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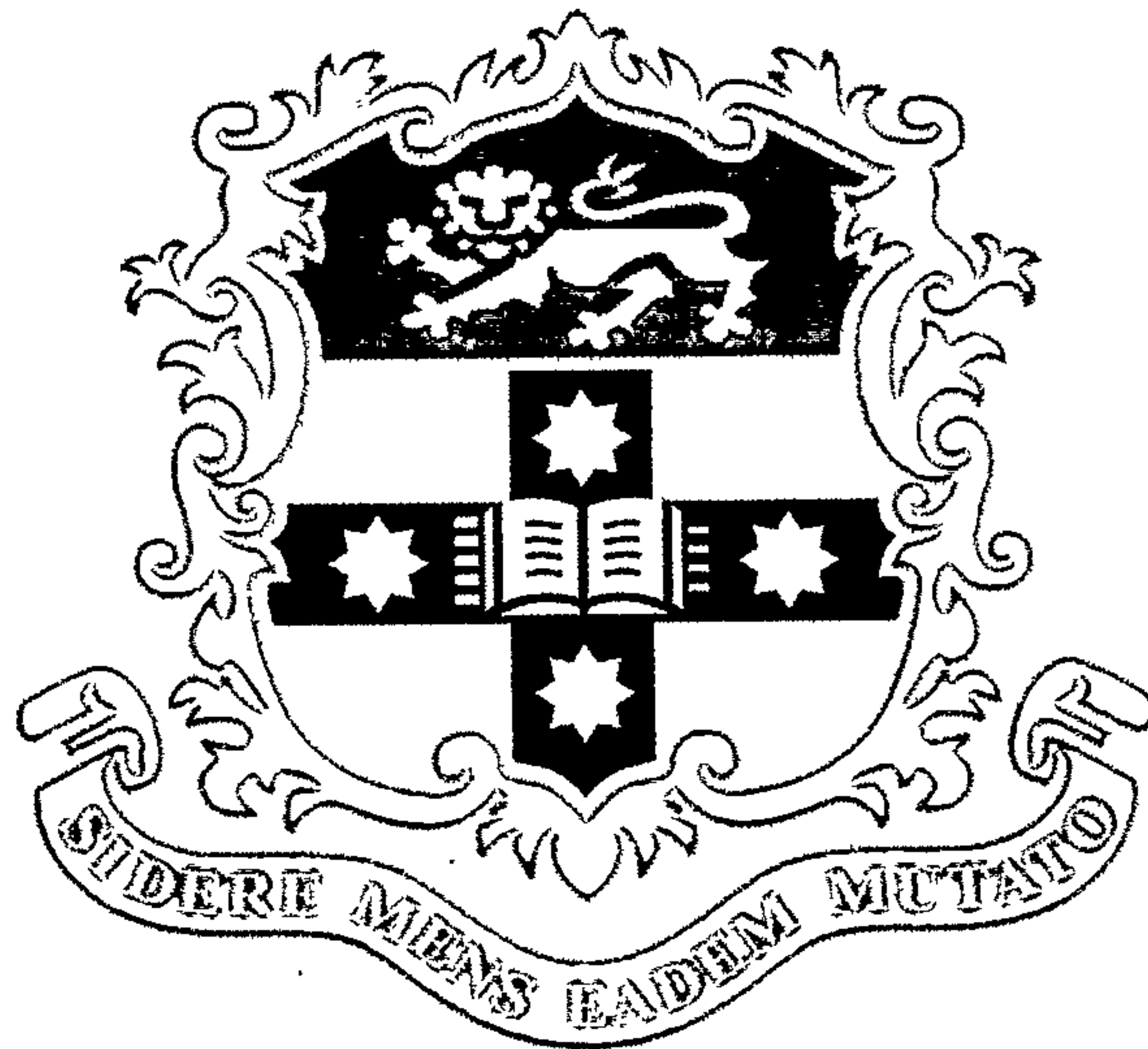
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A Primary Culture System for Human Odontoblasts



Juliette M. Scott BDS(hons), FRACDS

**A thesis submitted in partial fulfilment of the requirements
for the degree of**

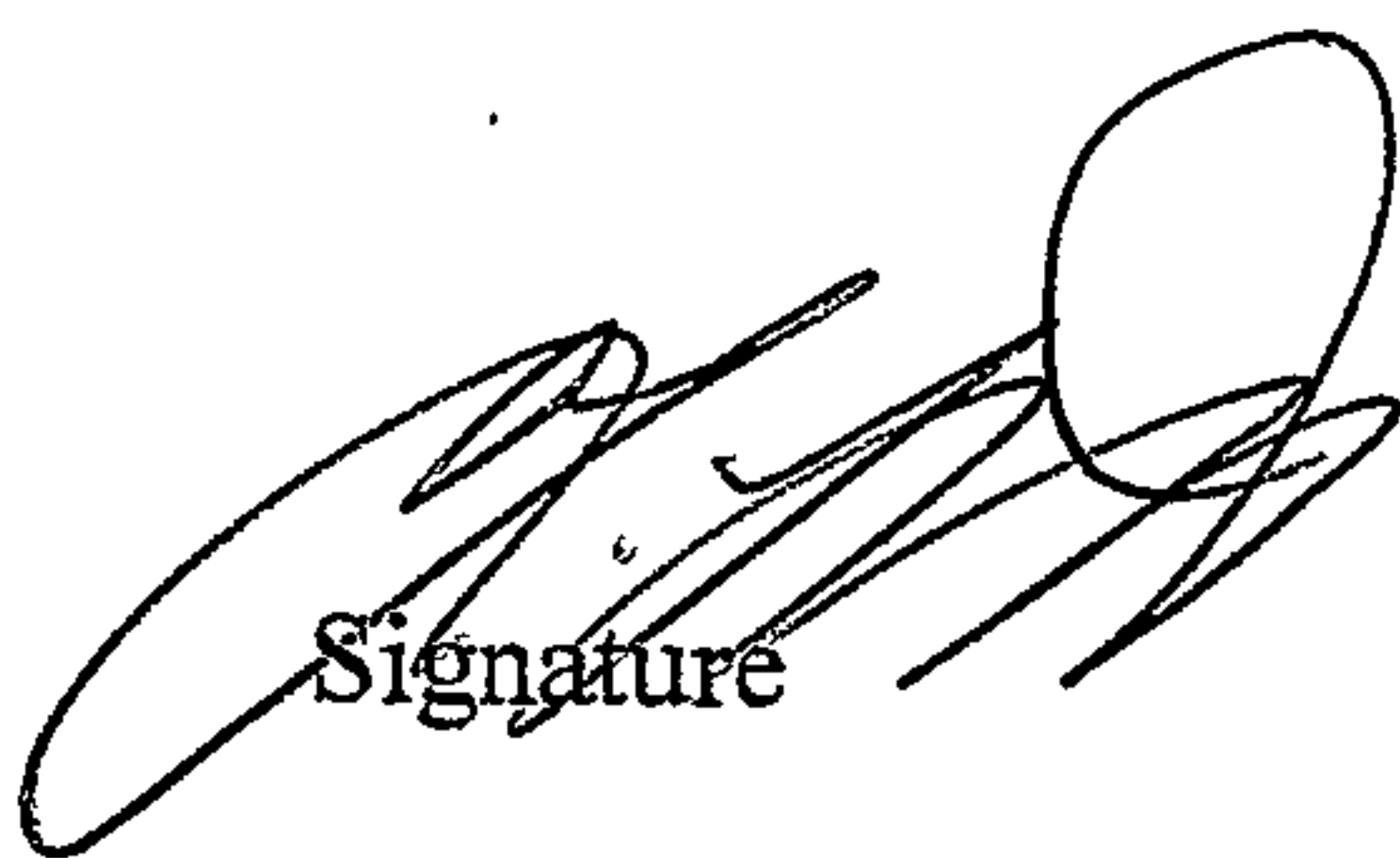
MASTER OF DENTAL SCIENCE (Paediatric Dentistry)

**Department of Paediatric Dentistry
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September 2004

Declaration

This thesis describes the work done in the Department of Physiology, Faculty of Science, and the Department of Paediatric Dentistry at the Westmead Centre for Oral Health, Faculty of Dentistry in the University of Sydney between February 2002 and June 2004. The research is entirely my own and has not been submitted in whole or in part for a degree at this or any other university. To my best knowledge it does not contain any material published or written by another person except as acknowledged in the text.



Signature

9/12/04.
Date

Juliette M. Scott

Acknowledgements

Completion of this thesis would not have been possible without the advice, help and support of many individuals.

First of all, my greatest thanks go to my supervisor Professor Rebecca Mason for giving me the opportunity to work in her laboratory. Her inspiration, dedication and support have been a tremendous help and greatly appreciated. I must make special mention of her enthusiasm during the difficult times, and her great ability to be always available for advice and encouragement. Also to Professor Mike Swain, for his unfailing optimism and encouragement. I further acknowledge the financial support of the National Health and Medical Research Council of Australia.

A great thankyou must go to all the members of the Mason lab. To Dr Meloni Muir, for her great patience and wonderful teaching. To Dharshi Siva for teaching me the intricacies of cell culture, and to Katie Dixon, Shivashni Deo, Shaimaa Atwa and Tara Brennan, for always been ready to help me, lend me cells, or just give me someone to talk to. To Dr Ramin Rohanizadeh for his expertise and invaluable assistance which was always given with such generosity, particularly with electron microscopy. Thankyou to Eleanor Kable for helping produce all the photos. Special thanks also must go to Dr Michael Slater, for his generosity with his time and expertise.

A special thankyou to the department of paediatric dentistry. To Associate Professor Richard Widmer, who was my inspiration to enter the world of paediatric dentistry.

You have never failed me as a mentor and have always kept me in good spirits. To Dr Erin Mahoney, for her amazing energy. Thankyou for always pushing me to do more. To Dr Angus Cameron, for all his help and support over the past three years. To Dr Sally Hibbert, who has kept me going through many a tough time, and to Dr Fiona Bell, for being a wonderfully supportive friend and colleague.

A great thankyou to my family – to my parents for their eternal support and encouragement, and to my brothers and sister for their ability to cheer me up. Lastly and most importantly, my deepest, dearest and everlasting thanks must go to my partner, Anthony, who has been truly amazing over the last three years. His support, encouragement and love have made this work possible.

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Abstracts Presented During the Candidature of this Thesis

Scott, JM; Swain, M; Mason, RS. "Development of a primary culture system for human odontoblasts". Faculty of Dentistry (University of Sydney) Research Day. October 2003, Sydney, Australia.

Scott, JM; Swain, M; Mason, RS. "Development of a primary culture system for human odontoblasts". Australian New Zealand Society of Paediatric Dentistry (ANZSPD), March 2004, Melbourne, Australia.

This presentation was the recipient of the Colgate-ANZSPD award for postgraduate research.

Scott, JM; Swain, M; Mason, RS. "Molecular approaches to dentinal caries. Development of a primary culture system for human odontoblasts". Royal Australasian College of Dental Surgeons, October 2004, Darwin, Australia.

This presentation was the recipient of the Royal Australasian College of Dental Surgeons Young Lecturer Award

Scott, JM; Swain M; Mason, RS. "A new primary culture system for human odontoblasts". Manuscript in preparation for publication.

Abbreviations

1,25(OH)₂D₃	1,25-Dihydroxyvitamin D ₃
ALP	Alkaline Phosphatase
ALK	Activin receptor-like kinase
ALK-3	Activin receptor-like kinase-3
βGP	Beta Glycerol Phosphate
BGP	Bone Gla Protein
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumin
BSP	Bone Sialoprotein
DEX	Dexamethasone
DGP	Dentine Gla Protein
DMEM	Dulbecco's' Modified Eagle's Growth Medium
DPP	Dentine Phosphoprotein
DPSC	Dentine Pulp Stem Cell
DSP	Dentine Sialoprotein
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetracetic Acid
EHDP	Etidronate Disodium
ETOH	Ethanol
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
HCL	Hydrochloric Acid
IGF	Insulin-like Growth Factor

IGF-I	Insulin-like Growth Factor I
IGF-II	Insulin-like Growth Factor II
MEM	Modified Eagle's Medium
NCP	Non-Collagenous Protein
OC	Osteocalcin
ON	Osteonectin
PBS	Phosphate Buffered Saline
PDL	Periodontal Ligament
PG	Proteoglycan
SHED	Stem Cells from Human Exfoliated Deciduous Teeth
SEM	Scanning Electron Microscopy
TCA	Trichloroacetic Acid
TGF	Transforming Growth Factor
TGF-β	Transforming Growth Factor Beta
TGF-β1	Transforming Growth Factor Beta 1

Abstract

Caries is one of the most prevalent of all human diseases. In recent years there has been a growing recognition that healing or remineralisation protocols for the treatment of carious lesions in dentine are possible, rather than just relying on surgical removal of the diseased tissue and placement of restorative materials. This project proposes to investigate a strategy for deliberately stimulating cellular reparative processes for the treatment of this disease. In order to do this, it is necessary to develop a cell culture system to develop and characterise the mineralising cells (odontoblast-like) from human dental explants. In order to examine a more readily available mineralising cell type, initial experiments were performed with human osteoblast cells.

Methods: An established laboratory cell line of human osteoblasts were cultured in a three-dimensional matrix (Gelfoam) and on Thermanox coverslips. Cell growth was examined by histological section. Potential influences on mineralisation were examined by alizarin red staining and tetracycline incorporation.

Freshly extracted primary teeth were sectioned and the pulps removed. The explants were then grown in flasks with culture medium (DMEM) supplemented with NaHCO_3 , l-ascorbic acid and l-glutamine, penicillin, streptomycin and fetal calf serum. Once the cultures reached confluence the cells were passaged. After four weeks in culture, cells were plated onto Thermanox coverslips and maintained for 1 week. At confluence they were fixed in 4% paraformaldehyde. Cells were characterised by immunohistochemistry to detect the presence of osteonectin, a

dentine-matrix protein. An established cell line of fibroblasts was used as a control. Other cultures were examined for alkaline phosphatase activity (an enzyme important in mineralisation) using an enzymatic assay. Human primary fibroblasts were used as a negative control, while human osteoblasts (bone forming cells) were used as a positive control. Cell morphology of dental pulp explants was examined under SEM.

Results: Osteoblasts grew strongly in Gelfoam, and showed infiltration and proliferation within the matrix. Infiltration and proliferation was enhanced by treatment with $1,25(\text{OH})_2\text{D}_3$. Both a source of phosphate (βGP) and calcium were found to be required for optimal mineralisation of osteoblasts in culture, compared to cells cultured in routine growth medium ($p < 0.0001$). The addition of $1,25(\text{OH})_2\text{D}_3$ and dexamethasone did not have a significant effect on mineralisation ($p > 0.05$).

An effective and efficient method was developed to obtain dental pulp explants that were suitable for culture (sectioning of freshly extracted human primary teeth). The dental explants grew strongly in culture, and were able to be subcultured and grown on coverslips. Immunohistochemistry revealed the dental pulp cultures to be strongly positive for osteonectin compared to the fibroblast control. SEM examination did not allow differentiation between dental pulp cells and fibroblasts.

The dental pulp cultures showed strong alkaline phosphatase activity compared to a positive osteoblast control, whilst fibroblasts had very little alkaline phosphatase activity. The addition of βGP to the growth medium for 14 days produced a significant increase in alkaline phosphatase activity of dental pulp cells compared to

unsupplemented dental pulp cells ($p < 0.01$) and fibroblasts treated with β GP ($p < 0.01$). Alkaline phosphatase activity of osteoblasts was also increased ($p < 0.05$). During examination of growth factors which might stimulate the functional activity of odontoblasts, it was found that treatment with transforming growth factor-beta 1 for 14 days nearly doubled alkaline phosphatase activity in the dental pulp explant cells ($p < 0.05$), whilst an equivalent concentration of insulin-like growth factor-1 did not alter alkaline phosphatase activity in these cultures. The addition of TGF- β 1 and β GP to the growth medium produced a very significant increase in alkaline phosphatase activity of the dental pulp explants ($p < 0.01$). Addition of growth factors had no effect on fibroblast controls.

Conclusions: Mineralising cells show enhanced growth in a three-dimensional matrix, and a source of calcium and phosphate is important for mineral deposition. Dental pulps obtained from primary teeth appear to be able to provide a source of odontoblast-like cells. The effect of culture conditions and growth factors was seen to be consistent with phenotypic odontoblastic stem cells. These cells will be able to be used to further study the cellular differentiation processes involved in remineralisation.

Chapter I. Literature Review

Introduction

Despite a significant decline in caries experience over the last few decades, the dental health of children, the aged and people in developing countries as well as lower socio-economic groups in developed countries remains a major concern. The dominant paradigm in caries management has been one of surgical management – that is, to remove all the infected tissue and replace it with a restorative material. There is a growing recognition that healing or remineralisation protocols for the treatment of carious lesions in dentine are possible. If the inherent repair capacity of the vital pulp-dentine tissue can be enhanced and optimised, it may be possible to allow for atraumatic and ultimately more successful management of dental caries.

The first section of this literature review will briefly focus on the general structure of teeth, and then will consider the structure of dentine in detail. The formation and microstructure of dentine will be examined. The responses of dentine under normal and pathological conditions are discussed.

The second section will review in detail the cells responsible for producing dentine, the odontoblasts. The nature, structure and function of these cells are discussed. The response of odontoblasts to *in vivo* and *in vitro* environments is examined, with a detailed discussion of the effects of known growth factors. Various methods of obtaining, culturing and examining odontoblasts are examined.

The third section describes the carious process, and the social impact this disease has.

The importance of developing experimental systems is placed into a clinical context.

I.1 Structure of Teeth and Dentine

Teeth serve a number of functions in humans. They are most commonly associated with mastication, but also play an important role in speech production and facial aesthetics. Human teeth have significant variation in size and shape, however histologically they are similar (Ten Cate 1994b).

Anatomically teeth consist of a crown and a root (Fig.I.1). Teeth are composed of the mineralised tissues and structures known as enamel, dentine and cementum. The acellular enamel and the supporting dentine surround a specialised connective tissue known as the dental pulp. The anatomical crown is covered by enamel. (Ten Cate 1994b).

