cells by fluorescence activated cell sorting (FACS) using a monoclonal antibody directed against alkaline phosphatase. Using monoclonal antibodies directed against osteoblast cell surface antigens it would be possible to isolate populations of cells responsive to specific hormones and growth factors. The ability of BOP cells to express receptors for oestradiol or PTH has not been tested. Cells within the population expressing the oestrogen receptor could potentially be isolated for the study of oestrogen effects. Isolation of subpopulations of cells expressing certain integrins may be useful for studying cell adhesion and may assist in the identification of the ligands bound by specific integrins.

Primary cultures of human osteoblast-like cells were transfected with SV40 DNA and a series of cell lines obtained. The cells expressed many of the hallmarks of the parent cultures and were able to differentiate in response to 1,25D. The cells had a limited lifespan *in vitro* but were stable for 15 passages. The cells displayed the responses of normal osteoblasts to the cytokines IL-1 and TNF but produced aberrant levels of inactive TNF. The cell lines resembled parent cultures more closely than the human osteosarcoma cell lines MG-63 and HOS TE85 and may prove to be a useful model for studying osteoblast function.

REFERENCES

1. Aarden LA, DeGroot ER, Schaep OL, Lansdorp PM: Production of hybridoma growth factor by human monocytes. Eur J Immunol 17:1411, 1987

2. Abe E, Tanaka H, Ishimi Y, Miyaura C, Hayashi T, Nagasawa H, Tomida M, Yamaguchi Y, Hozumi M, Suda T: Differentiation-inducing factor purified from conditioned medium of mitogen-treated spleen cell cultures stimulated bone resorption. Proc Nat Acad Sci USA 83:5958, 1986

3. Aizawa S, Yaguchi M, Nakano M, Inokuchi S, Handa H, Toyama K: Establishment of a variety of human bone marrow stromal cell lines by the recombinant SV40-adenovirus vector. J Cell Physiol 148:245, 1991

4. Akatsu T, Takahashi N, Udagawa N, Imamura K, Yamaguchi A, Sato K, Nagata N, Suda T: Role of prostaglandins in interleukin 1 induced bone resorption in mice in vitro. J Bone Miner Res 6:183, 1991

5. Al-Humidan A, Ralston SH, Hughes DE, Chapman K, Aarden L, Russell RGG: Interleukin-6 does not stimulate bone resorption in neonatal mouse calvariae. J Bone Miner Res 6:3, 1991

6. Albelda SM, Buck CA: Integrins and other cell adhesion molecules. FASEB J 4:2868, 1990

7. Allan EH, Hilton DJ, Brown MA, Evely RS, Yumita S, Metcalf D, Gough NM, Ng KW, Nicola NA, Martin TJ: Osteblasts display receptors for and responses to leukemia-inhibitory factor. J Cell Physiol 145:110, 1990

8. Andreason GA, Evans GA: Optimization of electroporation for transfection of mammalian cell lines. Analyt Biochem 180:269, 1989

9. Aufmkolk B, Hauschka PV, Schwartz ER: Characterisation of human bone cells in culture. Calcif Tissue Int 37:228, 1985

10. Baron R: Anatomy and ultrastructure of bone, in Favus MJ: (ed): Primer on the metabolic bone diseases and disorders of mineral metabolism, Kelseyville, American Society for Bone and Mineral Research, 1990, p 3

11. Baron R, Neff L, Louvard D, Courtoy PJ: Cell-mediated extracellular acidification and bone resorption: evidence for a low pH in resorbing lacunae and localization of a 100-kD lysosomal membrane protein at the osteoclast ruffled border. J Cell Biol 101:2210, 1985

12. Baron R, Vignery A, Horowitz M: Lymphocytes, macrophages and the regulation of bone remodelling, in Peck WA: (ed): Bone and Mineral Research II, Elsevier, 1983, p 175

13. Beck SL, Ammann AJ, Aufdemorte TB, Deguzman L, Xu Y, Lee WP, McFatridge LA, Chen TL: In vivo induction of bone by recombinant human transforming growth factor β_1. J Bone Miner Res 6:961, 1991a

14. Beck SL, Deguzman L, Lee WP, Xu Y, McFatridge LA, Gillett NA, Amento EP: TGF-β_1 induces bone closure of skull defects. J Bone Miner Res 6:1257, 1991b

15. Bellows CG, Sodek J, Yao KL, Aubin JE: Phenotypic differences in subclones and long-term cultures of clonally derived rat bone cell lines. J Cell Biochem 31:153, 1986

16. Beresford JN: Osteogenic stem cells and the stromal system of bone and marrow. Clin Orthop Rel Res 240:270, 1989

17. Beresford JN, Fedarko NS, Fisher LW, Midura RJ, Yanagishita M, Termine JD, Gehron Robey P: Analysis of the proteoglycans synthesised by human bone cells in vitro. J Biol Chem 262:17164, 1987

18. Beresford JN, Gallagher JA, Poser JW, Russell RGG: Synthesis of osteocalcin by human bone cells in vitro: Effects of $1,25(OH)_2D_3$ and PTH and glucocorticoids. Metab Bone Dis Rel Res 5:229, 1984

19. Beresford JN, Gallagher JA, Russell RGG: 1,25-dihydroxyvitamin D3 and human bone-derived cells in vitro: effects on alkaline phosphatase, type I collagen synthesis and proliferation. Endocrinology 119:1776, 1986

20. Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR: Stimulation of bone resorption and inhibition of bone formation in vitro by human tumour necrosis factors. Nature 322:547, 1986

21. Beutler B, Cerami A: Cachectin: More than a tumor necrosis factor. New Eng J Med 316:379, 1987

22. Bhalla AK, Amento EP, Krane SM: Differential effects of 1,25-dihydroxyvitamin D3 on human lymphocytes and monocyte/macrophages. Inhibition of Interleukin 2 and augmentation of Interleukin 1 production. Cell Immunol 98:311, 1986

23. Bianco P, Fisher LW, Young MF, Kopp JB, Termine JD, Gehron Robey P: Expression of bone sialoprotein in human developing bone as revealed by immunostaining and in situ hybridisation. J Bone Miner Res 4(S1):513, 1989(abstr)

24. Bird TA, Saklatvala J: Identification of a common class of high affinity receptors for both types of porcine interleukin 1 on connective tissue cells. Nature 324:263, 1986

25. Blair HC, Teitelbaum SL, Ghiselli R, Gluck S: Osteoclastic bone resorption by a polarised vacuolar proton pump. Science 245:855, 1989

26. Bonewald LF, Mundy GR: Role of transforming growth factor beta in bone remodeling: a review. Connect Tissue Res 23:201, 1989

27. Boyde A, Maconnachie E, Reid SA, Delling G, Mundy GR: Scanning electron microscopy in bone pathology: Review of methods, potential and application. Scanning Electron Microscopy IV:1537, 1986

28. Bronckers ALJJ, Gay S, DiMuzio MT: Immunolocalisation of gamma-carboxyglutamic acid containing proteins in developing rat bones. Coll Relat Res 5:273, 1985

29. Bronckers ALJJ, Gay S, Finkelman R,D.: Developmental appearance of Gla proteins (osteocalcin) and alkaline phosphatase in tooth germs and bone of the rat. Bone Miner 2:361, 1987

30. Butler WT: The nature and significance of osteopontin. Connect Tissue Res 23:123, 1989

31. Canalis E: Interleukin 1 has independent effects on DNA and collagen synthesis in cultures of rat calvaria. Endocrinology 118:74, 1986

32. Canalis E: Effects of tumor necrosis factor on bone formation in vitro. Endocrinology 121:1596, 1987

33. Chambers TJ: The regulation of osteoclastic development and function, in Evered D, Harnett S: (eds): Cell and Molecular Biology of Vertebrate Hard Tissues, Ciba Foundation Symposium 136, Chichester, John Wiley and Sons Ltd., 1988, p 92

34. Chen TL, Cone TM, Holton M, Feldman D: $1\alpha,25$-dihydroxyvitamin D_3 receptors in cultured rat osteoblast-like cells. J Biol Chem 258:4350, 1983

35. Chenu C, Delmas PD: Platelets contribute to circulating levels of bone sialoprotein in human. J Bone Miner Res 7:47, 1992

36. Cherington V, Brown M, Paucha E, St.Louis J, Spiegelman BM, Roberts TM: Separation of simian virus 40 large-T-antigen-transforming and origin-binding functions from the ability to block differentiation. Molec Cell Biol 8:1380, 1988

37. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Analyt Biochem 162:156, 1987

38. Clezardin P, Malaval L, Morel M-C, Guichard J, Lecompte T, Trzeciak M-C, Dechavanne M, Breton-Gorius J, Delmas PD, Kaplan C: Osteonectin is an α-

granule component involved with thrombospondin in platelet aggregation. J Bone Miner Res 6:1059, 1991

39. Collins DA, Chambers TJ: Effect of prostaglandins E_1, E_2, and $F_{2\alpha}$ on osteoclast formation in mouse bone marrow cultures. J Bone Miner Res 6:157, 1991

40. Delmas PD, Schlemmer A, Gineyts E, Riis B, Christansen C: Urinary excretion of pyridinoloine crosslinks correlates with bone turnover measured on iliac crest biopsy in patients with vertebral osteporosis. J Bone Miner Res 6:639, 1991

41. Dieudonne SC, Foo P, Van Zoelen EJJ, Burger EH: Inhibiting and stimulating effects of TGF-β_1 on osteoclastic bone resorption in fetal mouse bone organ cultures. J Bone Miner Res 6:479, 1992

42. Egrise D, Martin D, Neve P, Verhas M, Schoutens A: Effects and interactions of 17β-estradiol, T_3 and 1,25(OH)$_2$D$_3$ on cultured osteoblasts from mature rats. Bone Miner 11:273, 1990

43. Endo H, Kiyoki M, Kawashima K, Naruchi T, Hashimoto Y: Vitamin D_3 metabolites and PTH synergistically stimulate formation of chick embryonic femur *in vitro*. Nature 286:262, 1980

44. Eriksen EF, Colvard DS, Berg NJ, Graham ML, Mann KG, Spelsberg TC, Riggs BL: Evidence of estrogen receptors in normal human osteoblast-like cells. Science 241:84, 1988

45. Ernst M, Heath JK, Rodan GA: Estradiol effects on proliferation, messenger ribonucleic acid for collagen and insulin-like growth factor-I, and parathyroid hormone-stimulated adenylate cyclase activity in osteoblastic cells from calvariae and long bones. Endocrinology 125:825, 1989

46. Ernst M, Schmid Ch, Froesch ER: Enhanced osteoblast proliferation and collagen gene expression by estradiol. Proc Nat Acad Sci USA 85:2307, 1988

47. Evans DB, Bunning RAD, Russell RGG: The effects of recombinant human interleukin 1β on cellular proliferation and the production of prostaglandin E2, plasminogen activator, osteocalcin and alkaline phosphatase by osteoblast-like cells derived from human bone. Biochem Biophys Res Commun 166:208, 1990

48. Evans DB, Smith AG, Williams MM, Rathjen PD, Heath JK, Gowen M: Regulation of leukemia inhibitory factor (LIF) production in human bone cells *in vitro*, in Christiansen C, Overgaard K: (eds): Osteoporosis 1990, Copenhagen, Osteopress ApS, 1990, p 324

49. Eyre DR, Oguchi H: The hydroxypyridinium crosslinks of skeletal collagens: Their measurement, properties and a proposed pathway of formation. Biochem Biophys Res Commun 92:403, 1980

50. Fang MA, Hahn TJ: Effects of interleukin-6 on cellular function in UMR-106-01 osteoblastlike cells. J Bone Miner Res 6:133, 1991

51. Fedarko NS, Bianco P, Vetter U, Gehron Robey P: Human bone cell enzyme expression and cellular heterogeneity: Correlation of alkaline phosphatase enzyme activity with cell cycle. J Cell Physiol 144:115, 1990