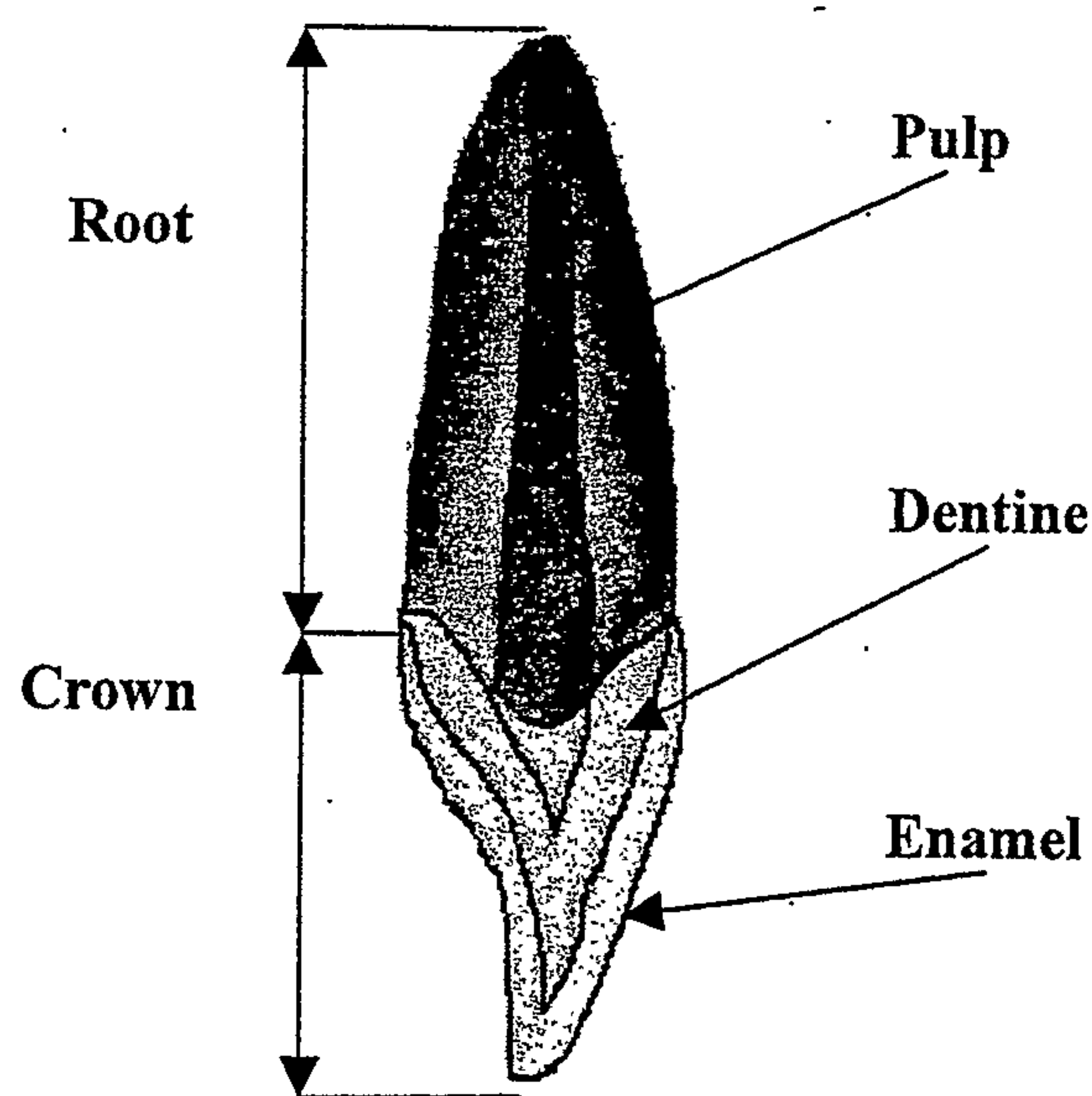


Figure I.1 Structure of tooth

Modified from Andreasen 1994 (Andreasen & Andreasen 1994)

The process of mineralisation is specific for each dental tissue. The mineralisation of enamel is a unique process, involving specific proteins not found elsewhere in the

body. Dentine mineralisation is part of a larger group of mineralised connective tissues that involves collagen and non-collagenous proteins. Within dentine as a whole there are differences between mantle and circumpulpal dentine, and intertubular and peritubular dentine (Goldberg et al. 1995).

Enamel is a hard, acellular inert material that is the most highly mineralised tissue in the body (Ten Cate 1994b). Enamel is produced by *ameloblasts* via the process of *amelogenesis*. Ameloblasts differentiate from the cells of the embryonic oral epithelium. During amelogenesis these cells secrete a mixture of enamel matrix proteins along their basal border. The protein is then immediately replaced with hydroxyapatite, to form an ordered structure of apatite crystals. The ameloblasts cover the entire surface of the enamel as it is formed, but are lost rapidly as the tooth erupts into the oral cavity (Eisenmann 1994). The loss of these cells means that enamel is a non-vital tissue that cannot be replaced when it is destroyed by wear or caries. However, enamel is permeable, and ionic exchange can occur between the enamel and the oral environment (Hume & Townsend 1998). Mature enamel consists of about 96% inorganic material (by weight) made up predominantly of hydroxyapatite crystals with traces of organic material. It is this high inorganic content that makes enamel susceptible to acid demineralisation by bacteria leading to dental caries.

I.1.1 Dentine

The brittle outer layer of enamel is supported by the dentine, which forms the bulk of the tooth structure. Dentine comprises of 70% mineral by weight (50% by volume), primarily hydroxyapatite crystals. 20% of dentine is made up of an organic matrix consisting primarily of the fibrous protein collagen. The remaining 10% is made up of

water (Goldberg et al 1995). Dentine is formed by *odontoblasts* via the process of *dentinogenesis*. The odontoblastic cell bodies are situated on the periphery of the dental pulp. The presence of these cells throughout life makes dentine a living tissue, with the capability to repair itself after injury (Ten Cate 1994b).

Dentine is a calcified connective tissue, and is similar to bone in its composition as well as in its mode of formation. However, there are a number of important differences between the two tissues. The arrangement of the protoplasmic cell processes and their tubules within dentine is unique. Unlike bone, dentine does not contain blood vessels, nor does it undergo remodelling. Dentine does not participate in calcium homeostasis, and it is not subject to gross resorption by cells under normal physiological conditions (except during exfoliation of deciduous teeth). As with all calcified tissues, the formation of dentine is a highly regulated and controlled process. The formation of dentine results in a complex tissue consisting of collagen, mucopolysaccharide ground substance and odontoblastic processes (Hume & Townsend 1998). Dentine is formed by two simultaneous processes in which the odontoblasts are instrumental: the formation of the proteinaceous dentine matrix, and the mineral crystallisation of this matrix.

I.2 Dentinogenesis

The mechanism of formation of mineralised tissues, the associated matrix components and their implication in the mineralising processes constitute the basis for many reviews (Goldberg et al 1995). Dentinogenesis occurs concurrently with amelogenesis. During mineralisation of most calcified tissues, a layer of cells forms

an organic matrix in which an inorganic calcium phosphate is deposited as mineral crystals. Dentine is produced by mesenchymally derived cells known as odontoblasts. The odontoblasts line the pulpal surface of the dentine during its formation (Linde & Lundgren 1995). When the odontoblasts mature, they secrete an extracellular matrix (ECM). The odontoblasts secrete both collagen and complex mucopolysaccharides from their outer end to form the dentinal matrix. The ECM is initially unmineralised, and is known as predentine. In the formation of primary dentine, the predentine ECM changes into dentine as the collagen fibrils are mineralised and additional unmineralised predentine continues to be laid down (Butler 1998). The collagen acts as a matrix for mineralisation, both during tooth formation and throughout life (Hume & Townsend 1998).

1.2.1 The Dentine Organic Matrix

The organic dentine matrix contains macromolecular components that are characteristic of many connective tissues. It also contains some components specific for mineralised tissue and dentine itself (Ten Cate 1994a). A primary function of the organic matrix is to determine the three-dimensional morphology of dentine. The organic matrix of circumpulpal dentine is synthesised and deposited extracellularly by the odontoblasts prior to mineral formation. The majority of the organic matrix is collagen, with non-collagenous proteins (NCPs) and some lipid-containing components making up the remainder (Linde & Lundgren 1995).

Collagens are the major components of dentine. They constitute 90% of the total dentine matrix, but are absent in peritubular dentine. Dentine collagen is mainly type I (as in bone), and it forms a dense, covalently cross-linked network in which the

mineral crystals are deposited (Ten Cate 1994a). Odontoblasts are also known to produce type V collagen (Sodek & Mandell 1982). Type V collagen has been identified in odontoblast cultures and tooth germs, and accounts for 3% of the collagen synthesised by odontoblasts and secreted in predentine. The presence of type III collagen is more controversial. It appears to be absent in normal predentine and dentine, but is present in some pathological conditions such as dentinogenesis imperfecta (Lund et al. 1998). Type IV collagen is only present at the onset of dentinogenesis, when a basement membrane isolates the first layer of unmineralised dentine from presecretory ameloblasts. Type VI has been identified in predentine as a minor component (Goldberg et al 1995).

The non-collagenous organic components of dentine include a complex mixture of proteoglycans, glycoproteins, sialoproteins, phosphoproteins and a variety of other molecules (Butler 1998). During dentinogenesis the extracellular matrix of dentine is mineralised by deposition of hydroxyapatite onto the fibrous matrix. After mineralisation, the non-collagenous dentine molecules remain trapped in the mineralised phase bound to matrix components or to hydroxyapatite crystals. These may be exposed or released as a consequence of injury to the periodontal ligament (PDL) and dental pulp.

I.2.2 Non-Collagenous Proteins of Dentine

There are a number of categories of dentine NCPs. One category includes those proteins that are found exclusively in dentine. These NCPs are only expressed by odontoblasts, and hence are known as *dentine specific proteins* (Butler & Ritchie 1995). Two proteins are considered to be dentine specific: dentine phosphoprotein

(DPP) and dentine sialoprotein (DSP). Dentine matrix protein-1 (Dmp1) was thought to be found only in dentine, but is now known to occur in bone as well (D'Souza et al. 1997). Another group of NCPs in dentine consists of ECM proteins found in bone, dentine and cementum, which are synthesised by the appropriate cells in each tissue. These include bone sialoprotein (BSP) and osteocalcin (bone Gla protein, BGP). This category is termed "mineralised tissue-specific proteins" (Butler & Ritchie 1995). Dentine contains other macromolecules synthesised and secreted by odontoblasts which are also synthesised by other cell types and are found in the ECM of several other tissues. This group includes osteopontin and osteonectin.

DPP and the Proteoglycans (PGs) seem to be the matrix constituents that are primarily involved in mineral induction and growth regulation during dentinogenesis. However, other NCPs, such as bone sialoprotein and dentine sialoprotein may play a role in the process (Butler et al. 1992; Chen et al. 1993).

1.2.2.1 Dentine Specific Proteins

The predominant NCP in all dentine species is made up of the highly phosphorylated phosphoproteins (often denoted as DPP, or **dentine phosphoprotein**), which make up half of the NCP fraction. DPP is found only in dentine and not in bone or any other tissues (Butler 1998). This is a strongly polyanionic type of protein. Depending on the species, several types of phosphoproteins have been extracted. Highly phosphorylated phosphoprotein (containing about 46% phosphoserine and 45% aspartic acid in bovine teeth and >40% phosphoserine in rat teeth) has been identified, and referred to as phosphophoryn (Dimuzio & Veis 1978). Moderately phosphorylated (25%

phosphoserine) and weakly phosphorylated (5-7% phosphoserine) phosphoproteins have also been identified (Goldberg et al 1995).

Immunolocalisation, *in situ* hybridisation and *in vitro* cell and organ cultures show that DPP are secreted by young, polarising odontoblasts and by mature odontoblasts (Butler 1998). Tagaki et al demonstrated the presence of DPP in all the mineralised dentine except the first external layer of mantle dentine through the use of "stains all" staining (Tagaki et al. 1986). Predentine remained unstained, as did the pulp cells. No staining was seen in secondary and reparative dentine. The same distribution has been found using immunocytochemical techniques (Rahima et al. 1988). As it is present in dentine and not predentine, DPP is believed to act as a mineral nucleator.

Since dentine phosphoproteins act as extra-cellular calcium-binding proteins, their binding properties with collagen and calcium have been investigated. When *in vitro* precipitation of calcium phosphate was carried out by one-dimensional double diffusion system in agar gel, the precipitation band was enhanced in the presence of DPP, but crystal growth was decreased. It appears that DPP allows the formation of new nuclei, but inhibits the growth of the already formed crystals. It was concluded that the possible role of DPP may be the regulation of crystal growth behind the mineralisation front (Fujisawa et al. 1987). At higher concentrations the growth of hydroxyapatite was inhibited, suggesting inhibition of secondary nucleation (Boskey et al. 1990). DPP attached to a surface induces mineral formation *in vitro*, whereas when free in solution, DPP acts as a mineral inhibitor (Lussi et al. 1988).

Dentine sialoprotein (DSP, non-phosphorylated sialoprotein) was initially called 95k glycoprotein, and also 53-kDa DSP (Goldberg et al 1995). It is a sialic acid-rich glycoprotein, with high carbohydrate content. It is synthesised by odontoblasts and secreted into predentine and dentine. The molecules appear in young odontoblasts before the mineralisation of dentine. Immunostaining is intense in the odontoblastic processes. Early predentine shows intense staining, whereas in the more mature stages staining is less intense and restricted to the odontoblastic processes. The cells and matrix of the dental pulp are also stained (Butler et al 1992). DSP has no attachment properties, and its precise role in mineralisation is unclear (Butler 1998).

I.2.2.2 Bone Sialoprotein

Bone Sialoprotein (BSP, Bone sialoprotein II) is also a glycoprotein. Like BSPI, it promotes attachment and spreading of cells, but to a lesser extent. BSP enhances type I collagen fibrillogenesis and small amounts have been found in dentine. In situ hybridisation with cDNA probes has shown no labelling at early stages of development, but by day 21 intense labelling is seen in the odontoblasts of foetal rat incisors. This protein may play a role in mediating the initial stage of mineralisation (Chen et al. 1992). Immunoreactivity has been noted in odontoblasts and their processes, and in the peritubular dentine of porcine dental tissues (Chen et al 1993).

I.2.2.3 Osteonectin

Osteonectin (ON, SPARC or BM 40) is a phosphorylated glycoprotein which inhibits hydroxyapatite growth, but promotes calcium and phosphate binding to denatured collagen. ON is virtually absent in rat dentine, but is present in porcine dentine. In

erupted porcine teeth, anti-ON staining in dentine was concentrated around the dentinal tubules, was weaker in the cells of the pulp, and intense in odontoblasts (Tung et al. 1985). In bovine dentine, ON constitutes about 4-6% of the total protein extractable from the tissue. In human teeth, the predentine, odontoblasts and their cell processes show a strong staining reaction (Reichert et al. 1992). In situ hybridisation studies with mRNA show an intense reaction over the odontoblasts. ON has been considered to be a positive marker of odontoblast phenotype (Bègue-Kirn et al. 1994).

I.2.2.4 Osteopontin

Osteopontin, also known as bone sialoprotein I (BSPI) is a 44kDa bone phosphoprotein also found in dentine. Immunostaining reveals faint staining in some young odontoblasts before mineralisation of the dentine, but no staining in preodontoblasts, mature odontoblasts and dentine. As a phosphorylated protein, osteopontin may promote the formation of mineral in dentine (Butler 1998).

I.2.2.5 Osteocalcin

Osteocalcin (OC, Dentine Gla Protein, DGP) is identical to the GLA-protein found in bone. At least 4 closely related fractions have been found in rodent dentine (Jontell & Linde 1983). This molecule has no effect on mineral formation but inhibits hydroxyapatite growth. Immunocytochemical information from rats shows intense staining in odontoblasts and dentine where it was seen to be either restricted to the odontoblast processes or entirely labelled (Bronkers et al. 1987). Only weak staining is seen in predentine (Camarda et al. 1987). In human and bovine teeth, only the mantle dentine reacts with anti-OC, and no staining is seen in odontoblasts or dentine.

1.2.2.6 Proteoglycans

Proteoglycans (PGs) make up another major dentine matrix component. The PGs in mineralised dentine belong to the class of small PGs with only one or two side chains, known as decorin and biglycan (Rahemtulla & Prince 1984). PGs are among the major components of the ground substance of dentine, and provide a medium for transport and diffusion (Goldberg et al 1995). PGs share the space between collagen fibres with phospholipids. In predentine, the appearance of the PGs is amorphous. In dentine, they are closely associated with the surface of collagen fibres or groups of collagen fibres. *In vivo*, PGs may act as a promoter of mineralisation, by functioning as a cation-exchanging reservoir (Hunter 1991). When immobilised on a solid support, PGs induce hydroxyapatite formation whereas in solution they inhibit hydroxyapatite formation and growth (Linde et al. 1989). PGs may have different functions at varying stages of development. In predentine, they may be important in transport and diffusion, and act as hydroxyapatite inhibitors. In contrast, in dentine, PGs adsorbed on collagen surfaces may promote hydroxyapatite initiation (Goldberg et al 1995).

In general, the biochemical composition of the predentine differs from that of dentine. Some constituents of predentine are not present in dentine, and vice versa. In predentine, in addition to type I collagen, there is PG, a small quantity of protein with a low degree of phosphorylation, and osteonectin. DPP and Gla-proteins of the osteocalcin type are not present in predentine (Jontell & Linde 1983). A main reason for the difference in composition of the mineralised dentine matrix to that of predentine seems to be that some macromolecular components (such as DPP, PG and

Gla-protein) are added at or just in advance of the mineralisation front. This is subsequent to intracellular transport along the odontoblast process followed by exocytosis (Linde 1985).

It is known that, in addition to inhibiting mineral formation, PGs may influence extracellular collagen fibrillogenesis. Linde and Lundgren (1995) postulate that the predentine PG may have a regulatory function in collagen fibril formation while at the same time preventing mineral from forming in this zone (Linde & Lundgren 1995). Since the highest concentrations of osteonectin in non-mineralised tissues are observed in collagen-rich tissues, it may be also that this protein has some role in organising the extracellular matrix.

1.2.3 Extracellular Mineral Formation

Dentinogenesis is a complex, highly controlled process. Odontoblasts initially secrete a fibrillar network known as predentine, which is composed primarily of unmineralised type I collagen. The predentine layer is transformed to dentine about 15-20µm from the cell border, as the odontoblasts continue to produce more predentine extracellular matrix material. In this way the cells retreat pulpally, but are still connected to the tissue that they are creating via the odontoblastic processes. The conversion of predentine to dentine involves several key regulatory events. The collagenous fibres are converted from a non-calcifying matrix to one in which plate-like apatite crystals grow within and around collagen fibrils (Landis 1996). The mechanisms involved in the mineralisation of predentine to form dentine are not well understood.