52. Fisher LW, Hawkins GR, Tuross N, Termine JD: Purification and partial characterisation of small proteoglycans I and II, bone sialoproteins I and II, and osteonectin from the mineral compartment of developing human bone. J Biol Chem 262:9702, 1987

53. Fisher LW, McBride OW, Termine JD, Young MF: Human bone sialoprotein: Deduced protein sequence and chromosomal localization. J Biol Chem 265:2347, 1990

54. Franceschi RT, Romano PR, Park K-Y: Regulation of type I collagen synthesis by 1,25-Dihydroxyvitamin D3 in human osteosarcoma cells. J Biol Chem 263:18938, 1988

55. Franceschi RT, Young J: Regulation of alkaline phosphatase by 1,25-dihydroxyvitamin D3 and ascorbic acid in bone-derived cells. J Bone Miner Res 5:1157, 1990

56. Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV: Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 16:381, 1966

57. Fromm M, Berg P: Deletion mapping of RNA regions required for SV-40 early region promoter function in vivo. J Mol Appl Genet 1:457, 1982

58. Garrett IR, Black KS, Mundy GR: Interactions between interleukin-6 and interleukin-1 in osteoclastic bone resorption in neonatal mouse calvariae. Calcif Tissue Int 46 S2:A 37, 1990(abstr)

59. Garrett IR, Boyce BF, Oreffo ROC, Bonewald L, Poser J, Mundy GR: Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. J Clin Invest 85:632, 1990

60. Gauldie J, Richards C, Harnish D, Lansdorp P, Baumann H: Interferon B2/ B-cell stimulatory factor type 2 shares identity with monocyte derived hepatocyte stimulatory factor and regulates the major acute phase protein response in liver cells. Proc Nat Acad Sci USA 84:7251, 1987

61. Gehron Robey P: The biochemistry of bone. Endocrinol Metab North America 18:859, 1989

62. Gehron Robey P, Findley DM, Young DF, Beresford JN, Stubbs J, Fisher LW, Termine JD: Regulation of osteonectin production by 1,25-dihydroxyvitamin D3 in human bone cells. J Bone Miner Res 1:288A, 1986(abstr)

63. Gehron Robey P, Termine JD: Human bone cells in vitro. Calcif Tissue Int 37:453, 1985

64. Gehron Robey P, Young MF, Fisher LF, McClain TD: Thrombospondin is an osteoblast-derived component of mineralised extracellular matrix. J Cell Biol 108:719, 1989

65. Gehron Robey P, Young MF, Flanders KC, Roche NS, Kondaiah P, Hari Reddi A, Termine JD, Sporn MB, Roberts AB: Osteoblasts synthesise and respond to transforming growth factor-type beta in vitro. J Cell Biol 105:457, 1987

66. Giacherio D, Hager LP: A poly(dT)-stimulated ATPase activity associated with simian virus 40 large T antigen. J Biol Chem 254:8113, 1979

67. Globus RK, Patterson-Buckendahl P, Gospodarowicz D: Regulation of bovine bone cell proliferation by fibroblast growth factor and transforming growth factor beta. Endocrinology 123:98, 1988

68. Gottesburen D, Rau B, Schulz A, Fischer LW, Gehron Robey P, Termine JD: Immunoelectron microscopy of osteoclastic bone resorption. Calcif Tissue Int 48(S):43A, 1991(abstr)

69. Gowen M: Actions of IL-1 and TNF on human osteoblast-like cells: Similarities and synergism, in Oppenheim J, Dinarello C, Kluger M, Powanda M: (eds): Monocytes and other non-lymphocytic cytokines, Allen R. Liss Inc., 1988, p 261

70. Gowen M: Interleukin 1 and tumor necrosis factor, in Gowen M: (ed): Cytokines and bone metabolism, London, CRC Press, 1992, p 71

71. Gowen M, Chapman K, Littlewood AJ, Hughes D, Evans DB, Russell RGG: Production of tumor necrosis factor by human osteoblasts is modulated by other cytokines, but not by osteotropic hormones. Endocrinology 126:1250, 1990

72. Gowen M, MacDonald BR, Russell RGG: Actions of recombinant human γ-interferon and tumor necrosis factor a on the proliferation and osteoblastic characteristics of human trabecular bone cells in vitro. Arthritis Rheum 31:1500, 1988

73. Gowen M, Mundy GR: Actions of recombinant interleukin 1, interleukin 2 and interferon γ on bone resorption *in vitro*. J Immunol 136:2478, 1986

74. Gowen M, Wood DD, Ihrie EJ, McGuire MK, Russell RGG: An interleukin 1 like factor stimulates bone resorption in vitro. Nature 306:378, 1983

75. Gowen M, Wood DD, Russell RGG: Stimulation of the proliferation of human bone cells in vitro by human monocyte products with interleukin-1 activity. J Clin Invest 75:1223, 1985

76. Graessmann M, Graessmann A: "Early" simian-virus-40-specific RNA contains information for tumor antigen formation and chromatin replication. Proc Nat Acad Sci USA 73:366, 1976

77. Graham FL, van der Eb AJ: A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456, 1973

78. Gray TK, Mohan S, Linkhart TA, Baylink DJ: Estradiol stimulates *in vitro* the secretion of insulin-like growth factors by the clonal osteoblastic cell line, UMR106. Biochem Biophys Res Commun 158:407, 1989

79. Gundberg-Carpenter C, Lian JB: Circulating osteocalcin: A bone-specific marker of osteoblast activity, in Lindh E, Thorell JI: (eds): Clinical impact of bone and connective tissue markers, London, Academic Press, 1989, p 315

80. Hilton DJ, Metcalf D, Nicola NA: Purification of a murine leukemia inhibitory factor from Krebs ascites cells. Anal Biochem 173:359, 1988