There is evidence that the transformation involves the secretion of NCPs at the mineralisation front, resulting in the initiation and control of mineralisation by the NCPs. It has been suggested that the secretory pathway for collagen differs from that for other NCPs, such as dentine phosphoproteins. This selectivity of secretion routes may be a key controlling factor in specifying the site of mineralisation and the transformation of predentine to dentine. Other possible factors involved in the predentine-dentine conversion include the enlargement and maturation of collagen fibrils, degradation and loss of proteoglycans and transport of calcium through the cellular layer (Butler & Ritchie 1995). Odontoblasts are thus involved in both a direct and indirect manner during dentinogenesis.

As described earlier, the mineral phase of dentine consists of hydroxyapatite crystals $[\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6]$. The dentine mineral does not have as perfect a crystalline structure as enamel, is somewhat calcium-deficient, and contains other ions such as carbonate and fluoride (Linde & Lundgren 1995). The mineral crystals in dentine are associated with the collagenous matrix, and are largely arranged with their c-axis parallel to the collagen fibres. Initiation of mineralisation at the dentine layer takes place within membrane-limited bodies known as "matrix vesicles". During dentinogenesis, calcium and phosphate ions are transferred from the vascular bed between the odontoblasts into the organic matrix of dentine to be incorporated into the dentine mineral (Linde & Lundgren 1995). Phosphatases, such as alkaline-phosphatase, are found in the matrix and promote mineralisation by hydrolysing phosphate from phosphoesters for incorporation into forming mineral (Anderson 1995; Goldberg et al 1995).

Primary Dentine constitutes the majority of the dentine mass. Primary dentine is formed at a relatively high rate during odontogenesis. The most peripheral layer is known as the *mantle dentine*, and this is seen in the zone beneath the enamel where dentine mineralization initially started (Linde & Lundgren 1995).

The dentine that surrounds the pulp is referred to as circumpulpal dentine. During the production of this region of dentine, a layer of *predentine* is present between the odontoblasts and the mineralized dentine. Predentine consists of a non-mineralized organic matrix (primarily collagenous) and is 10-40µm in width. Predentine acts as a zone for the formation and maturation of the collagen scaffolding of the dentine matrix (Linde & Lundgren 1995). Circumpulpal dentinogenesis occurs by two simultaneous processes. The organic predentine is formed adjacent to the odontoblasts, and is subsequently mineralised at the “mineralisation front” (Linde 1985). There is a time lag between the two processes, and this is seen morphologically as predentine. The time lag is due to the time needed to arrange the collagen molecules into a fibrous web. The difference in the composition of predentine from the dentine matrix indicates that some components are added at or just in advance of the mineralisation front (Linde & Lundgren 1995).

Induction of the mineral crystals during mineralisation is facilitated by a process referred to as heterogenous nucleation. ECM components carry chemical groups that are arranged in a specific way that mimics the crystal planes of the crystal phase that is to be formed. The energy barrier is lowered so that a solid phase of calcium phosphate is formed. The mineral nuclei formed at these nucleation sites eventually grow and fuse to become mineral crystals (Linde & Lundgren 1995).

When calcium phosphate minerals form, the type of crystals and their rate of formation are highly dependent on variables such as pH, the concentrations of calcium, phosphate and other physiological ions, as well as the presence of charged macromolecules. In dentine, the pH at the mineralisation front is 7.0 (Lundgren et al. 1992).

As the hydroxyapatite crystals in dentine and bone are seen in a specific relationship to collagen fibres, much interest has been focussed on the properties of collagen from mineralised tissues. Chemically, they differ very little from soft tissue collagens. During calcification, the initial mineral nuclei seem to be formed on or in the collagen fibres. However, collagen is not an efficient mineral nucleator, and tends to function as a means for orientation and a stable support for other macromolecules, such as NCPs and PGs, and the mineral crystals (Linde & Lundgren 1995).

In view of this, the NCPs and PGs have been the focus of interest as candidates for the induction and regulation of mineral formation. This is due to their Ca^{2+} binding capacity and their specific chemical properties that allow them to influence mineral induction and growth *in vitro* (Linde & Lundgren 1995). PG and DPP have been shown to be capable of inducing an apatitic phase under *in vitro* conditions, similar to those *in vivo*, where spontaneous mineral precipitation would not otherwise occur (Lussi et al 1988). This provides experimental evidence that dentine NCPs can induce mineral formation. It has also been shown that DPP and PG from rat incisor dentine can induce mineral under actual *in vivo* conditions (Lussi & Linde 1993).

As matrix secretion continues, most of the odontoblasts body withdraws towards the pulp. However a single process that projects from its cell body remains as a thin, continuous tube of protoplasm, and is known as the odontoblastic process. This results in the development of the dentine tubule. The tubule is non-calcified, and extends from the odontoblast cell body to the dentino-enamel junction. The tubules are tapered towards the enamel, with the diameter reducing by about one-half. The cell process may only occupy the inner one-third to one-half of the tubule, but the tubule remains patent and filled with tissue fluid. The tubule can act as a pathway through which fluid, chemicals and bacteria can move. The presence of dentine tubules in mature dentine determines much of the form, nature and properties of the tissue (Hume & Townsend 1998).

Calcification of the dentine matrix is most rapid in the months following its secretion, but some calcification continues slowly throughout life. The dentine immediately adjacent to the tubule lumen becomes more heavily calcified, and the tubule diameter decreases as more hydroxyapatite precipitates from the supersaturated dentinal fluid. The increasing thickness of the peritubular dentine increases the density of the whole tissue (Ten Cate 1994a).

Dentine continues to be slowly laid down throughout the life of a tooth, and is referred to as **secondary dentine**. Over time, this dentinal deposition causes a reduction in the size of the pulp cavity. This secondary dentine is primarily laid down on the roof and floor of the pulp chamber, producing a change in its size and shape (Hume & Townsend 1998).

Under certain conditions, **tertiary dentine** can be formed. This is seen as a thickening of the dentine, which occurs more rapidly when the dentine encounters abnormal stimulation. This can be as a result of exposure of the dentine surface to the oral environment by tooth fracture or wear, or when the odontoblast comes into contact with the by-products of bacterial metabolism. In a slowly advancing carious lesion or underneath a leaking restoration these bacterial by-products are present at a low level. Under these circumstances, the pulp attempts to protect itself by the odontoblast laying down additional dentine relatively rapidly. This tissue is known as “tertiary reparative dentine” (Smith et al. 2002).

If sufficient damage occurs to kill the odontoblasts but the adjacent pulpal tissue survives, new dentine-forming cells can differentiate from the pulpal ectomesenchyme. The resultant tissue is called **irregular reparative dentine** and may lack the usual tubular structure but include cell bodies (Hume & Townsend 1998).

I.3 The Dentine-Pulp Complex

The dental pulp is a soft connective tissue that is found in the central pulp chamber of the tooth (Ten Cate 1994b). In general terms, the dental pulp responds to damage in ways similar to other connective tissues – it can undergo inflammation, healing, or death. The pulp is, however, unique in that it is entirely enclosed in dentine and it has processes that extend throughout the dentine. The dental pulp and the dentine share many embryological, histological and functional characteristics. In view of this, the pulp and dentine should not be regarded as separate entities, but rather as a structural and functional biological unit (Hume & Massey 1998).

The functions of the dental pulp may be summarised as being:

1. Formative – it produces dentine
2. Nutritive – it nourishes the avascular dentine
3. Protective – it carries neural supply to dentine to allow for sensitivity
4. Reparative – it is capable of producing new dentine when required.

All these functions of the dental pulp relate to the functions of dentine (Torneck 1994).

The outer layer of the pulp, which is also the inner layer of the dentine, is composed of odontoblastic cell bodies. Immediately beneath this layer is a relatively cell-free zone, which is rich in sensory nerve endings and blood capillaries. The bulk of the remaining central pulp tissue is similar to connective tissue elsewhere in the body. It is made up of mesenchymal cells, defence cells and fibroblasts, collagen fibres, ground substance, blood vessel networks, lymphatics, sensory nerve trunks and free sensory endings. This tissue provides metabolic support for the odontoblasts during rapid dentinal deposition, both in initial growth and during repair. If odontoblasts die but the remainder of the pulpal tissues survive then new odontoblasts can differentiate from the pulp ectomesenchyme to lay down irregular reparative dentine (Hume & Townsend 1998; Ten Cate 1994b).

The blood supply of the pulp is rich, and probably represents the high metabolic activity levels of the odontoblasts. It also helps the tissue to overcome chemical and bacterial insult. With increasing age, a number of changes occur in the pulp. There is a decrease in cellularity and an increase in the incidence of pulp stones and diffuse

calcification. As the size of the pulp chamber decreases with continued deposition of dentine, the degree of vascularity decreases and so does the capacity of the pulp to withstand various insults (Hume & Townsend 1998).

I.3.1 The Dentine-Pulp Complex in Disease

The pulp can be damaged in a wide variety of ways, but usually three primary mechanisms predominate – dental caries, leakage of restorations and mechanical, thermal or chemical trauma. Most commonly, the dental pulp becomes inflamed (and possibly dies) as a result of the advance of the carious process through dentine (Torneck 1994).

As a vital tissue, dentine has a limited capacity for its own defence. The healing pattern of the pulp-dentine complex may be dependent on the type and extent of tissue injury, and the effect of the associated defence reaction on the structural and functional integrity of the pulp-dentine border (Tziafas 2004). The dentinal tubules are a potential pathway for the diffusion of noxious chemicals from the external environment to the pulp, and for the inward movement of micro-organisms. The tubules can, however, reduce their diameter or totally close by a number of mechanisms. About et al (2001) have suggested that the success or failure of restorative treatments may depend upon the extent of odontoblastic injury during operative procedures, as the vitality and dentine repair capacity of the dental pulp is dependent upon odontoblast survival (About et al. 2001).

Dentinal sclerosis is the narrowing of the tubules by the formation of peritubular dentine. This is a dense calcific material laid down through an active, odontoblastic,

metabolic process. Sclerosis may progress relatively rapidly beneath an advancing carious lesion, or in dentine that has been exposed to the oral environment. Sclerosis occurs slowly throughout life as a normal physiological process in ageing (Hume & Massey 1998).

Reparative dentine is formed locally in response to noxious stimuli such as caries, attrition, abrasion and restorative procedures. As long as the odontoblasts remain alive and viable, they will retain the capacity to produce additional dentine in response to injury. There are other cells in the pulp that can form new dentinogenic cells if the odontoblasts die, so long as the remainder of the pulp remains vital. After destruction of the odontoblast layer, increased mitoses occur in the underlying pulp, and undifferentiated pulp cells migrate to the region of necrosis from the deeper region of the pulp, and differentiate into odontoblasts. These cells then form reparative dentine, which has all the constituents of dentine, but may vary in form (Tziafas 1995).

The response of the pulp-dentine complex to injury depends to a large extent on the severity of the insult. Following a mild injury, most odontoblasts beneath the damaged area survive. The odontoblast layer is stimulated to form reparative dentine beneath the injury, while peritubular dentine formation is seen in the dentine tubules (Bjørndal & Mjör 2001). Reparative dentine, containing relatively normal tubules will be laid down at the pulp-dentine interface in the area of damage as a continuation of the main body of dentine. Reparative dentine shows many anatomical, biochemical and functional similarities to primary and secondary dentine, and can effectively oppose exogenous destructive stimuli to the pulp (Tziafas 2004). The formation of

this type of dentine represents up regulation of the biosynthetic activity of the primary odontoblasts, restricted to those cells affected by the injury.

If more odontoblasts die because of a more severe injury, the pulpal cells will produce reparative hard tissue of widely varying types. The form will depend on the nature and stage of differentiation of the cells that produce the primary calcific repair process. A cascade of inflammatory and healing events occurs rapidly in the area of the degenerating odontoblasts (Kim 1990). As part of the connective tissue healing process, pulpal cells proliferate and migrate towards the circumpulpal dentine. Secondary odontoblast-like cells are derived from the cell-rich subodontoblastic layer. This layer contains a high proportion of both fibroblasts and primitive mesenchymal cells, both of which may act as precursors of the replacement cells. Initially, fibroblast-like cells align themselves against the dentine, and atubular fibrodentine is laid down at the pulp-dentine border. As a new generation of odontoblast-like cells differentiate, tubular reparative dentine may be laid down in a polar predentine-like pattern (Bjørndal & Darvann 1999). In some cases cells in the area of calcification may become entrapped and die, leaving substantial defects in the reparative tissue. Under clinical conditions, the matrix formed at the pulp-dentine interface often comprises reactionary dentine, reparative dentine and fibrodentine. It is not possible to distinguish these processes at the *in vivo* level (Tziafas 2004).

The most severe injury to the dental pulp is an actual exposure of the pulp. Pulpal exposure due to caries shows a very limited potential for pulpal healing due to the prolonged bacterial infection of the pulp (Bergenholtz 2001). Favourable conditions for pulpal repair after exposure require a bacteria-free environment, the absence of

severe haemodynamic changes and the absence of severe inflammatory cell infiltration. Whether subsequent reactions lead to pulpal healing and repair or to generalised pulpal inflammation and necrosis will depend on the extent of the defence reactions (Trowbridge 1981). As a part of the wound healing process in a damaged dental pulp, the dentinogenic potential of pulpal cells can be expressed (Tziafas 2004). Proliferation, migration and differentiation of progenitor cells can give rise to a new generation of reparative dentine-forming cells (odontoblast-like cells) reconstituting the lost continuum at the pulp-dentine border (Mjör et al. 1991).

In the case of a chronically inflamed pulp, diffuse calcification may occur, presumably as a result of the activation, differentiation and calcific matrix secretion by mesenchymally derived cells. Relatively well-organised dentine may also be laid down within a chronically inflamed pulp to form pulp stones (Hume & Massey 1998).

I.4 Odontoblasts

Odontoblasts are mesenchymal cells derived from the neural crest. During tooth development, inductive epithelial-mesenchymal interactions lead to the differentiation of mesenchymal pulp cells into odontoblasts (About et al. 2000a).

Odontoblasts normally remain during the life of the tooth, with their cell bodies on the inner surface of predentine and their cell processes extending into it (Fig.I.2). The cell bodies form a monolayer of cell bodies densely packed at the periphery of the dental pulp (Goldberg et al 1995). They retain their capacity to secrete matrix protein and to form additional dentine (Hume & Townsend 1998).

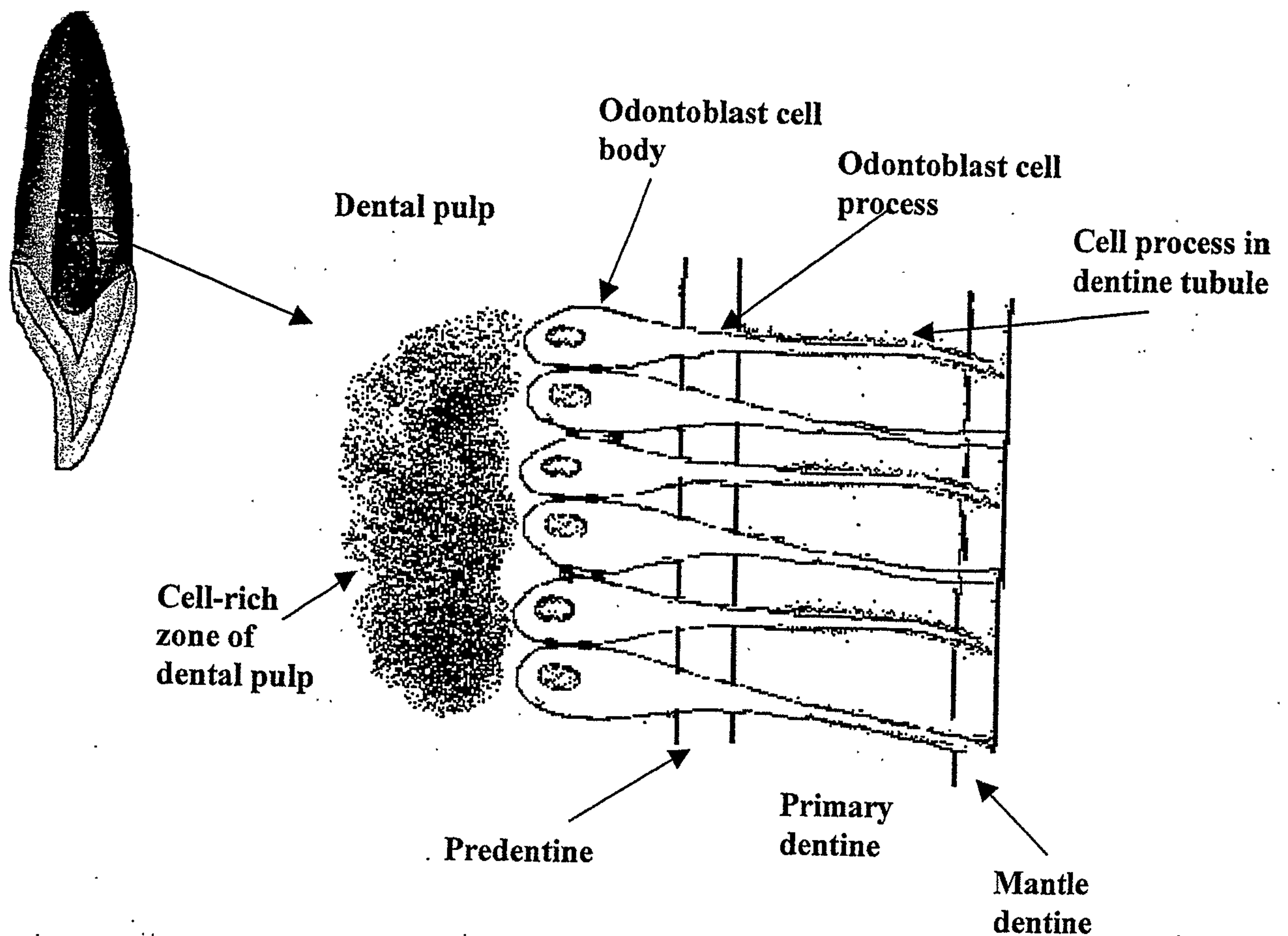


Figure I.2 Structural arrangement of odontoblast cell bodies and processes
 Modified from Ten Cate (1994) (Ten Cate 1994b)

Mature, functional odontoblasts synthesise and secrete the extracellular matrix constituents of predentine and dentine. The odontoblast cell bodies are responsible for producing collagen and non-collagen matrix molecules (Goldberg et al 1995). Odontoblasts that are actively producing dentine are columnar cells, and display characteristics of actively synthesising and secreting cells. The cells have long cytoplasmic processes at the distal zone of the cell body, which contain a rich network of longitudinally arranged microtubules, cytoplasmic filaments and endocytic/exocytic vesicles. They lack major organelles (Garant 1972; Sasaki & Garant 1996). The processes penetrate through the predentine into the dentine in the dentinal tubules (Fig I.3).

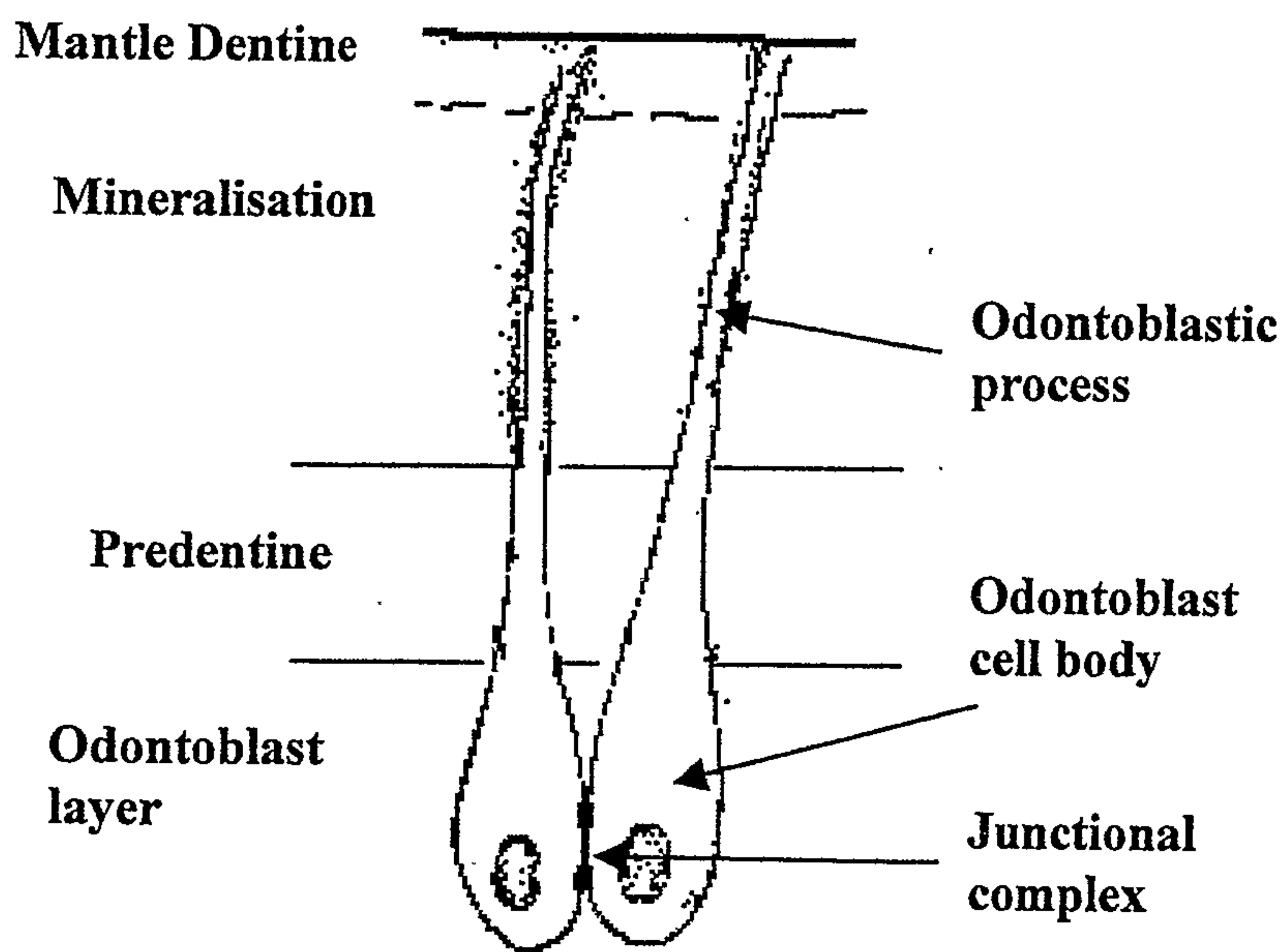


Figure I.3 Mature odontoblasts in continuing dentinogenesis.

Modified from Ten Cate 1994 (Ten Cate 1994a)

At the proximal part, the odontoblast has an extensive, well-developed rough-surfaced endoplasmic reticulum, many mitochondria, a prominent Golgi apparatus, multiple secretory vesicles, lysosome-like dense bodies, centrioles and prominent ciliar structures (Fig.I.4) (Jones & Boyde 1984; Sasano & Kagayama 1997). Adjacent odontoblasts are held together by numerous macular adherens, gap junctions and a well-developed junctional complex concentrated at the distal end of the cell body close to the predentine layer (Jones & Boyde 1984).

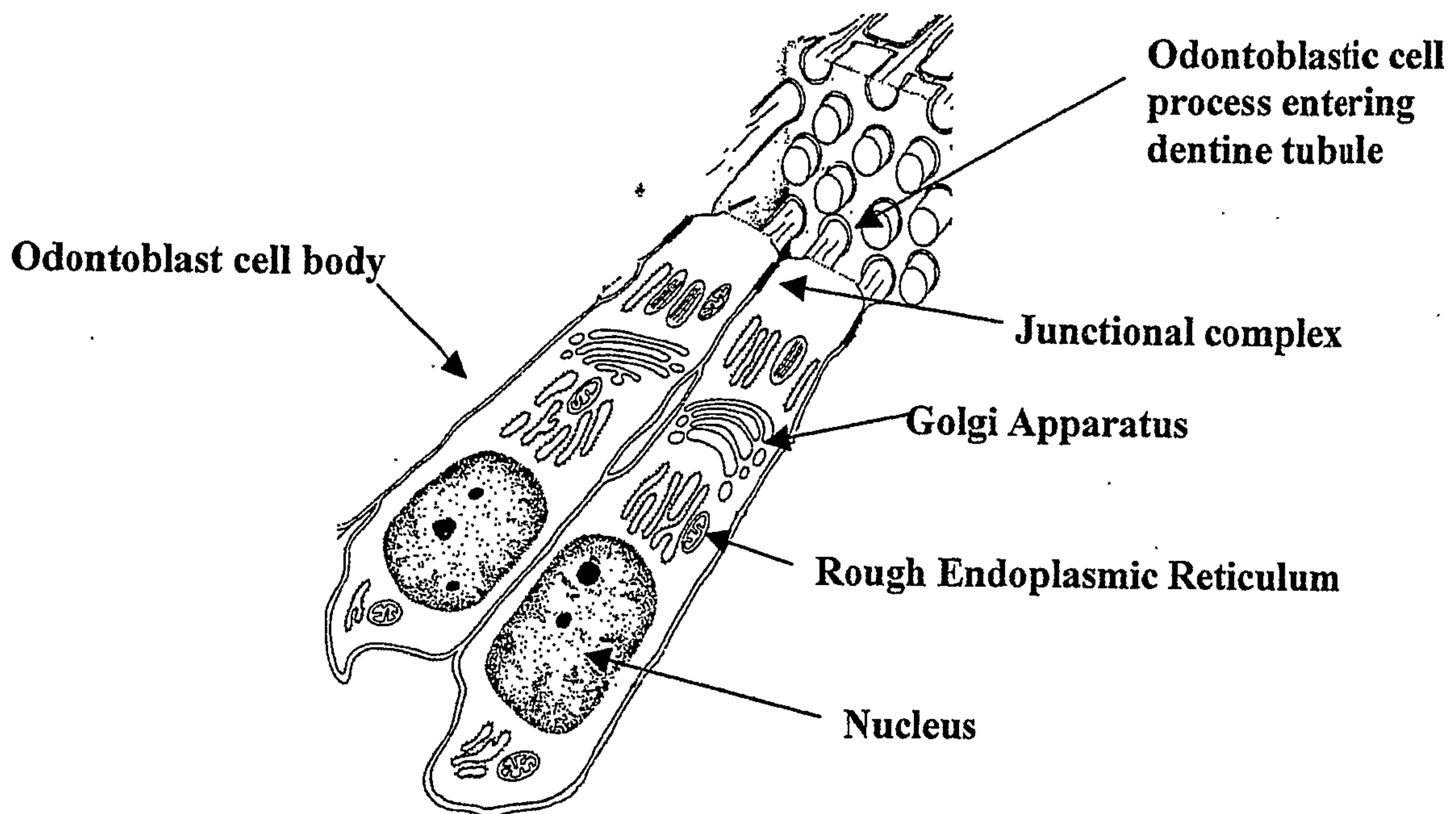


Figure I.4 Cellular structure of odontoblasts.

Modified from Ten Cate (1994) (Ten Cate 1994a)

Odontoblasts synthesise type-1 collagen (the major component of the dentine matrix) into the predentine at the proximal part of the odontoblast process (Weinstock & Leblond 1974). Subsequent mineral deposits are closely associated with the collagen fibres, and give rise to the mantle dentine. Initiation of mineralisation at the dentine layer takes place within membrane-limited bodies known as “matrix vesicles”. Phosphatases, such as alkaline-phosphatase, are found in the matrix and promote mineralisation by hydrolysing phosphate from phosphoesters for incorporation into forming mineral (Anderson 1995; Goldberg et al 1995).

During dentinogenesis, odontoblasts play an important role in transporting Ca^{2+} ions from the vascular network to be incorporated into the mineral phase. The Ca^{2+} ions are transported through the cells by transmembraneous ion-transporting mechanisms

(Linde & Lundgren 1995). This central role of the odontoblasts means that they are in control of the extracellular ionic environment in the predentine zone.

Several studies have shown that alkaline-phosphatase activity is linked to the mineralisation of calcified tissues, such as cartilage, bone and dentine (Anderson & Reynolds 1973; Weiss et al. 1989; Whyte 1994). In the developing tooth, the alkaline-phosphatase activity is concomitant with the onset of cell differentiation, at the late bell stage of embryonic tooth development (Fig I.5) (Woltgens et al. 1995). At the ultrastructural level, a strong enzymatic reaction for alkaline-phosphatase has been observed in “matrix vesicles” (Sasaki & Garant 1996).

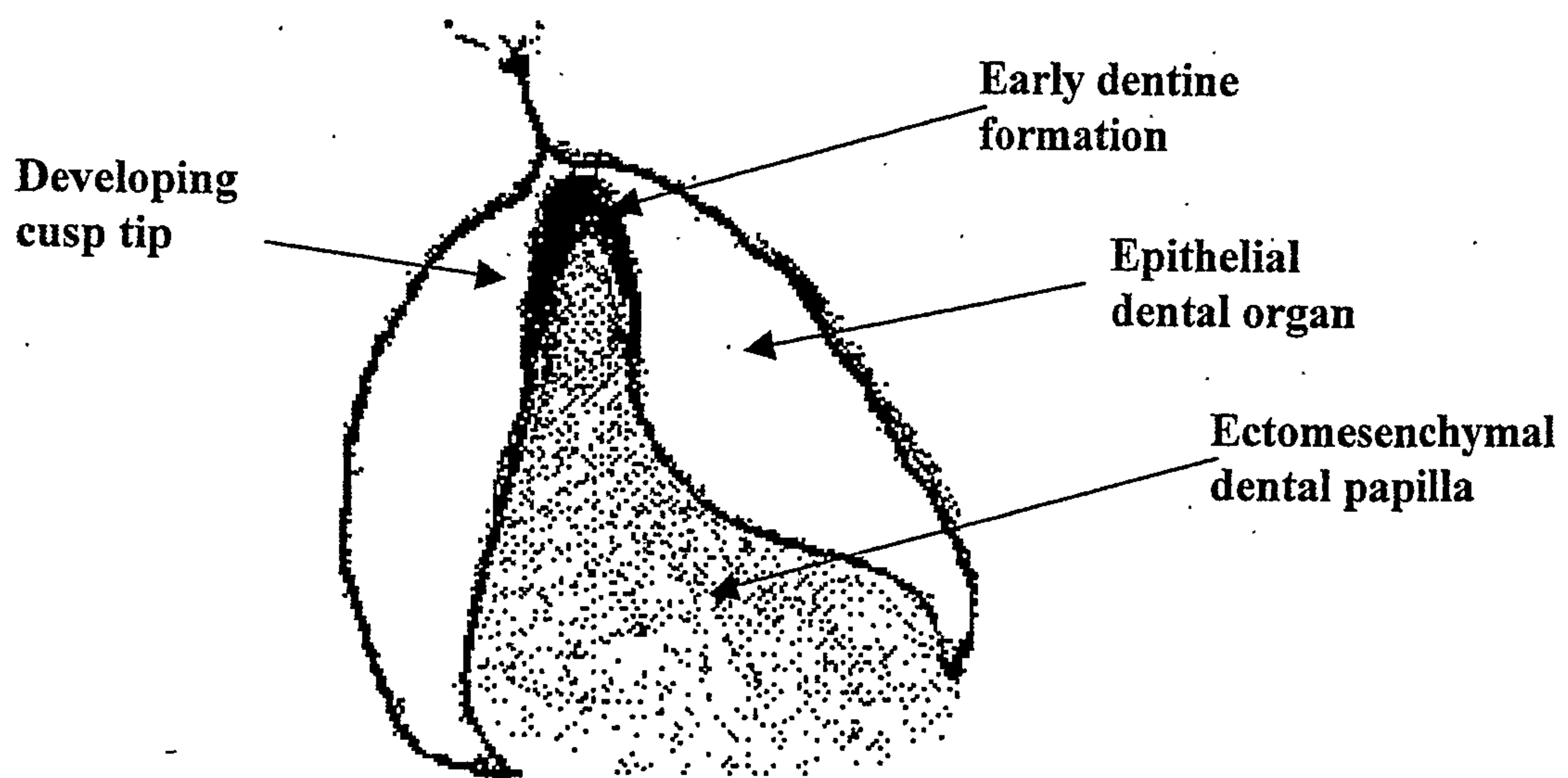


Figure I.5 Late Bell stage of tooth development

Early dentine formation is commencing at the tip of the developing cusp. Modified from Ten Cate 1994 (Ten Cate 1994b).

I.4.1 Odontoblast Differentiation

Odontoblast differentiation is dependent upon the interaction of pre-odontoblasts with the adjacent inner dental epithelium through a specific basement membrane. Growth

factors in the extracellular matrix may play a role in tooth initiation, morphogenesis and cell differentiation (Thesleff & Sahlberg 1996). During odontogenesis, extracellular matrix remodelling is required in both the enamel organ and predentine prior to and during mineralisation. Degradation of the basement membrane separating the initial layer of predentine and pre-secretory ameloblasts is a requisite for enamel formation (Linde & Goldberg 1993).

It has been suggested that terminal differentiation of odontoblasts could only be achieved after a minimum number of cell cycles, and that the inner dental epithelium exerts control over this terminal differentiation through basement membrane mediated interactions (Ruch 1985). It is not clear whether distinct odontoblast and pulp progenitor cell populations exist in the dental papilla. All the neural crest derived cells may be potential odontoblasts, although only some of them overtly differentiate (Bègue-Kirn et al. 1992; Ruch et al. 1995).

The biological properties of the dentine matrix have been studied by many investigators. It has been shown to contain proteins with mitogenic properties (Finkelman et al. 1990). The biological effects of these proteins on dental mesenchymal cells have been investigated *in vivo* (Tziafas et al. 1995a), and have been shown to directly induce odontoblast-like cell differentiation and matrix synthesis may indirectly further control cell polarization (Bègue-Kirn et al 1992).

Expression of several fibroblast growth factors (FGFs) has been localised in developing teeth. FGF1 and FGF2 have been detected in epithelial and mesenchymal components of the tooth by immunohistochemistry (Cam et al. 1992). The biological

effects of FGFs are mediated through high affinity tyrosine-kinase receptors. Expression of FGF-receptor-1 in differentiated and secretory odontoblasts suggests that FGFs play an important role in these events (Kettunen et al. 1998). Transforming growth factor beta 1 (TGF- β 1) has been shown to have high expression in polarised odontoblasts, but decreased expression in functional and mature odontoblasts (Bègue-Kirn et al 1994).

It has been observed that certain combinations of growth factors belonging to the TGF, FGF and insulin-like growth factor (IGF) families are able to mimic the action of the inner enamel epithelium and induce the *in vitro* differentiation of odontoblasts (Bègue-Kirn et al 1992; Bègue-Kirn et al 1994; Bègue-Kirn et al. 1998a; Martin et al. 1998). Bègue-Kirn et al (1992) reported the effects of dentine proteins on the morphological features of isolated mouse dental papillae embedded in semi-solid medium and grown *in vitro* (Bègue-Kirn et al 1992). This paper was the first to demonstrate that in the presence of dentine matrix proteins and other growth factors, the initiation of odontoblast differentiation can be obtained, despite the absence of inner enamel epithelium and a competent basement membrane. TGF- β 1 and bone morphogenetic protein 2 (BMP2) were both seen to stimulate odontoblast differentiation and matrix secretion by dental papillae cells. A TGF- β -like molecule was thought to be present in dentine that could act as a modulator on the initiation of the cytological and functional differentiation of odontoblasts. The phenotype of these experimentally induced cells appears to be similar to *in vivo* odontoblasts. Normal odontoblasts and *in vitro* induced odontoblast-like cells expressed transcripts encoding for TGF- β 1 and 3, BMP 2 and 4, BSP and osteonectin (Bègue-Kirn et al 1994). Further investigation examined the expression patterns of DSP and DPP in

developing mouse dental papillae. Both molecules were detected in developing odontoblasts, and odontoblast differentiation was induced with TGF- β (Bègue-Kirn et al. 1998b).

Unda et al (2000) cultured isolated dental papillae from 17-day-old mice in the presence of combinations of FGF1, FGF2, and TGF- β 1, and evaluated the cytodifferentiation by electron microscopy (Unda et al. 2000). This study was based on earlier investigations (Martin et al 1998). They also looked at the capability of the cells to produce an extracellular matrix, indicative of the dentine mineralisation process, in terms of secretion of type-1 collagen and alkaline-phosphatase activity. The cells were maintained in culture for 6 days. Only the dental papillae that were treated with FGF1 and TGF- β 1 contained differentiated odontoblast-like cells at the periphery of the explants, and these cells secreted an extracellular matrix similar to predentine. Treatments with FGF2 and TGF- β 1 induced cell polarization at the surface of the explants, but no matrix secretion was observed. This study concluded that in their culture system, exogenous combinations of FGF1, FGF2 and TGF- β 1 interact with preodontoblasts and induce cell polarisation or differentiation. FGF1 and TGF- β 1 appeared to have a synergistic effect to promote morphological and functional features of differentiated odontoblasts. FGF2 appeared to modulate the action of TGF- β 1, causing morphological polarisation of preodontoblasts but limiting the functional activity of the cells. The importance of FGF in developing teeth has been shown, however its significance in mature teeth is not clear.