81. Haley KP, Overhauser J, Babiss LE, Ginsberg H,S., Jones NC: Transformation properties of type 5 adenovirus mutants that differentially express the E1A gene products. Proc Nat Acad Sci USA 81:5734, 1984

82. Hamilton JA, Lingelbach SR, Partridge NC, Martin TJ: Stimulation of plasminogen activator in osteoblast-like cells by bone resorbing hormones. Biochem Biophys Res Commun 122:230, 1984

83. Hauschka PV, Lian JB, Gallop PM: Direct identification of the calcium-binding amino acid, γ-carboxyglutamate, in mineralized tissue. Proc Nat Acad Sci USA 72:3925, 1975

172

84. Heath JK, Rodan SB, Yoon K, Rodan GA: Rat calvarial cell lines immortalised with SV-40 large T antigen: Constitutive and retinoic acid inducible expression of osteoblastic features. J Bone Miner Res 124:3060, 1989

85. Helfrich MH, Nesbitt SA, Dorey EL, Horton MA: Rat osteoclasts adhere to a wide range of RGD (Arg-Gly Asp) peptide-containing proteins, including the bone sialoproteins and fibronectin, via a β_3 integrin. J Bone Miner Res 7:335, 1992

86. Helle M, Brakenhoff JPJ, DeGroot ER, Aarden LA: Interleukin 6 is involved in interleukin 1 induced activities. Eur J Immunol 18:957, 1988

87. Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, Kachiwamura S, Nikajima K, Koyama K, Iwamatsu A, Tsunaswa S, Sakiyama F, Matsui H, Takahara Y, Taniguchi T, Kishimoto T: Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature 324:73, 1986

88. Hoffmann O, Klaushofer K, Gleispach H, Leis HJ, Luger T, Koller K, Peterlik M: Gamma interferon inhibits basal and interleukin 1-induced prostaglandin production and bone resorption in neonatal mouse calvaria. Biochem Biophys Res Commun 143:38, 1987

89. Holtrop ME, Raisz LG: Comparison of the effects of 1,25-dihydroxycholecalciferol, prostaglandin E_2 and osteoclast-activating factor with parathyroid hormone on the ultrastructure of osteoclasts in cultured long bones of foetal rats. Calcif Tissue Int 29:201, 1979

90. Horton JE, Raisz LG, Simmons HA, Oppenheim JJ, Mergenhagen SE: Bone resorbing activity in supernatant fluid from cultured human peripheral blood leukocytes. Science 177:793, 1972

91. Horton MA, Rimmer EF, Moore A, Chambers TJ: On the origin of the osteoclasts: The cell surface phenotype of rodent osteoclasts. Calcif Tissue Int 37:46, 1985

92. Houssiau FA, Devogelaer J-P, van Damme J, Nagant de Deuxchaisnes C, van Snick J: Interleukin 6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides. Arthritis Rheum 31:784, 1988

93. Humphries MJ: The molecular basis and specificty of integrin-ligand interactions. J Cell Sci 97:585, 1990

94. Huschtscha LI, Holliday R: Limited and unlimited growth of SV40 transformed cells from human diploid MRC-5 fibroblasts. J Cell Sci 63:77, 1983

95. Ishimi Y, Miyaura C, Jin CH, Akatsu T, Abe E, Nakamura Y, Yamaguchi A, Yoshiri S, Matsuda T, Hirano T, Kishimoto T, Suda T: IL-6 is produced by osteoblasts and induces bone resorption. J Immunol 145:3297, 1990

96. Izumi T, Scully SP, Heydemann A, Bolander ME: Transforming growth factor β_1 stimulates type II collagen expression in cultured periosteum-derived cells. J Bone Miner Res 7:115, 1992

97. Keeting PE, Rifas L, Harris SA, Colvard DS, Spelsberg TC, Peck WA, Riggs BL: Evidence for interleukin-1β production by cultured normal human osteoblast-like cells. J Bone Miner Res 6:827, 1991

98. Keeting PE, Scott RE, Colvard DS, Anderson MA, Oursler MJ, Spelsberg TC, Riggs BL: Development and characterisation of a rapidly proliferating, well-differentiated cell line derived from normal adult human osteoblast-like cells transfected with SV40 large T antigen. J Bone Miner Res 7:127, 1992

99. Keeting PE, Scott RE, Colvard DS, Han IK, Spelsberg TC, Riggs BL: Lack of a direct effect of estrogen on proliferation and differentiation of normal human osteoblast-like cells. J Bone Miner Res 6:297, 1991

100. Kehrl JH, Roberts AB, Wakefield LM, Jakowlew S, Sporn MB, Fauci AS: Transforming growth factor beta is an important immunomodulatory protein for human B lymphocytes. J Immunol 3855, 1986

101. Kellerman O, Buc-Caron MH, Marie PJ, Lamblin D, Jacob F: An immortalized osteogenic cell line derived from mouse teratocarcinoma is able to mineralize in vivo and in vitro. J Cell Biol 110:123, 1990

102. Klein DC, Raisz LG: Prostaglandins; Stimulation of bone resorption in tissue culture. Endocrinology 86:1436, 1970

103. Klein-Nulend J, Pilbeam KC, Raisz LG: Effect of 1,25-dihydroxyvitamin D_3 on prostaglandin E_2 production in cultured mouse parietal bones. J Bone Miner Res 6:1339, 1991

104. Komm BS, Terpening CS, Benz DJ, Graeme KA, Gallegos A, Korc M, Greene GL, O'Malley BW, Haussler MR: Estrogen binding, receptor mRNA and biologic response in osteoblast-like osteosarcoma cells. Science 241:81, 1988

105. Kream BE, Jose M, Yamada S, DeLuca HF: A specific high affinity binding molecule for 1,25-dihydroxyvitamin D_3 in fetal bone. Science 197:1086, 1977

106. Kream BE, Rowe D, Smith MD, Maher V, Majeska R: Hormonal regulation of collagen synthesis in a clonal rat osteosarcoma cell line. Endocrinology 119:1922, 1986