TGF- β 1 transcripts are expressed in preodontoblasts (Bègue-Kirn et al 1994). The exogenous addition of TGF- β 1 to the dental papillae cultures could have interacted

with competent preodontoblasts, and regulated cell differentiation and matrix production.

The role of the IGF family in the growth and differentiation of dental pulp cells has been considered, primarily in combination with other growth factors. In mouse dental papillae *in vitro*, odontoblast-like cells obtained in the presence of IGF1 did not show strong odontoblastic traits (they did not express TGF- β 1) (Bègue-Kirn et al 1994).

I.4.2 Odontoblast Regulation in Mature Teeth

Mature cells in dental pulp are considered to be capable of differentiating into odontoblasts (or odontoblast-like cells), as they are capable of responding to injury by producing reparative dentine *in vivo* (Fitzgerald et al. 1990), as well as forming hard tissue *in vitro* (Nakashima 1991). At a critical point in this biological change, many growth factors and hormones could be involved. Dentine represents a significant storage site for growth factors, including IGF-I, IGF-II, platelet-derived growth factor, epidermal growth factor, vascular endothelial growth factor, FGF, metalloproteinase and TGF- β super family members such as TGF- β 1 (Silva et al. 2004).

The molecular processes underlying reactionary dentinogenesis have been approached in experimental models *in vitro* and in carious human teeth *in vivo* (Magliore et al. 1996; Sloan & Smith 1999). Our understanding in this field has advanced with the appreciation that the dentine extracellular matrix does not represent an inert material, but contains many bioactive molecules that are potentially available for release during pulp healing and repair (Magliore et al. 2001). Carious demineralisation of the dentine

may release bioactive molecules, which signal the associated dentinogenic events (Smith 2002).

The debate on the origin of odontoblast-like cells and the associated signalling mechanisms controlling migration, orientation, attachment and cytodifferentiation of pulpal cells remains to be resolved (Tziafas 2004). It has been strongly suggested that the exposed dentine surface and the accumulated bioactive molecules might provide the necessary signals that determine the underlying cell function (Heritier et al. 1990). Tziafas (2004) suggests two possible mechanisms for reparative dentinogenesis: direct induction of odontoblast-like cells by the dentine matrix or indirect differentiation of odontoblast-like cells on an intermediate fibrodentinal matrix (Tziafas 2004). The dentinogenic activity of the dentine matrix might be attributed to the soluble fraction of dentine components.

The dentinogenic activity of this dentine fraction has been demonstrated *in vitro* by Bègue-Kirn et al (1992) (Bègue-Kirn et al 1992). It was shown that the dentinogenic activity could be abolished by preincubation of the components with an antibody that blocked the biological activity of TGF- β molecules. Similarly, any effect of antibody-treated dentine on pulpal cells was completely or partially lost *in vivo*, indicating that the dentinogenic activity of dentin matrix can at least be partially ascribed to TGF- β (Tziafas 1995). It seems that the endogenous pools of TGF- β s and other growth factors in the dentine matrix may provide a natural delivery system. This system may regulate reparative dentinogenesis after the destruction of primary odontoblasts in deep dentinal cavities (Tziafas 2004). Further studies are required to clarify whether

the biological effect of dentine matrix on initiation of reparative dentinogenesis could be triggered therapeutically.

The mechanisms affecting the activity of mature odontoblasts as a response to external stimuli are not well-understood. The molecular basis of odontoblast differentiation is unknown. As has been seen, most cell culture systems are concerned with developing cells. Experimental investigations in mature teeth have shown that a network of extracellular matrix molecules and growth factors signal tertiary dentinogenesis. Dentine contains several growth factors, including IGF-I, IGF-II, and TGF- β (Finkelman et al 1990). Of these, TGF- β has been suggested as a major candidate for regulating the pulp-dentine complex response to caries (Smith et al. 1995). The BMP group are also thought to play an integral role (Bègue-Kirn et al 1992; Bègue-Kirn et al 1994).

BMPs are soluble, locally acting signalling molecules which bind to specific receptors on the surface of cells. These receptors activate signal transduction mechanisms leading to the induction of particular genes. *In vivo*, BMPs act primarily as differentiation factors, acting on responsive mesenchymal cells (Silva et al 2004). The importance of the BMP group is supported by studies which examined the effect of direct application of recombinant BMP-2, BMP-4 and BMP-7 on amputated pulp. This application induced the formation of large amounts of reparative dentine (Nakashima 1994). About et al (2000) examined the effect of the application of BMP-4 on human dental pulp explants. Application of BMP-4 was seen to up-regulate the expression of nestin. Nestin is thought to play a role in odontoblast differentiation in developing teeth, and in mature teeth under pathological conditions. It was suggested

that BMP-4 is involved in nestin up-regulation, and that it may be important in regulating protective mechanisms of the dental pulp (About et al. 2000b).

TGF- β superfamily members and their receptors play an important part in the differentiation of mesenchymal pulp cells into odontoblasts during reparative dentinogenesis. This has been shown in a bovine primary dental pulp cell culture, which was used as an *in vitro* model to explore the proliferation and differentiation of pulp cells into preodontoblasts (Toyono et al. 1997). The functions of the TGF- β family are mediated by specific cell-surface receptors. These receptors are type I serine/threonine kinase receptors (termed activin receptor-like kinases, or ALK) and type II receptors. ALK-3 mRNA is highly expressed in the odontoblast layer in the bell stage of odontogenesis (Ikeda et al. 1996). Although much is known about the presence and probable biological action of the TGF- β family receptors in embryonic tooth development, there is much less information about their activity in mature pulp tissue and during reparative dentinogenesis. ALK-3 can be isolated from rat dental pulp cell lines (Toyono et al 1997), but other receptors for the TGF- β s and BMPs have not yet been identified in adult pulp tissue.

Toyono et al (1997) used the identification of type I and type II receptors for TGF- β and BMPs expressed in bovine adult dental pulp cells to study the inductive response of pulp cells in reparative dentine formation (Toyono et al 1997). Progressive differentiation of cultured dental pulp cells into preodontoblasts was observed.

Insulin is an important hormone for the growth and proliferation of a variety of somatic cells in culture (Straus 1984). Early studies indicated that it may be involved

in the regulation of cell growth in dental pulp explants (Nagata et al. 1989). An *in vitro* experiment using rat dental pulp cells showed that insulin could be involved in the differentiation of pulp cells in dentinogenesis (Liang et al. 1992). As the biological effects of insulin and IGF-I and IGF-II are similar to each other (LeRoith & Roberts Jr 1993), the insulin-like growth factors were considered to play an important part in the growth and differentiation of dental pulp cells (Onishi et al. 1999).

IGF-I has been shown to enhance the alkaline phosphatase activity of bone-derived cells (Chenu et al. 1990). However, IGF-I and IGF-II had no effect on DNA synthesis or the alkaline phosphatase activity of bovine pulp cells (Nakashima 1992). Onishi et al (1999) examined the effects of IGF-I, IGF-II and insulin on the proliferation and alkaline phosphatase activity of dog dental pulp cells (Onishi et al 1999). Insulin was found to generally promote the growth of the pulp cells, but had no direct effect on their differentiation. IGF-I and IGF-II were found to have a weak enhancing effect on the DNA synthesis and alkaline phosphatase activity. The conclusion that IGF-I might have a primary role in the growth and differentiation of pulp cells has not been supported by other investigators.

I.4.3 Direct Induction of Reparative Dentinogenesis

In pathological conditions (such as carious lesions) reparative dentine is produced by stimulated odontoblasts. It has been suggested that this stimulation may be due to signalling molecules such as TGF- β 1 and BMP-2, which are released from the dentine during demineralisation (About et al 2000a; Bègue-Kirn et al 1994). If the insult to the pulp is severe, the odontoblasts may be destroyed. Newly formed odontoblast-like cells replace the destroyed odontoblasts, and continue producing reparative dentine

(Trowbridge 1981). It has been suggested that these cells may originate from fibroblasts in the dental pulp (Fitzgerald et al 1990). Signaling molecules such as TGF- β 1 and BMPs seem to be necessary to stimulate proliferation and differentiation of pulp cells (About et al 2000a).

In the clinical management of the deep carious lesion, direct pulp treatment is frequently required. The ultimate goal of a regenerative treatment strategy in direct pulp treatment is to induce differentiation of odontoblast-like cells forming reparative dentine at the pulp-capping material interface and to stimulate the biosynthetic activity of the surrounding primary odontoblasts (Mjör 2002). The optimal end result is the reconstitution of the dentinal defect with a bridge of reparative dentine in direct continuity with the reactionary dentine formed around the pulp exposure. The signalling mechanisms regulating reparative dentine after pulp capping have not been fully understood. It has been postulated that a network of interactions between extracellular matrix molecules, including fibronectin (Tziafas et al. 1995b) and growth factors regulates odontoblast-like cell differentiation and reparative dentinogenesis in the repairing pulp environment (Bègue-Kirn et al 1994). The presence of a mechanical support seems to be of critical importance. An intermediate fibrodentinal matrix may act as the basement membrane does for odontoblast differentiation during tooth formation (Ruch 1985).

Application of biologically active growth and morphogenetic factors and extracellular matrix molecules has resulted in hard tissue formation in animal models. BMPs such as BMP-2, BMP-4 and BMP-7 (osteogenic protein-1) induced formation of osteodentine in large amounts followed by tubular reparative dentine (Nakashima

1994). Pulp capping with IGF-I has demonstrated complete dentinal bridging and occasional tubular reparative dentine formation (Lovschall et al. 2001). Osteodentine followed by homogenous and well-mineralised atubular reparative dentine was seen after pulp capping with bone sialoprotein (Decup et al. 2000). Hard tissue formation at a distance from the capping was found after placement of enamel matrix derivatives in the exposed pulp (Nakamura et al. 2001).

Intrapulpal implantation of Millipore filters containing either EDTA-soluble dentine components (Tziafas et al 1995a) or human TGF- β 1 (Tziafas et al. 1998) induced specific dentinogenic events in close proximity to the implants. Cytological differentiation of odontoblast-like cells and reparative dentine formation were seen around the filters. Implantation of Millipore filters containing other growth factors such as basic FGF or IGF-II showed increased dentinogenic effect at a distance from the implant (Tziafas et al 1998). The filters that were immersed in TGF- β 1 were seen to be surrounded by a thick zone of tubular matrix lined with polarized columnar cells (Tziafas & Papadimitriou 1998). When biomatrices were incubated with TGF- β neutralizing antibody, the demineralised dentine completely lost its inductive ability (Tziafas & Papadimitriou 1998). Tziafas and colleagues concluded that pulp cells can express their dentinogenic potential in response to an appropriate surface containing exogenous TGF- β 1. They postulated that the dentinogenic activity of the dentine matrix may be at least partly ascribed to TGF- β 1 molecules (Tziafas & Papadimitriou 1998).

It is clear that a broad spectrum of biological substances stimulate reparative dentine formation in the exposed pulp. However it is important to recognize that in the

majority of cases reparative dentinogenesis proceeds via formation of a fibrodentine matrix. Formation of fibrodentine could imply an indirect effect, e.g. stimulation of the biosynthetic activity of pulpal cells, which is later superseded by the tissue-specific dentinogenic response (Tziafas 2004).

I.5 Systems for Studying Odontoblasts

Odontoblast differentiation *in vivo* comprises sequential and highly integrated mechanisms. Increasingly it is been recognised that odontoblast differentiation is linked to a number of known growth factors. Terminal differentiation of odontoblasts consists of a series of cytological and functional changes, including withdrawal from the cell cycle, cytoplasmic polarization, and finally secretion and mineralisation of predentine/dentine (Ruch et al 1995). Experimental data related to the function of mature odontoblasts, without the confounding effects of the pulpal response, are not readily available or attainable because of the nature and location of the cells.

When dental pulp tissue is injured, reparative dentine is formed by newly differentiated odontoblasts that arise either from mesenchymal cells situated perivascularly or from mesenchymal cells that form the subodontoblast layer in the dental pulp tissue (Torneck 1994). The mechanisms affecting the secretory activity of mature odontoblasts as a response to external stimuli are not well-understood. There is an increasing body of research about this differentiation process, with investigators reporting that it is controlled by hormones (Young et al. 1995) and growth factors (Bègue-Kim et al 1994; Cam et al 1992; Kettunen et al 1998; Thesleff & Sahlberg 1996). However, much about the mechanism of odontoblast differentiation remains to

be clarified. The understanding of this mechanism is fundamental to the development of treatments for damaged or diseased vital teeth.

The use of cell culture systems is a useful approach for investigation of the odontoblast differentiation process. Several *in vitro* approaches have been developed to study the differentiation process, and to examine the potential ability of dental pulp cells to differentiate into odontoblasts and to produce a mineralising matrix (Couble et al. 2000). Dental root tissues have been used to produce a “co-culture” of epithelial and mesenchymal cells (Farges et al. 1995). Epithelial-mesenchymal nodules were formed, and mineralising areas observed within aggregations of cells. These cells were described as “odontoblast-like”. It was suggested that cultured cells were able to maintain the ability to reorganise into odontogenic tissues *in vitro* (Couble et al 2000).

A variety of methods have been used to study isolated cells from dental pulp/papilla mesenchyme in the absence of epithelial components (Couble et al 2000). Dental pulp cell cultures have consisted of clonal cell lines (Kasugai et al. 1988; Kawase et al. 1995) and primary cultures of cells isolated from teeth (About et al 2000a; Nakashima 1991; Young et al 1995). These cells retain various odontoblastic phenotypes, and the characteristic nature of the cells is beneficial to the investigation of the differentiation of the odontoblasts. However, to improve clinical therapies, it is necessary to be able to examine dentine formation in a culture system. Cell polarisation and the appearance of a cellular process (the main morphological features of odontoblast terminal differentiation) have not been convincingly reported in human studies (Couble et al 2000).

Attempts have been made to reproduce the differentiation of pulp cells into odontoblast-like cells *in vitro*, so that *in vivo* mechanisms may be better understood. Dental pulp cells have been successfully cultured in bovine and rat models (Nakashima 1991; Yokose et al. 2000). It has been demonstrated that cultured pulp cells from human deciduous tooth germs are able to form mineralisation nodules in culture (Hao et al. 1997). The nodules seemed to be composed of hydroxyapatite, however the characteristics of the cells and the nodules were not examined. About et al set out to design an *in vitro* method for examining the differentiation of human dental pulp cells into odontoblasts, and to examine the nature of any mineralisation nodules produced (About et al 2000a).

About et al (2000) found that the dental pulp cells were observed to proliferate and adhere to the culture dish within 3 days. When the cells were cultured in the presence of beta glycerol phosphate (β GP), regular fiber-like structures started to appear at the explant border after 3 weeks, and were seen to extend towards the peripheral parts. This was followed by the deposition of mineral crystals along and within the fibrous structures. The cultures were maintained for 8 weeks, and the mineralisation front continued to expand during this time. Newly formed nodules were also observed. The cells that were in direct contact with the nodules showed a polarized morphology, similar to that seen *in vivo*. The formation of dentinal tubules was not observed. These nodules were seen to be calcium-positive by Von Kossa staining. In control cultures without β GP no mineralisation was observed, and the morphology of the cells was more fibroblastic. When the cells were subcultured, they again quickly adhered to the culture surface, and developed a spindle-shaped morphology. In some areas the cells developed a multilayered appearance. This study used Fourier transform infrared

spectroscopy to determine the nature of the mineralisation nodules. A great analogy was seen in the mineral composition of the nodules with *in vivo* dentine (About et al 2000a).

Couple et al (2000) described a similar method, in an attempt to develop a simple culture system to examine the mechanisms involved in human dentine formation. The morphology of the cells was observed to be a mixture of fibroblast-shaped and polygonal-shaped cells. After 5-6 weeks in culture, the cells grown in media containing β GP showed more polarized, polygonal cells. Cells around the pulp explant showed an eccentric position of the nucleus, and some had an elongated process. The younger cells at the periphery of the cell outgrowth had a more fibroblastic shape. At the ultrastructural level, the polarized cells displayed well-developed intracytoplasmic organelles, a rudimentary cell process and gap junctions between cells (Couple et al 2000). Bundles of ECM were deposited around the explant after 2-3 weeks in culture, and areas of mineralisation were detected (only in cultures containing β GP). Human dental pulp cells grown in explant cultures were shown to develop odontoblastic features. The presence of β GP in the culture medium was related to an increase in more odontoblast-like cells. β GP is routinely used to induce and/or increase mineralisation of collagenous matrices produced *in vitro* (Boskey et al. 1996; Couple et al 2000). This molecule may have a possible role in pulp cell differentiation *in vitro*, but this remains to be proven (Couple et al 2000).

Alliot-Licht et al (2001) were able to demonstrate the presence of mineralisation without the addition of β GP to the culture medium (Alliot-Licht et al. 2001). However, this study maintained the cells in culture for two months, and demonstrated

the presence of calcium and phosphorous under scanning electron microscopy. The increased culture period may have contributed to the process of mineralisation.

1.5.1 Development of Odontoblast Systems

Although the exact mechanisms are not known, it is accepted that dental pulp mesenchymal cells have the ability to differentiate into odontoblast-like cells (Torneck 1994). Since odontoblasts develop from mesenchymal tissue and produce a collagenous extracellular matrix as do osteoblasts, it is reasonable to speculate that odontoblasts have functions and characteristics similar to those of osteoblasts. For these reasons, culture systems for osteoblasts can be adapted to be suitable for the *in vitro* culture of odontoblast-like cells.

As odontoblasts are relatively difficult to obtain in large quantities, initial studies on techniques and methods are more practically done using osteoblasts. Osteoblasts develop as relatively undifferentiated mesenchymal stem cells carried within the trabeculae of human bone. This bone can be harvested in surgical procedures. Under culture conditions that provide ascorbate and phosphate, the cells differentiate into well characterised osteoblasts (Slater et al. 1994a; Slater et al. 1994b). The cells grow well even in the presence of relatively high concentrations of antibiotics (Slater et al 1994a). When the cells are cultured on a cell-adherent substrate, they produce a well-organised collagen matrix into which hydroxyapatite is deposited (Slater et al 1994a; Slater et al 1994b). The processes of cell proliferation, extracellular matrix deposition and subsequent mineralisation are markedly enhanced by the provision of growth factors, including mixed growth factor preparations derived from platelet preparations (Slater & Mason 1995).

I.5.2 Harvesting Odontoblasts

Odontoblasts have proved problematic to obtain in large quantities. Some authors have stated that odontoblasts cannot be cultured by traditional cell culture methods, thus restricting *in vitro* studies (Tjäderhane et al. 1998). This is due to the fact that mature odontoblasts are terminally differentiated cells that are incapable of proliferating. The location of the cells within the tooth root means they are not easily available or attainable. Extracting odontoblasts from dentine disrupts the connection between the cell body and the cell process, resulting in damage to the cell morphology or cell death (Scheiss et al. 1966). Mature odontoblasts harvested from teeth may not possess the functional activities of the mature odontoblasts attached to dentine in a vital tooth.

In view of these obstacles, a number of methods have been described in the literature for obtaining dental pulp and dental papillae mesenchymal cells. Animal studies are common. Cell culture methods have been described for inducing dental papilla mesenchymal cell differentiation into odontoblasts. Unda et al (2000) studied the differentiation process using dental papillae isolated from the first molars of 17-day-old mice (Unda et al 2000). Lower molars from 17-day-old Swiss mouse embryos were dissected and trypsinised to dissociate the epithelial and mesenchymal components. Each dental papilla was embedded in semisolid medium. This method allowed for good growth of cells, and for ultrathin preparations for transmission electron microscopy and histochemistry.

Yokose et al (2000) used dental pulp cells enzymatically isolated from growing rat incisors to establish a cell culture system (Yokose et al 2000). This method was

designed to establish a cell culture system that reflected dentine formation in dental pulp tissue. The mandibles were removed from 5 week old rats and placed into 70% ethanol and then saline. The incisor teeth were extracted, and scored sagittally with a sterile diamond bur flushed with sterile saline. The teeth were then cracked open; the pulp tissues pulled out and cut into several pieces. The tissue fragments were then enzymatically treated with collagenase, trypsin and EDTA.

Guo et al (2000) describe a similar method for isolating mature odontoblasts from rat incisors that would be suitable for study by patch-clamp electrophysiological techniques (Guo et al. 2000). Freshly extracted rat incisors were placed into cold saline and cleaned with sterile gauze. The teeth were clamped and sectioned by a low speed saw whilst under constant saline irrigation. 500µm sections containing pulp tissue were then enzymatically degraded by collagenase and protease.

Even though the cells obtained in these studies show some functional and structural resemblance to mature odontoblasts, they can be considered only as odontoblast-like cells. Tjäderhane et al (1998) suggest that the use of these models is mainly related to differentiation studies of the precursor cells and cannot be directly applied to mature odontoblasts (Tjäderhane et al 1998). In view of this, an organ culture system was developed to study odontoblast function in the rat incisor. Extracted rat incisors were cut longitudinally, the pulp "sac" removed, and the odontoblasts remaining attached to enamel were cultured (Heywood & Appleton 1984).

Although animal studies are essential in developing many techniques, there is an increasing realisation that human samples are also important for odontoblast culture

(Tjäderhane et al 1998). Use of human teeth offers a number of advantages over rodent teeth. Rodent teeth are continuously growing, whereas in human teeth the length of primary dentinogenesis is limited. In addition, there are significant differences between the species, as exhibited by marked difference between the protein composition of dentine (Linde & Goldberg 1993).

Tjäderhane et al (1998) describe a method of obtaining human dental pulps from human third molars. Freshly extracted, intact third molars of young patients (ages from 18 to 25 years) were used so that cultures could be obtained with a minimal history of external irritation and large pulp chambers (Palosaari et al. 2001; Tjäderhane et al 1998). The teeth were stored in sterile PBS, and prepared for culturing by swabbing with 70% alcohol. A horizontal groove 2mm deep was cut around the root surface about 5mm apically from the cemento-enamel junction, using a thin-sectioning machine. The pulp chamber was apparently not breached. The root was dissected from the crown using pliers, and the crown placed in PBS or culture medium. In a sterile laminar flow hood, the pulps were removed from the crowns with forceps, and placed into culture wells. The crowns were embedded in to agarose gel to attempt to culture any remaining odontoblasts on the pulp chamber walls. The cells were cultured for up to 10 days.

About and colleagues developed a simple *in vitro* system for studying the differentiation and mineral production of dental pulp cells (About et al 2000a). This study used immature third molars extracted during "normal treatment" of sixteen year old patients. The teeth were collected immediately after extraction and swabbed with 70% alcohol, washed with sterile PBS and transferred to a sterile laminar flow hood.

The apical portions of the teeth were removed with scalpels and the dental pulps removed with forceps. No specific information was given about how much of the apical portion was removed, or exactly how immature the roots were. The pulps were minced, rinsed with PBS and cultured in dishes (About et al 2000a). Odontoblast-like cells were able to be demonstrated in culture through morphological and molecular criteria.

Couple et al (2000) used a similar method. Human third molars from young patients (aged 14 to 16 years) were obtained, and more accurately described as tooth “germs”, rather than whole teeth (Couple et al 2000). The whole pulp was removed through the apical end of the tooth germ, and the apical portion removed to prevent contamination by periodontal fibroblasts. Each pulp gave rise to 20-30 explants of about 3-4mm² that were grown in culture dishes.

Alliot-Licht et al (2001) also describe the use of young third molar teeth. In this study, unerupted third molar teeth at the three-quarter root development stage were surgically removed. The importance of this stage of root development was emphasised, in order to exclude pulp cells in the process of embryological differentiation into odontoblasts. The teeth were “cracked open” and the coronal pulps removed (Alliot-Licht et al 2001).

Until recently, there were no known reports on obtaining odontoblasts for cell culture from primary teeth. Miura et al (2003) describe the isolation of dental pulp cells from exfoliated primary teeth (Miura et al. 2003). The time frame between exfoliation and tooth collection is not stated, nor are the storage conditions. The remnant pulps were

separated from the crowns, and enzymatically digested by collagenase. These cells were treated as pluripotential stem cells, and were shown to be able to differentiate into odontoblasts *in vivo*. Primary teeth are significantly different from permanent teeth in terms of their developmental processes, tissue structure and function (Miura et al 2003).

The majority of studies use developing tooth buds or immature pulps to obtain and study odontoblasts. Although these studies give invaluable information as to the nature and behaviour of these complex cells, it must be questioned how close these situations are to an *in vivo* situation. The developing tooth bud and root sheath have multiple interacting growth factors that are not present in a mature, functioning tooth. It is important to establish experimental conditions that closely mimic the *in vivo* environment if clinically applicable treatments are to be developed.

1.5.3 Culture Conditions for Odontoblasts

Odontoblasts have been grown under a variety of culture conditions, but most are similar. Gagari et al (2003) describe “differentiating” and “non-differentiating” conditions (Gagari et al. 2003a). “Differentiating” medium is supplemented with 50µg/ml of ascorbic acid and 50mM of βGP. The cells were cultured for 26 days.

Unda et al (2000) supplemented their solid medium with 20% foetal calf serum, ascorbic acid (0.18 mg/ml), L-glutamine (20µM) and kanamycin (0.1mg/ml). The cells were cultured for 6 days (Unda et al 2000).

Yokose et al (2000) placed their cell populations into α -modified minimum essential medium (α -MEM), supplemented with 10% foetal calf serum and antibiotics (100 μ g/ml Penicillin G and 100IU/ml streptomycin). 1mM β GP and ascorbic acid (50 μ g/ml) were added when the cells reached a subconfluent state. The cells were incubated up to day 20 (Yokose et al 2000).

Tjäderhane et al (1998) used osteoblast medium to culture the teeth in their initial studies. This consisted of Dulbecco's modified Eagle's medium (DMEM) mixed with Ham's F12 medium (1:1), and was supplemented with 10% foetal bovine serum (FBS), 1 μ g/ml vitamin K1, 50 μ g/ml vitamin C, 100 IU/ml penicillin G, 100 μ g/ml streptomycin and 0.25 μ g/ml amphotericin B (Tjäderhane et al 1998).

About et al (2000) used modified eagle's medium (MEM) with no antibiotic supplementation. Some cultures were supplemented with 2mM β GP. Trypsinisation was by 0.2% trypsin and 0.02% EDTA (About et al 2000a). Couble et al (2000) used Eagle's basal medium, supplemented with 50 μ g/ml ascorbic acid, 100 IU/ml penicillin and 50 μ g/ml streptomycin. Some cells received 10% foetal calf serum with or without 10mM β GP, and some received 15% foetal calf serum with or without β GP (Couble et al 2000).

I.5.4 Identification of Odontoblasts in Culture

A variety of experimental methods have been derived to obtain and culture odontoblasts. Human dental pulp cell cultures are a heterogenous population of cells, possibly at different stages of differentiation. The majority of methods consist of culturing odontoblasts (or odontoblast-like cells) from dental papillae or dental pulps,

both of which have a mixed population of cells, including odontoblasts, fibroblasts and undifferentiated mesenchymal cells. In view of this, it is essential that there are techniques which enable the putative odontoblasts to be identified. This has been done through immunohistochemistry, electron microscopy, alkaline phosphatase activity and molecular biology.

Miller et al (1976) proposed that the acquisition of alkaline-phosphatase activity may be a requisite for the differentiation and possible specialisation of pulp cells *in vivo*, as the activity was higher in the subodontoblastic layer than in the odontoblastic layer, and highest in the pulp cells just before their differentiation into odontoblasts (Miller et al. 1976). This enzyme can be used as an early marker for odontoblast differentiation in dental pulp cell culture.

Unda et al (2000) used electron microscopy to determine cytodifferentiation and histochemical identification of odontoblast markers to identify differentiated cells (Unda et al 2000). They looked at the ability of the cells to produce an extracellular matrix, indicative of the dentine mineralisation process, by detecting the secretion of type-1 collagen and alkaline-phosphatase activity. The differentiated odontoblasts-like cells exhibited cytological features of functional odontoblasts with matrix vesicle secretion and mineral formation, positive alkaline-phosphatase activity and type-I collagen production. Cells that were cultured under different growth factor conditions and did not differentiate fully were seen to have cell polarisation and long thin cell processes containing matrix vesicle. However, type-1 collagen secretion was not detected and alkaline-phosphatase activity was completely inhibited (Unda et al 2000).

Several studies have shown that alkaline-phosphatase activity is linked to the mineralisation of calcified tissues, such as cartilage, bone and dentine (Anderson & Reynolds 1973; Weiss et al 1989; Whyte 1994). In the developing tooth, the alkaline-phosphatase activity is concomitant with the onset of cell differentiation, at the late bell stage (Woltgens et al 1995). At the ultrastructural level, a strong enzymatic reaction for alkaline-phosphatase has been observed in “matrix vesicles” (Sasaki & Garant 1996). This has been used by a number of investigators to determine cell differentiation, and to measure the effects of various growth factors (Onishi et al 1999). Gagari et al (2003) found that alkaline phosphatase activity was significantly enhanced in human dental pulp cultures that were grown in medium supplemented with ascorbic acid and β GP (Gagari et al 2003a).

In the study by Unda et al (2000), alkaline-phosphatase activity was located along the whole surface of the control dental papillae (Unda et al 2000). It was suggested that this may have been linked to the uptake of nutrients or phosphate from the culture medium, as has been considered previously (Woltgens et al 1995). However, alkaline-phosphatase activity was reduced and restricted in the presence of FGF2 (which was seen to limit the functional differentiation of the odontoblasts) (Unda et al 2000).

Varying effects of TGF- β 1 on alkaline-phosphatase activity have been observed. In cultures of adult bovine dental pulp cells, TGF- β 1 inhibited the enzyme (Toyono et al 1997). In osteosarcoma cell cultures, TGF- β 1 stimulated the activity of enzymes associated with calcification, including alkaline-phosphatase (Bonewald et al. 1990).

Growth factor action depends on the cell type and on the interaction with other factors present.

Histochemistry and immunohistochemistry have been extensively used by investigators to demonstrate the differentiation of dental pulp cell cultures. The presence of non-collagenous proteins found in dentine can be used to identify cell types and differentiation in dental pulp cell cultures. Yokose et al (2000) used the detection of osteocalcin and Dentine Matrix Protein-1 to determine whether nodule formation was associated with dentine formation. Osteocalcin is one of the Gla-proteins related to matrix mineralisation in dentine, and is secreted at the mineralisation front (Linde 1985). The amount of osteocalcin released into the culture medium increased as calcific nodules were formed. This data corresponded to the level of mRNA expression (Yokose et al 2000).

About et al used immunohistochemistry to demonstrate strong and uniform type I collagen reactivity in all their β GP treated cells and in the mineralisation nodules (About et al 2000b). Osteonectin was expressed in the same cells and nodules, and was seen to be higher in the cells contacting the nodules. Nestin was observed to be expressed in the dental pulp cells, and was also more intense in the cells contacting the nodules. Nestin has been seen to be expressed in the developing teeth of rats (Terling et al. 1995), and in developing human teeth. In mature odontoblasts nestin expression is down regulated, but dental injury up-regulates its expression (About et al 2000b). The expression of these molecules showed that cultured human dental pulp cells are able to differentiate into odontoblast-like cells.

Couple et al (2000) showed strong staining for type I collagen in all cells and ECM cultured in the presence of β GP (Couple et al 2000).

The use of molecular biology can allow for the detection of specific proteins. Specific proteins can be detected in dental pulp cell cultures by demonstrating the presence of mRNA. Osteocalcin mRNA has been shown to be expressed in fully mature odontoblasts at a high level during dentinogenesis (Bidder et al. 1998). Yokose et al (2000) used Northern blot analysis to demonstrate osteocalcin expression, and Reverse Transcriptase-PCR to detect DMP-1 mRNA expression in the dental pulp cells (Yokose et al 2000).

In situ hybridisation techniques can be used to demonstrate the presence of small amounts of protein. This technique has been used to demonstrate that DMP-1 was expressed in odontoblasts, ameloblasts, cementoblasts and osteoblasts (MacDougall et al. 1998). This study noted that DMP-1 was expressed not only in dentine, but also in other mineralising tissues. Couple et al (2000) made use of in situ hybridisation to reveal a high and continuous expression of the α 1(1) collagen gene in all polarised cells and around the explants cultured with β GP. The DSPP gene was weakly expressed in the cultured cells before matrix mineralisation, and more strongly expressed by cells localised in the mineralising areas (Couple et al 2000). The DSPP gene encodes for the two dentine-specific proteins, DSP and DPP (Couple et al 2000). Guo et al (2000) considered BSP to be a suitable marker for mineralized tissues in general because of its relative specificity for these tissues, but also looked at the expression of DSP, osteocalcin and type I collagen by in situ hybridization to characterize the odontoblastic phenotype of isolated cells (Guo et al 2000).

I.6 Stem Cells

Stem cell research has recently been attracting much attention in the popular press. Postnatal stem cells have been isolated from a wide variety of tissues, including bone marrow, brain, skin, skeletal muscle and dental pulp (Gronthos et al. 2000; Miura et al 2003). It has been noted that postnatal stem cells may be used to give rise to tissues that are quite different to the original source of the cells. For example, neural stem cells may contribute to blood and skeletal muscle. The full developmental potential of postnatal stem cells is not yet fully understood (Miura et al 2003).

Human dental pulp stem cells (DPSCs) have been isolated from human adult dental pulp (Gronthos et al 2000). When transplanted into mice, the DPSCs generated a dentine-like structure that was lined with odontoblast-like cells surrounding a pulp-like tissue. The dental pulps were obtained from extracted human third molar teeth, and enzymatically digested by collagenase. When examined by immunohistochemistry, the DPSCs expressed a variety of markers associated with endothelium, smooth muscle, bone (alkaline phosphatase, type I collagen, osteonectin, osteopontin and osteocalcin) and fibroblasts (type III collagen and FGF2). BSP was not detected, nor were the dentine specific proteins DSP and DPP. This indicates that the initial DPSC culture represents an undifferentiated phenotype (Gronthos et al 2000).

In long term cultures of 5-6 weeks supplemented with ascorbic acid and dexamethasone, the DPSCs formed condensed nodules with high calcium levels. When the cells were transplanted into mice, a dentine/pulp-like structure was generated. These transplants were seen to express transcripts for BSP, osteocalcin and DSP/DPP when examined by reverse transcription-PCR and in situ hybridisation (Gronthos et al 2000).

Miura et al (2003) emphasise the importance of accessing resources that may provide a source of high-quality human postnatal stem cells. They describe how a distinctive population of multipotent stem cells was isolated from the remnant pulp of human exfoliated primary teeth. The stem cells were termed SHED (stem cells from human exfoliated deciduous teeth) (Miura et al 2003). These cells were seen to be able to differentiate into neural cells, adipocytes and odontoblasts. After *in vivo* transplantation, they were seen to induce bone formation, dentine formation and express neural markers in the brains of mice. The cells were considered to be a population of highly proliferative, clonogenic cells (Miura et al 2003).

When grown in culture, the SHED showed a higher proliferation rate than adult bone marrow stromal cells or adult dental pulp stem cells. They were found to express the cell-surface molecules STRO-1 and CD146 around the blood vessels of the pulp remnant. These are early mesenchymal stem-cell markers. This indicates that SHED may have originated from a perivascular environment (Miura et al 2003).

When SHED were supplemented with ascorbic acid, dexamethasone and inorganic phosphate, calcium accumulation was noted. Dentine matrix proteins BSP, DSP and

DPP were noted to be up-regulated. When BMP-4 was added to the culture, osteocalcin expression was noted. These results indicate that SHED have the ability to differentiate into functional odontoblasts *in vitro* (Miura et al 2003). When transplanted into mice, the SHED yielded odontoblasts that were directly associated with a dentine-like structure that was immunoreactive to dentine-specific DSPP antibody. However, they were unable to regenerate a complete dentine-pulp complex.

Miura et al (2003) are extremely optimistic about the future potential of SHED. They consider that primary teeth may provide an ideal resource of stem cells to repair damaged tooth structure, as well as inducing bone regeneration. However, as they conclude, the biological significance of the existence of these cells requires further determination (Miura et al 2003).

Gagari et al presented their findings at a recent conference, although they are not yet published (Gagari et al 2003a; Gagari et al. 2003b). They claim that primary cultures of human dental pulp cells may possess some features of stem cells. However, primary human dental pulp cell cultures are a heterogenous population of cells, possibly at different stages of differentiation. Stem cell factor is a potent hematopoietic growth factor. In long-term cultures of human dental pulp cells, supplementation of the culture medium with ascorbic acid and β GP resulted in higher amounts of stem cell factor been produced. This team of researchers suggest that further investigation of this event may give further insight into the possible "stem cell function" of human dental pulp cell cultures.

It has long been recognised that during development, the differentiation of ameloblasts and odontoblasts is dictated by interactions between epithelial cells of the inner enamel organ and mesenchymal cells of the dental papilla (Linde & Goldberg 1993). It has also become clear that although the mineralised dental tissues do undergo remodelling as a routine process, dentine can respond to noxious threats by producing reparative dentine (Tziafas 1995). It has been thought that progenitor cells are recruited from the dental pulp to become odontoblast-like cells and produce dentine when the original odontoblasts are lost (Smith et al 1995). However, despite increasing amounts of research in this field, the origin and nature of these cells has not been characterised, and usually assumed to be unknown without speculation on what further investigation may be necessary (Tziafas 2004). With ever-increasing knowledge regarding the sources and behaviour of stem cells, the suggestion of Gronthos et al (2000) that the dental pulp may be a source of stem cells seems to be not only reasonable, but increasingly probable (Gronthos et al 2000).