107. Lajeunesse D, Frondoza C, Schoffield B, Sacktor B: Osteocalcin secretion by the human osteosarcoma cell line MG-63. J Bone Miner Res 5:915, 1990

108. Land H, Parada LF, Weinberg RA: Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304:596, 1983

109. Lian JB, Friedman PA: The vitamin K dependent synthesis of γ-carboxyglutamic acid by bone microsomes. J Biol Chem 253:6623, 1978

110. Linkhart TA, Linkhart SG, MacCharles DC, Long DL, Strong DD: Interleukin-6 messenger RNA expression and Interleukin-6 protein secretion in cells isolated from normal human bone. J Bone Miner Res 6:1285, 1991

111. Littlewood AJ, Aarden LA, Evans DB, Russell RGG, Gowen M: Human osteoblastlike cells do not respond to Interleukin-6. J Bone Miner Res 6:133, 1991a

112. Littlewood AJ, Russell J, Harvey GR, Hughes DE, Russell RGG, Gowen M: The modulation of the expression of IL-6 and its receptor in human osteoblasts *in vitro*.. Endocrinology 129:1513, 1991b

113. Lorenzo JA, Raisz LG, Hock JM: DNA synthesis is not necessary for osteoclastic responses to parathyroid hormone in cultured fetal rat long bones. J Clin Invest 72:1924, 1983

114. Lotz M, Jirik F, Kabouridis P, Tsoukas C, Hirano T, Kishimoto T, Carson DA: B cell stimulating factor 2/interleukin 6 is a costimulant for human thymocytes and T lymphocytes. J Exp Med 167:1253, 1988

115. Lowe C, Cornish J, Callon K, Martin TJ, Reid IR: Regulation of osteoblast proliferation by leukemia inhibitory factor. J Bone Miner Res 6:1277, 1991

116. Lowik C, van der Pluijm G, Hoekman K, Aarden L, Bijvoet O, Papapoulos S: Parathyroid hormone (PTH) and PTH-like protein (PLP) stimulate interleukin 6 production by osteogenic cells: A possible role for interleukin 6 in osteoclastogenesis. Biochem Biophys Res Commun 162:1546, 1989

117. Lowik CWGM: Differentiation inducing factors: Leukemia Inhibitory Factor and Interleukin 6, in Gowen M: (ed): Cytokines and bone metabolism, London, CRC Press, 1992,

118. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the folin phenol reagent. J Biol Chem 193:265, 1951

119. Lucas PA, Price PA, Caplan AI: Chemotactic response of mesenchymal cells, fibroblasts and osteoblast-like cells to bone Gla protein. Bone 9:319, 1988

120. Metcalf D, Gearing DP: Fatal syndrome in mice engrafted with cells producing high levels of the leukemia inhibitory factor. Proc Nat Acad Sci USA 86:5948, 1989

121. MacDonald BR, Gallagher JA, Ahnfelt-Ronne I, Beresford JN, Gowen M, Russell RGG: Effects of bovine parathyroid hormone and 1,25-dihydroxyvitamin D_3 on the production of prostaglandins by cells derived from human bone. FEBS letters 169:49, 1984

122. MacDonald BR, Gallagher JA, Russell RGG: Parathyroid hormone stimulates the proliferation of cells derived from human bone. Endocrinology 118:2445, 1986

123. Majeska RJ, Rodan SB, Rodan GA: Parathyroid hormone responsive clonal cell lines from rat osteosarcoma. Endocrinology 107:1494, 1980

124. Malaval L, Darbouret B, Preaudat C, Jolu J-P, Delmas PD: Intertissular variations in osteonectin: A monoclonal antibody directed to bone osteonectin shows reduced affinity for platelet osteonectin. J Bone Miner Res 6:315, 1991

125. Mangan DF, Welch GR, Wahl SM: Lipopolysaccharide, tumor necrosis factor-α, and IL-1β prevent programmed cell death (apoptosis) in human peripheral blood monocytes. J Immunol 146:1541, 1991

126. Manolagas SC, Burton DW, Deftos LJ: 1,25-Dihydroxyvitamin D_3 stimulates the alkaline phosphatase activity of osteoblast-like cells. J Biol Chem 256:7115, 1981

127. Marusic A, Kalinowski JF, Lorenzo JA: Regulated production of leukemia inhibitory factor mRNA and bioactivity by bone cells. J Bone Miner Res 5(Suppl. 1):S152, 1990(abstr)

128. Matsuyama T, Lau KHW, Wergedal JE: Monolayer cultures of normal human bone cells contain multiple subpopulations of alkaline phosphatase positive cells. Calcif Tissue Int 47:276, 1990

129. Mawer EB, Hayes ME, Still PE, Davies M, Lumb.G.A. , Palit J, Lennox Holt PJ: Evidence for nonrenal synthesis of 1,25-Dihydroxyvitamin D in patients with inflammatory arthritis. J Bone Miner Res 6:733, 1991

130. Mayne LV, Priestley A, James MR, Burke JF: Efficient immortalisation and morphological transformation of human fibroblasts by transfection with SV-40 DNA linked to a dominant marker. Exp Cell Res 162:530, 1986

131. McSheehy PMJ, Chambers TJ: Osteoblastic cells mediate osteoclastic responsiveness to parathyroid hormone. Endocrinology 118:824, 1986

132. McSheehy PMJ, Chambers TJ: 1,25-Dihydroxyvitamin D3 stimulates rat osteoblastic cells to release a soluble factor that increases osteoclastic bone resorption. J Clin Invest 80:425, 1987

133. Merry KH, Russell J, Dodds RA, Gowen M: The transcriptional control of TGFβ is distinct from IL-1 in human osteoblasts. Cytokine 1991(in press)

134. Miller SA, Dykes DD, Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215, 1988

135. Moreau JF, Donaldson DD, Bennett F, Witek-Giannotti J, Clark SC, Wong GG: Leukemia inhibitory factor is identical to the myeloid growth factor Human Interleukin for DA cells. Nature 336:690, 1988