1.7 Dental Caries

Dental caries is one of the most common diseases to affect humans (Robinson et al. 2000). For many years, caries was considered to be a continuous progressive demineralisation of enamel crystals, followed by degradation of dentine, resulting in formation of a cavity. The current concept of the nature and progression of caries recognises that it is a multifactorial process, and emphasises the cyclical nature of the demineralisation-remineralisation chemical reactions that occur on tooth structure. Caries is perceived to result from a prolonged imbalance in the oral cavity such that

factors favouring demineralisation of enamel and dentine overwhelm factors that favour remineralisation and repair of those tissues. Approaching caries as a disease arising from an imbalance places greater emphasis on the enhancement of the natural host protective factors, rather than simply controlling the infection (McIntyre 1998;Rugg-Gunn 2001).

At the most basic level, dental caries is caused by interaction between oral bacteria, their access to fermentable carbohydrates and a vulnerable tooth surface (Wilding & Solomon 1996). A high level of acid concentration and a high frequency of contact will over time lead to demineralisation of the tooth surface. In most cases, natural protective factors and repair mechanisms can be enhanced to control the problem. Normal salivary flow, good oral hygiene and fluoride exposure are the most significant components contributing to this balance (McIntyre 1998).

The mineral component of enamel and dentine is hydroxyapatite, $[\text{Ca}_{10}(\text{OH})_2(\text{PO}_4)_6]$ (Robinson et al 2000). After eruption the tooth minerals are subject to interactions with the oral environment, Saliva and plaque possess components that can be beneficial or harmful to the tooth. In a neutral environment, hydroxyapatite is in equilibrium with the local aqueous environment. Under normal conditions, saliva is supersaturated with calcium and phosphate ions, but this does not result in any significant mineral deposition into sound, plaque-free surfaces. When fermentable carbohydrates are eaten, bacteria present in the dental plaque form acid and the pH drops. The solubility of the enamel mineral is pH-dependent, and at a low pH, more calcium and phosphate are needed in the surrounding fluids to maintain a supersaturated condition. Below a pH of 5.5 (also known as the “critical pH”), the

mineral becomes undersaturated and begins to dissolve. The dissolution of the outer layer of enamel releases hydroxy and phosphate groups, and these molecules, combined with the continual washing of the surface by saliva drive the pH to return to neutral. After the pH crosses the point for saturation, mineral starts to reprecipitate back on to the remaining apatite crystals (McIntyre 1998;ten Cate & van Loveren 1999).

The chemical composition of this reformed mineral depends on what is available in the surrounding fluid. This can result in a mineral that has a different composition and possibly a lower solubility. If carbonate is washed out of the structure and fluoride is incorporated, this first cycle of dissolution and reprecipitation leads to a mineral that is more resistant to acid dissolution. The critical pH is then slightly lowered (Larsen & Pearce 1997). Fluoride also stimulates and enhances the remineralisation by affecting the kinetics of precipitation, driving precipitation of mineral back in to the tooth surface (Shellis & Duckworth 1994).

The chemical basis of the demineralisation-remineralisation process is similar for enamel and dentine. However, the different structures and relative quantity of the mineral and organic tissue content of each of these materials causes significant differences in the nature and progress of the carious lesion. Enamel caries is clearly a dynamic process, but dentinal caries is a vital process, involving a tissues that is able to defend itself (Wilding & Solomon 1996).

The initial enamel lesion develops when the pH level at the tooth surface exceeds that which can be counterbalanced by remineralisation, but is not low enough to inhibit

surface remineralisation. This lesion is reversible. If the demineralisation-remineralisation imbalance continues, the surface of the incipient lesion collapses through dissolution of apatite or fracture of the weakened crystals, resulting in cavitation. Plaque can now be retained within the depths of the cavity, and so the remineralisation process is more difficult and less effective. The dentine-pulp complex may start to be involved at this stage (McIntyre 1998).

As the lesion progresses, bacteria invade dentine and the process continues to be driven by dietary substrate. Cariogenic bacteria produce acid to dissolve the hydroxyapatite of deeper dentine, so there is a front of demineralisation in advance of the bacterial invasion. Although there may be some bacteria in or even beyond the area of demineralisation, they are not clinically relevant. If the lesion is left to extend through the dentine the enamel will become progressively undermined and weakened. Collapse of the unsupported enamel may eventually result in a wide open cavity that is relatively self-cleansing and less plaque retentive. The caries process may then slow down, leading to the development of a hard leathery floor on the cavity which is more or less inactive. If the caries is rapidly progressing the pulp is at risk of irreversible damage because the remineralizing and sclerosing process which normally seals the tubules will be unable to keep pace with the rate of advancement of the lesion (McIntyre 1998; Soames & Southam 1993).

I.7.1 Social Impact of Dental Caries

Despite a significant decline in caries experience over the last few decades, the dental health of children, the aged and people in developing countries as well as lower socio-economic groups in developed countries remains a major concern. The impact of this

issue can be seen by considering the dental health of children. In Australia, 45% of 5 and 12 year old children have dental caries. Furthermore, 40% of all decayed teeth in young children are untreated (Slade et al. 1996). Untreated dental decay may result in pain and infection, and can lead to serious medical complications such as facial cellulitis. Subsequent hospital admission, intra-venous antibiotics and extraction of carious teeth under general anaesthetic with associated economic, social and psychological costs are common sequelae (Alcaino et al. 2000; Tennant et al. 2000). In addition, the longevity of restorations in primary teeth is highly variable (Kilpatrick 1993).

I.7.2 Management of Dental Caries

For over 100 years the management of dental caries has been essentially surgical, involving the removal of all carious tissue as well as the destruction of sound tooth tissue. This surgical approach was based on an inadequate understanding of the carious process. Dental restorations ultimately fail for a number of reasons. These include the inherent weakness of the residual tooth structure once cavity preparation is complete, a lack of biocompatibility between the tooth tissue and the restorative material, and iatrogenic damage to the pulpal tissues (Mount & Hume 1998; Pitts 2004). Minimal removal of tooth tissue during cavity preparation is therefore advantageous. However carious dentine is not only heavily infected but also weak. Failure to address either the bacterial infection or the inadequate mechanical support will inevitably lead to failure of any superimposed restorative material. Hence the focus of cariology and restorative research is leading towards devising methods of treating (as opposed to removing) carious dentine. If such a method could be

developed that both disinfects and encourages mineralisation, the long term survival of the restored tooth would be improved.

As previously discussed, demineralisation and remineralisation of enamel and dentine is an everyday natural occurrence in the oral cavity. However, the enhanced dietary intake of acidic beverages and sugar-laden food coupled with limited oral hygiene predisposes teeth to demineralisation and the onset of caries development. This situation is exacerbated in isolated communities or regions or in developing countries by the absence of water fluoridation and minimal oral health care facilities.

In Australia there have been two major developments in the last decade where it has been proposed that remineralisation of tooth enamel can be achieved using calcium phosphate solutions. Reynolds (1997) has pioneered the use of casein phosphopeptides (CPP) as a rich source of calcium and phosphate ions (Reynolds 1997). When incorporated into saliva or plaque in proximity to the tooth surface, these ions promote enhanced enamel remineralisation. A recent clinical trial has shown the anti-caries efficacy of these approaches (Hay & Thomson 2001). However these developments have minimal documentation of the property changes of the treated enamel and dentine.

As the treatment of dental caries has moved towards a more conservative, or minimal intervention approach, the use of material such as glass ionomer cements has become a subject of discussion, particularly for use in children's teeth. Glass Ionomer Cements contain a fluoroaluminosilicate ion-leachable glass, and release fluoride from their set structure (Combe et al. 1999). Many investigators have demonstrated

the ability of glass ionomer to increase the fluoride content in enamel and dentine adjacent to restorations. This uptake of fluoride would theoretically increase resistance to acid demineralisation and prevent caries formation around restorations (Dunne et al. 1996; Tyas 1991), although not all authors have found this. Fluoride release from glass ionomers has also been shown to have an anti-microbial effect against *Streptococcus mutans* in plaque (Svanberg et al. 1990).

It is generally believed that long-term fluoride release from glass ionomer leads to a local cariostatic environment, however the effectiveness of this in preventing secondary caries has been questioned (Qvist et al. 1997). Although a recent *in vitro* study clearly demonstrates the remineralizing effect of glass ionomer on adjacent carious lesions (Jang et al. 2001), these effects are not so apparent in clinical trials: Comparative studies of amalgam and glass ionomer cement in class II lesions in primary teeth have shown low frequencies of secondary caries, and no significant differences between the cariostatic ability of the two materials (Qvist et al. 1997; Welbury et al. 1991). It has been suggested that this may be because caries is related to each patient's caries activity, and as such several factors play a role in explaining the discrepancy between expectations and results. The presence of fluoride, although beneficial, cannot overcome poor plaque control and repeated carbohydrate challenges (Rugg-Gunn 2001; Scott & Mahoney 2003).

The carious process can ultimately cause damage to the dental pulp, by direct bacterial invasion, or the through bacterial products and toxins. Vital pulp therapy aims to treat reversible pulpal injury and maintain pulp vitality and function. A successful outcome for vital pulp therapy depends on a number of factors, including

the type and location of pulpal injury, the age of the tooth, the treatment modality and the integrity of the final restoration (Hume & Mount 1998). The successful outcome of restorative treatments is ultimately dependent upon the natural repair responses of the dental pulp cells (About et al 2001).

The available strategies to treat caries have not been shown to have a significant effect on carious dentine. It is well-known that odontoblasts are capable of responding to a carious lesion with proliferation and matrix synthesis (About et al 2000b). There is considerable potential to enhance this innate odontoblastic response. If this can be coupled with chemically and biologically enhanced remineralisation, it may be possible to allow for atraumatic repair of carious lesions.

I.8 Aims

In order to develop improved methods of treatment for dental caries, the mechanisms of odontoblast differentiation and response to injury must be more closely examined. The understanding of these mechanisms is fundamental to the development of treatments for damaged or diseased vital teeth. A close review of the current literature as outlined above has revealed a number of areas that need further investigation and clarification.

The odontoblasts that have been studied in culture systems so far have been obtained either from animals or immature human teeth. There are limited studies on human odontoblasts. The effect of growth factors on odontoblasts and the dental pulp has been the subject of investigation, but there is no clarity in relation to their effect.

There has been little discussion on the establishment of optimal culture conditions for odontoblasts.

There is a need for the development of a system for obtaining human odontoblast from readily available material through an efficient and practical technique. Culture conditions then need to be established, so that optimal methods for stimulating dental remineralisation may be studied.

Hence the aims of this thesis are:

- 1. To optimise cell culture techniques using mineralising cells (osteoblasts initially)**
- 2. To establish methods for obtaining dental pulps from primary teeth**
- 3. To optimise cell culture conditions for the development of odontoblast-like cells from human dental pulp explants**
- 4. To characterise the odontoblast-like cells from human dental pulp explants**
- 5. To examine the effects of growth factors on the odontoblast-like cells**

Chapter II. Materials and Methods

II.1 Materials

II.1.1 Cell Culture Materials

Dulbecco's modified Eagles' medium (DMEM) with calcium, DMEM without calcium and phosphate buffered saline (PBS) were purchased from Trace Scientific Ltd. (Sydney, Australia). FBS was purchased from Commonwealth Serum Laboratories (CSL) Limited (Parkville, VIC, Australia). L-ascorbic acid phosphate (phosphoascorbate), 1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃), B-glycerophosphate, dexamethasone, Dimethylsulfoxide (DMSO), ethylene diamine tetraacetic acid (EDTA), L-glutamine, D-glucose, Insulin-like growth factor-1 (IGF-1), NaHCO₃, Penicillin G, Streptomycin sulphate, Transforming Growth Factor-β (TGF-β) and trypsin were acquired from Sigma (St Louis, MO, USA), along with all other chemicals unless otherwise stated. Didronel[®] tablets containing 200mg of Etidronate disodium were obtained from Norwich Eaton.

Plastic cell culture flasks, multi-well plates and glass coverslips were obtained from Nunc Inter Med (Roskilde, Denmark) and Becton-Dickson Labware (Franklin Lakes, NJ, USA). Sterile ampoules for the freezing and storage of cells were obtained from Nunc Inter Med (Roskilde, Denmark). Thermanox[®] coverslips were obtained from Nunc Inc. (Illinois, USA). Gelfoam was obtained from Pharmacia & Upjohn Pty Ltd. (Sydney, Australia).

II.1.2 General Reagents

Trichloroacetic acid (TCA) and absolute ethanol were obtained from Ajax Chemicals (Sydney, Australia). Alizarin red S, cetylpyridinium chloride and alkaline phosphatase reagents were purchased from Sigma (St Louis, MO, USA). Betadine antiseptic solution was purchased from Livingstone (Sydney, Australia). Glutaraldehyde was obtained from Alltech Associates Australia (Sydney, Australia).

II.1.3 Histological and Immunological Reagents

The osteonectin (polyclonal) antibody for immunohistochemical staining was obtained from Calbiochem® (San Diego, CA, USA). The DAKO LSAB Plus kit was purchased from DAKO (Glostrup, Denmark). Access to equipment and assistance for immunohistochemical procedures was provided by Dr Michael Slater, Department of Anatomy and Histology, University of Sydney.

Histological sample preparation and staining was carried out by Dr Jane Radford, Department of Pathology at the University of Sydney.

II.1.4 Radiochemicals and Scintillant

The radiochemical [^3H]-tetracycline (Tritium [^3H]; 0.5-1.0Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA, USA). Ultima Gold liquid scintillation cocktail was purchased from Packard Instruments Inc. (USA).

II.1.5 Miscellaneous Materials and Equipment

The water-jacketed incubator with controlled CO₂ environment was purchased from Forma Scientific (Sydney, Australia), and the biohazard laminar flow hood from Gelman Sciences Pty Ltd (Sydney, Australia).

The electronic balance (model FX-320) was purchased from A&D Co. Ltd (Tokyo, Japan). The combined red rod pH electrodes and the portable pH meter (model PHM80) were acquired from Radiometer Copenhagen (Paris, France). The NUOVA II stir plate and the Titertek plate shaker were obtained from Flow Laboratories (Sydney, Australia). The centrifuge was purchased from Hettich Zentrifugen (Tuttlngen, Germany), and the Vortex mixer from the Thermolyne Corporation (Dubuque, Iowa, USA). Cells were counted with a haemocytometer (Fuchs Rosenthall model) obtained from Weber Scientific International Ltd. (Sussex, England).

The inverted phase-contrast microscope was obtained from Olympus Optical Co Ltd (Tokyo, Japan). The photomicrographs on this microscope were taken with an Olympus SC-35 SLP photomicrographic camera. A Zeiss KS400 microscope purchased from Carl Zeiss Vision (Munich, Germany) and a Sony Progressive colour CCD camera (Tokyo, Japan) were used in image recording and analysis, and were located in the Electron Microscope Unit (The University of Sydney). An Olympus 4040 zoom digital camera (Tokyo, Japan) was used for photographic records of methods.

The liquid scintillation counter (Model 1900CA) was purchased from Packard Instruments Inc. (USA). The spectrometer (Polarstar galaxy) was obtained from BMG Labtechnologies (Australia).

All dental instruments and drills were kindly supplied on loan by the Westmead Centre for Oral Health (Westmead, Australia).

II.1.6 Composition of Culture Media

Osteoblast Growth Medium

Osteoblasts were cultured in DMEM with calcium, supplemented with ascorbic acid (0.044g/L l-ascorbic acid), l-glutamine (0.292g/L), NaHCO_3 (3.5g/L), and 10% v/v FBS, pH 7.0.

Fibroblast Growth Medium (FGM)

Fibroblasts were cultured in DMEM with calcium, 0.293g/L L-glutamine, 3.7 g/L NaHCO_3 , 0.03g/L penicillin, 0.05g/L streptomycin and 5% v/v FBS, pH 7.4.

Dental Pulp Cell Growth Medium

Dental pulp cells were cultured in basic osteoblast culture medium: DMEM with calcium supplemented with ascorbic acid (0.044g/L l-ascorbic acid), l-glutamine (0.292g/L) and NaHCO_3 (3.5g/L), and 10% v/v FBS. Streptomycin (50 $\mu\text{g/ml}$) and penicillin (100 IU/ml) were added to the basic medium.

II.1.7 Other Solutions

0.25% Trypsin: 0.3525g/L NaHCO₃, 2.5 g/L trypsin.

Dental pulp transport medium: DMEM with calcium, supplemented with ascorbic acid (0.044g/L l-ascorbic acid), l-glutamine (0.292g/L), NaHCO₃ (3.5g/L), streptomycin (50µg/ml) and penicillin (100 IU/ml)

II.2 Osteoblast Studies

II.2.1 Source

Established laboratory cell lines of human osteoblasts (SV40) were used, initially obtained from surgically derived human trabecular bone, as has been described previously (Slater et al 1994b). Cells had been stored under liquid nitrogen. When required for use, cells were thawed and transferred directly into a 75cm² culture flask containing the osteoblast growth medium. The medium was discarded and replaced on the following day to remove dimethyl sulfoxide.

II.2.2 Culture Conditions

All cultures were maintained in a water jacketed, humidified incubator with a 5% (v/v) carbon dioxide atmosphere mixed in air at 37°C. All cell culture materials and equipment were sterile and all cell culture procedures were performed aseptically to minimise bacterial, fungal and mycoplasmal contamination. Cell cultures were monitored for contaminations by visual examination with an inverted phase contrast

microscope. Cultures found to be contaminated with micro-organisms were immediately discarded.

Standard osteoblast growth medium was used. The culture medium was changed every second day. When cells were confluent, cultures were subcultured via trypsinisation. Osteoblasts were detached with 5ml of 0.25% trypsin and incubated at 37°C for 6 minutes. The resulting cell suspension was neutralised with growth medium containing FBS and centrifuged at 2000 rpm for 5 minutes to obtain a cell pellet. The cells were resuspended in osteoblast growth medium and counted using a haemocytometer. Cell suspensions were subsequently diluted in plating medium to the concentrations required for the respective study.

II.2.3 Basic Cell Culture and Examination of Growth of Cells in a Matrix

Gelfoam was used to mimic a biological three dimensional matrix for mineralising cells. It is a sterile, absorbable gelatin sponge used as a haemostatic agent in surgical procedures. Small pieces (approximately 8mm²) of Gelfoam were placed onto Thermanox coverslips in 24-well cell culture plates.