136. Mulkins MA, Manolagas SC, Deftos LJ, Sussman AH: 1,25-Dihydroxyvitamin D3 increases bone alkaline phosphatase isoenzymes in human osteogenic sarcoma cells. J Biol Chem 258:6219, 1982

137. Mundy GR: Bone resorbing cells, in Favus MJ: (ed): Primer on the metabolic bone diseases and disorders of mineral metabolism, Kelseyville, American Society for Bone and Mineral Research, 1990, p 18

138. Mundy GR, Altman AJ, Gondek M, Bandelin JG: Direct resorption of bone by human monocytes. Science 196:1109, 1977

139. Mundy GR, DeMartino S, Rowe DW: Collagen and collagen-derived fragments are chemotactic for tumor cells. J Clin Invest 68:1102, 1981

140. Myers RM, Rio DC, Robbins AK, Tjian R: SV40 gene expression is modulated by the cooperative binding of T antigen to DNA. Cell 25:373, 1981

141. Nemunaitis J, Andrews DF, Crittenden C, Kaushansky JK, Singer JW: Response of simian virus 40 (SV40)-transformed, cultured human marrow stromal cells to hematopoietic growth factors. J Clin Invest 83:593, 1989

142. Nijweide PJ, Van der Plas A, Scherft JP: Biochemical and histological studies on various bone cell preparations. Calcif Tissue Int 33:529, 1981

143. Noda M, Rodan GA: Transcriptional regulation of osteopontin production in rat osteoblast-like cells by parathyroid hormone. J Cell Biol 108:713, 1989

144. Noda M, Vogel RL, Hasson DM, Rodan GA: Leukemia inhibitory factor suppresses proliferation, alkaline phosphatase activity, and type I collagen messenger ribonucleic acid level and enhances osteopontin mRNA in murine osteoblast-like (MC3T3E1) cells. Endocrinology 127:185, 1990

145. Noda M, Yoon K, Prince CW, Butler WT, Rodan GA: Transcriptional regulation of osteopontin production in rat osteosarcoma cells by type β transforming growth factor. J Biol Chem 263:13916, 1988

146. Oldberg A, Franzen A, Heinegard D: Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. Proc Nat Acad Sci USA 83:8819, 1986

147. Oreffo ROC, Mundy GR, Seyedin SM, Bonewald LF: Activation of the bone-derived latent transforming growth factor beta complex by isolated osteoclasts. Biochem Biophys Res Commun 158:817, 1989

148. Oshima RG, Pellett OL, Robb JA, Schneider JA: Transformation of human cystinotic fibroblasts by SV40: Characteristics of transformed cells with limited and unlimited growth potential. J Cell Physiol 93:129, 1977

149. Oursler MJ, Cortese C, Keeting P, Anderson M, Bonde S, Riggs BL, Spelsberg TC: Modulation of TGFβ protein and mRNA levels in normal human osteoblast-like cells by 17β-estradiol and PTH. Endocrinology 129:3313, 1991

150. Owen M: Marrow stromal stem cells. J Cell Sci S10:63, 1988

151. Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS: Progressive development of the rat osteoblast phenotype in vitro: Reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 143:420, 1990

152. Pacifici R, Carano A, Santoro SA, Rifas L, Jeffrey JJ, Malone JD, McCracken R, Avioli LV: Bone matrix constituents stimulate interleukin 1 release from human blood mononuclear cells. J Clin Invest 87:221, 1991

153. Pacifici R, Rifas L, McCracken R, Vered I, McMurtry C, Avioli LV, Peck WA: Ovarian steroid treatment blocks a postmenopausal increase in blood monocyte interleukin 1 release. Proc Nat Acad Sci USA 86:2398, 1989

154. Pacifici R, Rifas L, Teitelbaum S, Slatopolsky E, McCracken R, Bergfeld M, Lee W, Avioli LV, Peck WA: Spontaneous release of interleukin 1 from human blood monocytes reflects bone formation in idiopathic osteoporosis. Proc Nat Acad Sci USA 84:4616, 1987

155. Parfitt AM: The cellular basis of bone remodelling: The quantum concept reexamined in light of recent advances in the cell biology of bone. Calcif Tissue Int 36:S37, 1984

156. Parker BA, Stark GR: Regulation of simian virus 40 transcription: Sensitive analysis of the RNA species present early in infections by virus or viral DNA. J Virol 31:360, 1979

157. Partridge NC, Alcorn B, Michelangeli VP, Kemp BE, Ryan CB, Martin TJ: Functional properties of hormonally responsive cultured normal and malignant rat osteoblastic cells. Endocrinology 108:213, 1981

158. Peacock M, Jones S, Clemens TI, Amento EP, Kurnick J, Krane SM, Holick MF: High affinity 1,25(OH)$_2$ vitamin D$_3$ receptors in a human monocyte-like cell line (U937) and in a cloned human T lymphocyte, in Norman AS, Schaefer K, Herrath DV, Grigoleit HG: (eds): Vitamin D: Chemical, biochemical and clinical endocrinology of calcium metabolism , Berlin, Walter de Gruyter, 1982, p 83

159. Pfeilschifter J, Chenu C, Bird A, Mundy GR, Roodman GD: Interleukin-1 and tumor necrosis factor stimulate the formation of human osteoclast-like cells in vitro. J Bone Miner Res 4:113, 1989

160. Pfeilschifter J, Seyedin SM, Mundy GR: Transforming growth fator beta inhibits bone resorption in fetal rat long bone cultures. J Clin Invest 82:680, 1988

161. Pinet F, Corvol M-T, Dench F, Bourguignon J, Feunteun J, Menard J, Corvol P: Isolation of renin-producing human cells by transfection with three simian virus 40 mutants. Proc Nat Acad Sci USA 82:8503, 1985

162. Pircher R, Lawrence DA, Jullien P: Latent beta-transforming growth factor in nontransformed and Kirsten sarcoma virus-transformed normal rat kidney cells, clone 49F. Cancer Res 44:5538, 1984

163. Price PA, Baukol SA: 1,25-dihydroxyvitamin D$_3$ increases synthesis of the vitamin K dependent bone protein by osteosarcoma cells. J Biol Chem 255:11660, 1980

164. Price PA, Otsuka AS, Poser JW, Kristaponis J, Raman N: Characterization of a g-carboxyglutamic acid-containing protein from bone. Proc Nat Acad Sci USA 73:1447, 1976

165. Price PA, Williamson MK, Haba T, Dell RB, Jee WSS: Excessive mineralization with growth plate closure in rats on chronic warfarin treatment. Proc Nat Acad Sci USA 79:7734, 1982

166. Radna RL, Caton Y, Jha KK, Kaplan P, Li G, Traganos F, Ozer HL: Growth of immortal Simian Virus 40 tsA-transformed human fibroblasts is temperature dependent. Mol Cell Biol 9:3093, 1989

167. Raisz LG: Hormonal Regulation of bone growth and remodelling, in Evered D, Harnett S: (eds): Cell and molecular biology of vertebrate hard tissues, Chichester, John Wiley and Sons, 1988, p 226

168. Raisz LG: Local and systemic factors in the pathogenesis of osteoporosis. New Eng J Med 318:818, 1988

169. Raisz LG, Trummel CL, Holick MF, DeLuca HF: 1,25-dihydroxycholecalciferol: a potent stimulator of bone resorption in organ culture. Science 175:768, 1972

170. Reinholt FP, Hultenby K, Oldberg A, Heinegard D: Osteopontin- a possible anchor of osteoclasts to bone. Proc Nat Acad Sci USA 87:4473, 1990

171. Rickard D, Russell RGG, Gowen M: Oestradiol inhibits the release of tumour necrosis factor but not interleukin 6 from adult human osteoblasts in vitro. Osteoporosis Int 2:94, 1992

172. Rigby WFC, Stacy T, Fanger MW: Inhibition of T cell mitogenesis by 1,25-Dihydroxyvitamin D_3 (calcitriol). J Clin Invest 74:1451, 1984

173. Riggs BL, Jowsey J, Goldsmith RS, Kelly PJ, Hoffman DL, Arnaud CD: Short and long-term effects of estrogen and synthetic anabolic hormone in postmenopausal osteoporosis. J Clin Invest 51:1659, 1972

174. Robins SP, Stewart P, Astbury C, Bird HA: Measurement of the cross linking compound, pyridinoline, in urine as an index of collagen degradation in joint disease. Ann Rheum Dis 45:969, 1986

175. Rodan GA, Heath JK, Yoon K, Noda M, Rodan SB: Diversity of the osteoblastic phenotype, in Evered D, Harnett S: (eds): Cell and molecular biology of vertebrate hard tissues, Chichester, John Wiley and Sons, 1988, p 78

176. Romberg RW, Werness PG, Riggs BL, Mann KG: Inhibition of hydroxyapatite crystal growth by bone-specific and other calcium-binding proteins. Biochemistry 25:1176, 1986

177. Roodman GD: Interleukin-6: An osteotropic factor? J Bone Miner Res 7:475, 1992

178. Roodman GD, Ibbotson KJ, MacDonald BR, Kuehl TJ, Mundy GR: $1,25(OH)_2$ vitamin D_3 causes formation of multinucleated giant cells with osteoclast characteristics in cultures of primate marrow. Proc Nat Acad Sci USA 82:8213, 1985

179. Rosenblum MG, Donato NJ: Tumor necrosis factor alpha: a multifaceted peptide hormone. Crit Rev Immunol 9:21, 1989

180. Rosin A, Freiberg H, Zajicek G: The fate of rat bone marrow, spleen and periosteum cultivated in vivo in the diffusion chamber with specific reference to bone formation. Exp Cell Res 29:176, 1963

181. Sabatini M, Boyce B, Aufdemorte T, Bonewald L, Mundy GR: Infusions of recombinant human interleukins 1α and 1β cause hypercalcemia in normal mice. Proc Nat Acad Sci USA 85:5235, 1988

182. Saklatvala J: Tumour necrosis factor α stimulates resorption and inhibits synthesis of proteoglycan in cartilage. Nature 322:547, 1986

183. Sambrook J, Fritsch EF, Maniatis T: Molecular cloning: A laboratory manual (ed 2nd). New York, Cold Spring Harbor Laboratory Press, 1989, p 16.30

184. Sato K, Kasono K, Fujii Y, Kawakami M, Tsushima T, Shizume K: Tumor necrosis factor type α (cachectin) stimulates mouse osteoblast-like cells (MC3T3-E1) to produce macrophage-colony stimulating activity and prostaglandin E_2. Biochem Biophys Res Commun 29:323, 1987

185. Schall TJ, Lewis M, Koller KJ: Molecular cloning and expression of a receptor for human tumor necrosis factor. Cell 61:361, 1990

186. Scott DM, MacDonald C, Brzeski H, Kinne R: Maintenance of expression of differentiated function of kidney cells following transformation by SV40 early region DNA. Exp Cell Res 166:391, 1986

187. Scott RE, Estervig DN, Tzen C-Y, Minoo P, Maercklein PB, Hoerl BJ: Nonterminal differentiation represses the neoplastic phenotype in spontaneously and simian virus 40-transformed cells. Proc Nat Acad Sci USA 86:1652, 1989

188. Seckinger P, Isaaz S, Dayer J-M: A human inhibitor of tumor necrosis factor α. J Exp Med 167:1511, 1988

189. Seyedin SM, Thomas TC, Thomson AY, Rosen DM, Piez KA: Purification and characterisation of two cartilage inducing factors from demineralised bone. Proc Nat Acad Sci USA 82:2267, 1985

190. Singer JW, Charbord P, Keating A, Nemunaitis A, Raugi G, Wight TN, Lopez JA, Roth GJ, Dow LW, Fialkow PJ: Simian virus 40-transformed adherent cells from human long-term marrow cultures: Cloned cell lines produce cells with stromal and hematopoietic charactertistics. Blood 70:464, 1987

191. Small MB, Gluzman Y, Ozer HL: Enhanced transformation of human fibroblasts by origin-defective simian virus 40. Nature 296:671, 1982

192. Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau JF, Stahl M, Rogers D: Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 336:688, 1988

193. Sodek J, Heersche JN: Uptake of collagenolytic enzymes by bone cells during isolation from embryonic rat calvaria. Calcif Tissue Int 33:255, 1981

194. Somerman MJ, Fisher LW, Foster RA: Human bone sialoprotein I and II enhance fibroblast attachment in vitro. Calcif Tissue Int 43:50, 1988

195. Sporn MB, Roberts AB, Wakefield LM, de Crombrugghe B: Some recent advances in the chemistry and biology of transforming growth factor beta. J Cell Biol 105:1039, 1987

196. Stanka P: Occurrence of cell junctions and microfilaments in osteoblasts. Cell Tissue Res 159:413, 1975

197. Stashenko P, Dewhirst FE, Peros WJ, Kent RL, Ago JM: Synergistic interactions between interleukin 1, tumor necrosis factor, and lymphotoxin in bone resorption. J Immunol 138:1464, 1987

198. Stein GS, Lian JB, Owen TA: Bone cell differentiation: a functionally coupled relationship between expression of cell-growh- and tissue-specific genes. Curr Opinion Cell Biol 2:1018, 1990

199. Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S: In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvariae. J Cell Biol 96:191, 1983

200. Tam CS, Heersche JNM, Murray TM, Parsons JA: Parathyroid hormone stimulates the bone apposition rate independently of its resorptive action: Differential effects of intermittent and continuous administration. Endocrinology 110:506, 1982

201. Tashjian AH, Voelkel EF, Lazzaro M, Singer FR, Roberts AB, Derynck R, Winkler ME, Levine L: α and β human transforming growth factors stimulate prostaglandin production and bone resorption in cultured mouse calvaria. Proc Nat Acad Sci USA 82:4535, 1985

202. Tatakis DN, Schneeberger G, Dziak R: Recombinant interleukin-1 stimulates prostaglandin E_2 production by osteoblastic cells: Synergy with parathyroid hormone. Calcif Tissue Int 42:358, 1988

203. Teitelbaum SL: Skeletal growth and development, in Favus MJ: (ed): Primer on the metabolic bone diseases and disorders of mineral metabolism, Kelseyville, American Society for Bone and Mineral Research, 1990, p 7

204. Tennekoon GI, Yoshino J, Peden KWC, Bigbee J, Rutkowski JL, Kishimoto Y, DeVries GH, McKhann GM: Transfection of neonatal rat Schwann cells with SV40 large T antigen gene under control of the metallothionein promoter. J Cell Biol 105:2315, 1987

205. Termine JD, Kleinman HK, Whitson SW, Conn KM, McGarvey ML, Martin GR: Osteonectin, a bone specific protein binding mineral to collagen. Cell 26:99, 1981

206. Thomson BM, Mundy GR, Chambers TJ: Tumour necrosis factors α and β induce osteoblastic cells to stimulate osteoclastic bone resorption. J Immunol 138:775, 1987

207. Thomson BM, Saklatvala J, Chambers TJ: Osteoblasts mediate interleukin 1 stimulation of bone resorption by rat osteoclasts. J Exp Med 164:104, 1986

208. Tjian R, Fey G, Graessmann A: Biological activity of purified simian virus 40 T antigen proteins. Proc Nat Acad Sci USA 75:1279, 1978

209. Tjian R, Robbins A: Enzymatic activities associated with a purified simian virus 40 T antigen-related protein. Proc Nat Acad Sci USA 76:610, 1979

210. Turksen K, Aubin JE: Positive and negative immunoselection for enrichment of two classes of osteoprogenitor cells. J Cell Biol 114:373, 1991

211. Wakefield LM, Smith DM, Masui T, Harris CC, Sporn MB: Distribution and modulation of the cellular receptor for transforming growth factor beta. J Cell Biol 105:965, 1987

212. Weiner S, Traub W: Organisation of hydroxyapatite crystals within collagen fibrils. FEBS letters 206:262, 1986

213. Whitley GS, Nussey SS, Johnstone AP: SGHTL-34,a thyrotrophin resposive immortalised human thyroid cell line generated by transfection. Mol Cell Endocrinol 52:279, 1987

214. Whyte MP, Teitelbaum SL, Murphy WA, Bergfeld MA, Avioli LV: Adult hypophosphatasia. Clinical, laboratory, and genetic investigation of a large kindred with review of the literature. Medicine 58:329, 1979

215. Williams RL, Hilton DJ, Pease S, Willson TA, Stewart TL, Gearing DP, Wagner EF, Metcalf D, Nicola NA, Gough NM: Myeloid leukemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 336:684, 1988

216. Wong GL, Cohn DV: Target cells in bone for parathormone and calcitonin are different: enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces. Proc Nat Acad Sci USA 72:3167, 1975

217. Yamamoto T, Ecarot B, Glorieux FH: In vivo osteogenic activity of isolated human bone cells. J Bone Miner Res 6:45, 1991

218. Yoon K, Buenaga R, Rodan GA: Tissue specificity and developmental expression of rat osteopontin. Biochem Biophys Res Commun 148:1129, 1987

219. Young MF, Day DD, Dominquez P, McQuillan CI, Fisher LW, Termine JD: Structure and expression of osteonectin mRNA in human tissue. Connect Tissue Res 24:17, 1990

220. Zaidi M, Moonga B, Moss DW, MacIntyre I: Inhibition of osteoclastic acid phosphatase abolishes bone resorption. Biochem Biophys Res Commun 159:68, 1989

221. van Damme J, Opdenakker G, Simpson RJ, Rubira MR, Cayphas S, Vink A, Billiau A, van Snick J: Identification of the human 26-kD protein, interferon β2 (IFN-β2), as a B cell hybridoma/plasmacytoma growth factor induced by interleukin 1 and tumor necrosis factor. J Exp Med 165:914, 1987