Cells were trypsinised and seeded onto the Gelfoam samples at 50×10^3 cells/well. 5mM β GP was added to the culture medium to encourage mineralisation. Media was changed every second day. At 7 days after plating when cells were confluent treatments were added for a further 10 days. Treatments consisted of an ethanol vehicle and 1,25(OH)₂D₃ at 1×10^9 concentration.

II.2.3.1 Histology

Two Gelfoam samples (one of each treatment) were prepared for histology. Samples were fixed in 10% neutral buffered formalin then stored in cold 70% ethanol. Samples were not decalcified, and were embedded for paraffin sectioning at a thickness of 10 μ m. Prepared sections were stained with hematoxylin and eosin (H&E).

II.2.4 Influences on Mineralisation

Cells were plated onto Thermanox coverslips in 96 well cell culture plates at 20 x 10³ cells per well. 5mM β GP was added to the culture medium to encourage mineralisation. Media was changed every second day. At 7 days after plating when cells were confluent treatments were added for a further 10 days. Treatments consisted of an ethanol vehicle, 1,25(OH)₂D₃ at 1 x 10⁹ concentration, and dexamethasone at concentration of 1 x 10⁷. Etidronate Disodium (EHDP) is a sequestering and chelating agent that inhibits the crystal growth of hydroxyapatite that was added as an inhibitory treatment.

II.2.4.1 Effect of Calcium and β GP in Media on Mineralisation.

Cells were plated onto Thermanox coverslips in 96 well cell culture plates at 20 x 10³ cells per well. Media was changed every second day. Treatments were added at the time of plating. Treatments consisted of osteoblast growth medium, osteoblast growth

medium without calcium and osteoblast growth medium with 5mM β GP. Cells were examined for evidence of mineralisation at 14 days and 21 days of treatment.

II.3 Mineralisation

Measurement of the rate of mineralisation in human bone cell cultures was carried out by Alizarin Red staining and by determining the uptake of the radiolabelled tracer [^{3} H]-tetracycline.

II.3.1 Alizarin Red Assay

The mineral content of cultures was determined by alizarin red S staining following the method of Chang et al (2002).

Cells were fixed in 10% neutral buffered formalin for 10 minutes. Fixative was removed by three 5-minute washes with PBS. Staining was by alizarin red S (2% wt/vol) for 10 minutes. Wells were washed 4 times with distilled water. The staining was eluted by cetylpyridinium chloride (10% wt/vol) for 1 hour with gentle agitation and quantified by A_{562} readings on the spectrometer.

II.3.2 Tetracycline Uptake

Tetracycline is an antibiotic that precipitates at calcifying fronts. (Milch et al. 1957)

The mineral content of cultures was determined by the measurement of tetracycline incorporation following the method of Patava (1996). The uptake of [^{3} H]-tetracycline into cultures of human bone-derived cells was measured by adding 100 000 cpm/well

of tracer for the final 24 hours of the tracer period. [³H]-tetracycline was extracted from the cultures following three 5-minute washes with 4°C PBS and dehydration with three 5-minute washes with absolute ethanol. Extraction was carried out using two 1 hour washes of 500µl of 10% TCA at 4°C. The extracts were pooled and added to 3mls of Ultima Gold liquid scintillation cocktail. The amount of tracer in the extract was measured in a Model 1900CA Packard liquid scintillation counter.

II.4 Odontoblast Studies

II.4.1 Source

Ethical approval for the collection of human teeth was obtained from the Western Sydney Area Health Service Human Research Ethics Committee (approval number HREC2002/9/4.11(1512)). Ethical approval was also obtained from the University of Sydney Human Ethics Committee (reference number 2975, protocol number 02/07/18).

In the department of Paediatric Dentistry at the Westmead Centre for Oral Health, New South Wales, many children present with carious primary teeth that require extraction under general anaesthesia as part of complete dental treatment and rehabilitation (Alcaino et al 2000). Each patient who presented to the department over a 12 month period (October 2002 to October 2003) who required extraction of carious primary teeth as part of their complete dental treatment was invited to take part in the study. Participation was voluntary and subjects were permitted to withdraw their permission without penalty or prejudice. The tooth samples were kept anonymously,

so consent could not be withdrawn once the tooth sample had been provided. Informed consent was obtained from the parent or legal guardian of the patient. A participant information sheet was given to all potential participants and a consent form signed if they agreed to take part in the study.

There are no protocols reported in the literature for obtaining human dental pulps for odontoblast culture from extracted primary teeth. The protocol for collection of teeth and subsequent collection of odontoblasts was based on the work of Couble et al (2000), About et al (2000) and Guo et al (2000) (About et al 2000a;Couble et al 2000;Guo et al 2000). Teeth selected required extraction due to extensive caries, but were judged to have a vital pulp on their clinical appearance. That is, there were no clinical signs of abscess formation or severe discolouration (Fig.II.1). A total of 47 upper incisor teeth were collected.



Figure II.1. Extracted tooth (after been in transport medium).

Note the extensive caries (arrow) and absence of periapical pathology.

II.4.2 Methods for Obtaining Odontoblastic Cells from Available

Material

A number of methods were tested to determine the most effective method of obtaining dental pulps as aseptically as possible. The initial working protocol for the clinical collection for odontoblasts was as follows:

1. Immediately following extraction, tooth is placed into cold (4°C) sterile transport medium. (Figs.II.2 (a), (b))
2. Sample labelled with date and time of extraction and extent of carious lesion (moderate or deep).
3. Transport to laboratory in cold medium.
4. Teeth wiped with Betadine. (Fig.II.3)
5. Sectioning of tooth with laboratory motor.
6. Removal of dental pulp with hand instruments.
7. Dental pulp explants placed into culture flasks in dental pulp cell growth medium. Small amount of media added to cover samples.

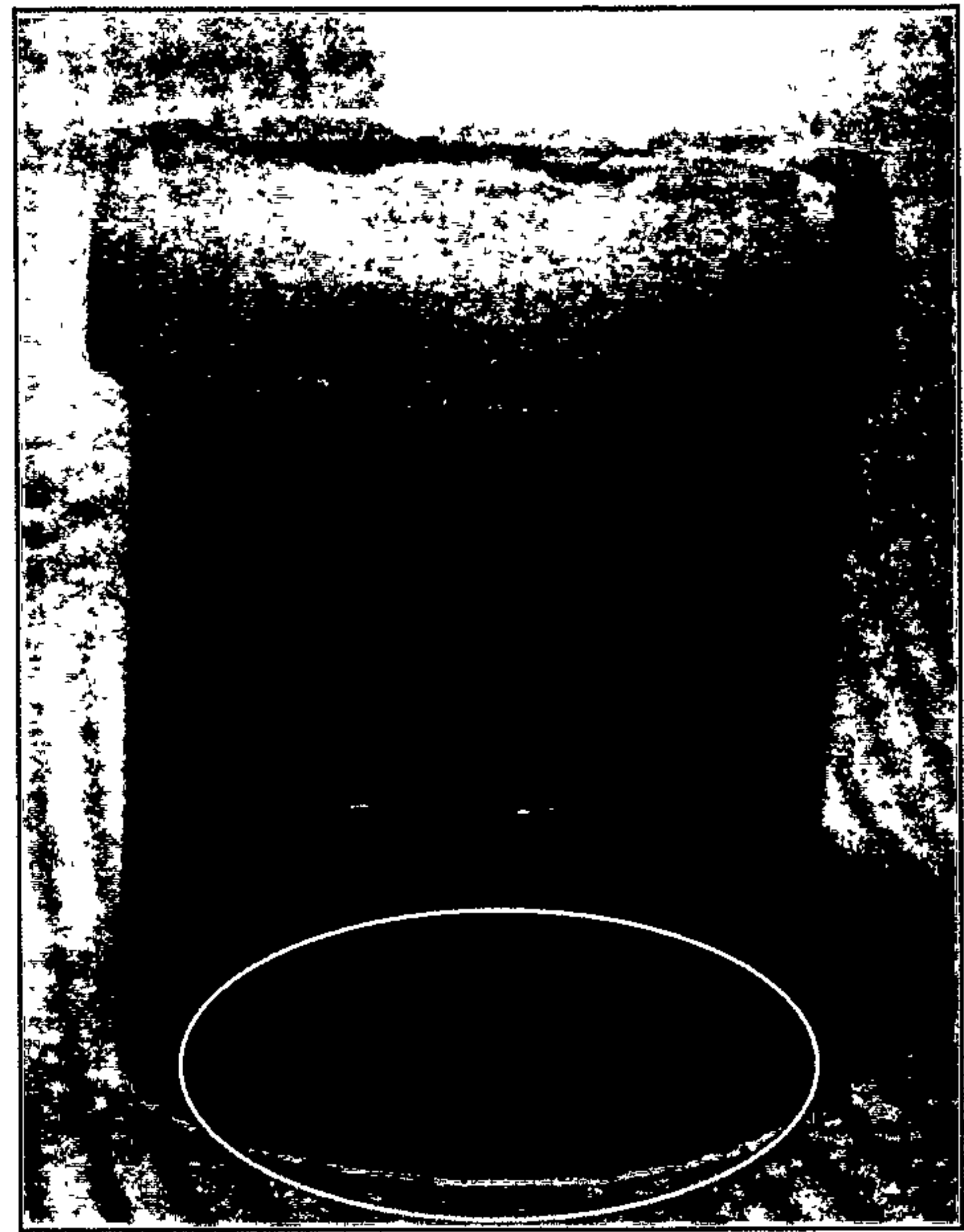
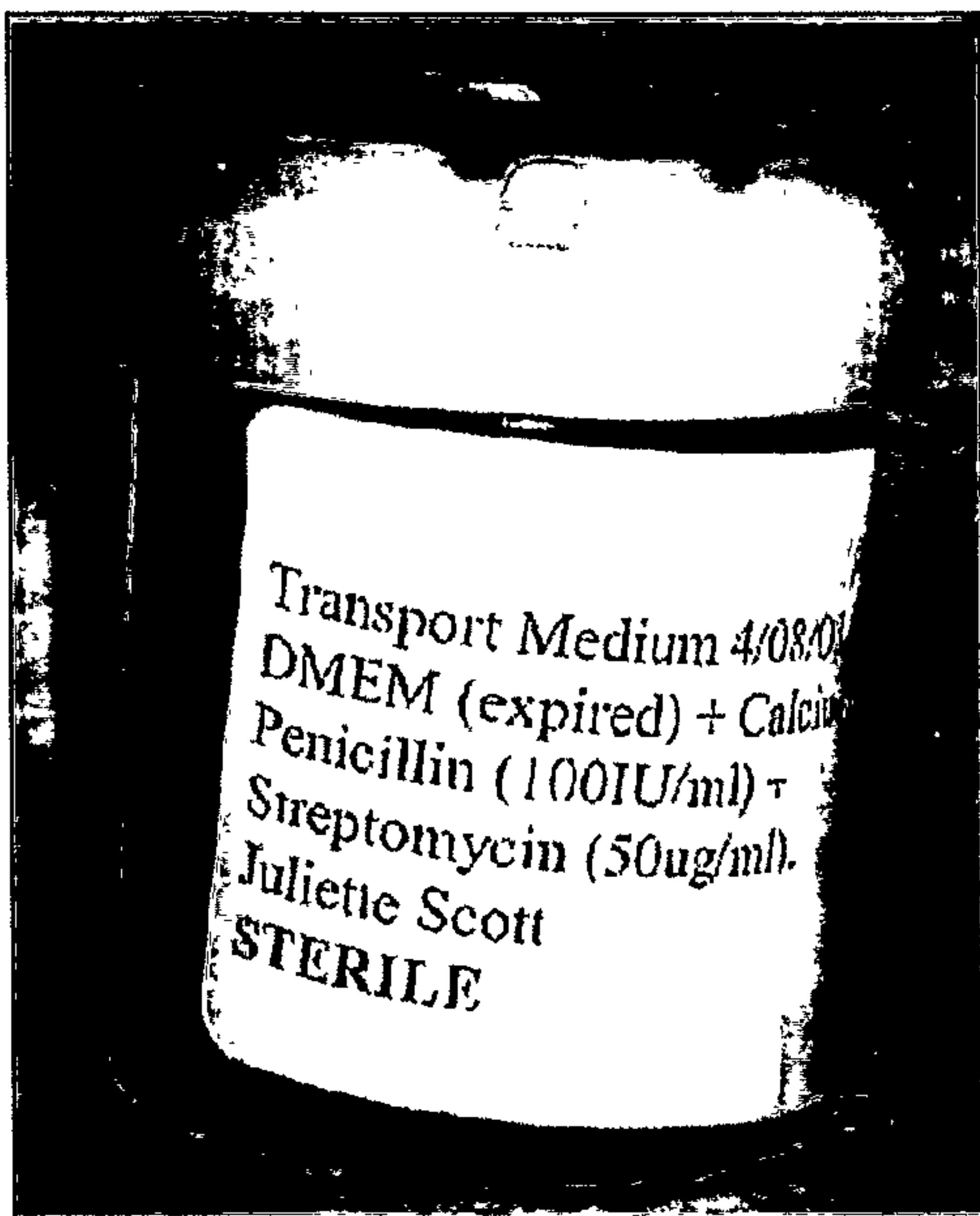


Figure II.2 (a) Transport medium in sterile container

(b) Teeth in transport medium



Figure II.3 Tooth being wiped with Betadine

After the collection of the first samples, the protocol was adapted to be:

1. Immediately following extraction, tooth is placed into cold (4°C) sterile transport medium.
2. Sample labelled.

3. Teeth wiped with Betadine.
4. Groove cut into root surface with high-speed dental drill and diamond bur, cooled with sterile saline. Groove reaches into pulp chamber.
5. Transport to laboratory in cold medium.
6. Sectioning of tooth with surgical hammer and chisel.
7. Dental pulp explants placed into culture flasks in culture medium. Small amount of media added to cover samples.

Following the collection of the second samples, the procedure was further adapted, to what became the final protocol. All materials and equipment are sterile, and the procedure takes place in a clean operating theater environment.

1. Immediately following extraction, the tooth is placed into cold (4°C) sterile transport medium.
2. Sample labelled.
3. Tooth wiped with Betadine to remove all traces of blood and soft tissue.(Fig.II.3)
4. Tooth partially sectioned with high speed drill cooled with sterile saline.(Figs.II.4, II.5)
5. Tooth placed into fresh cold transport medium and transported to laboratory
6. Sectioning of tooth completed with surgical chisel in sterile biohazard hood (Figs.II.6, II.7)
7. Pulp fragments removed with forceps and endodontic files. Inner surface of root thoroughly cleaned with hand instruments. (Figs.II.8, II.9)
8. Apical portion of pulp discarded

9. Dental pulp explants placed into culture flasks in culture medium. Small amount of media added to cover samples

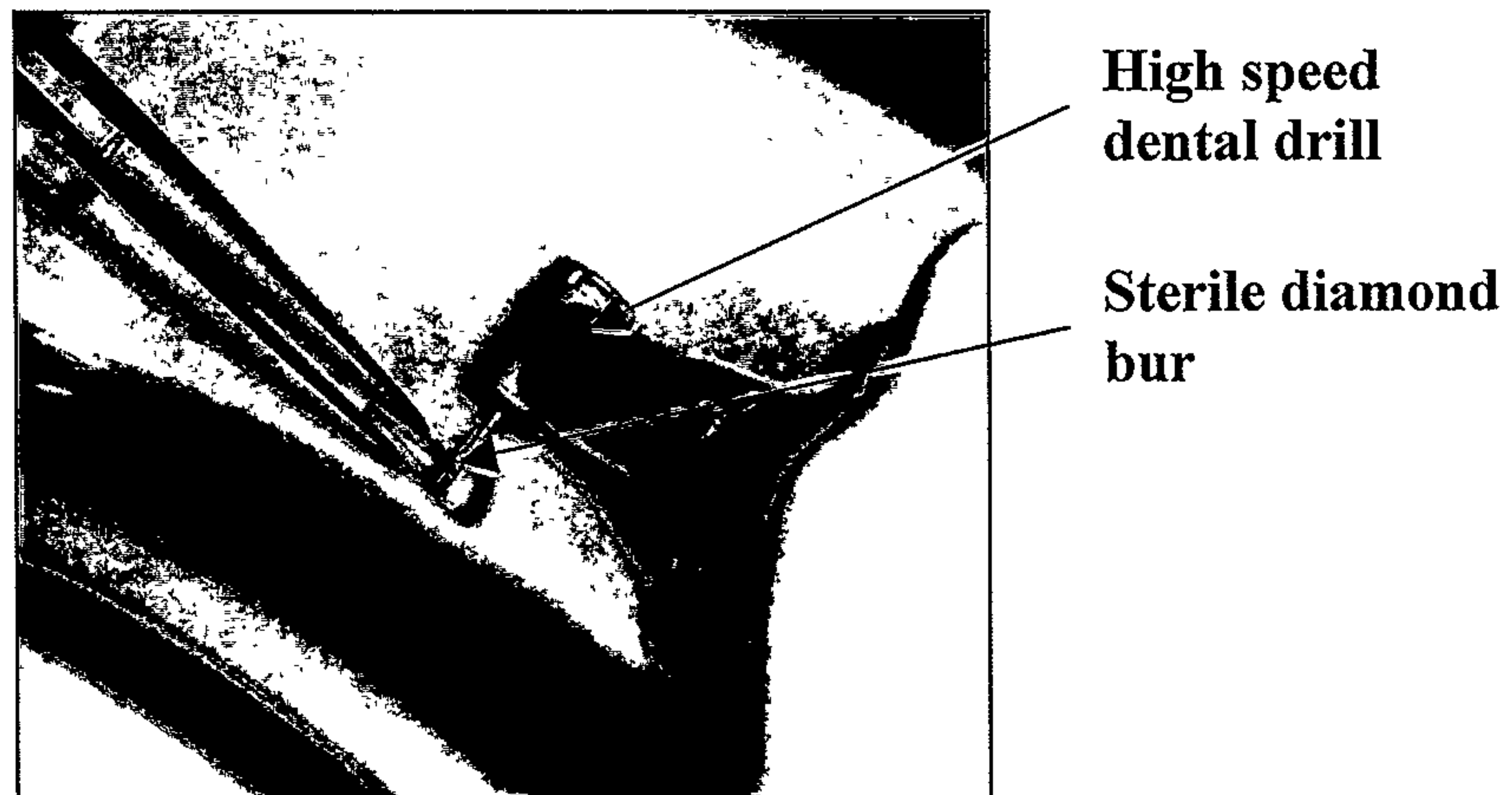


Figure II.4 Preparing to section tooth

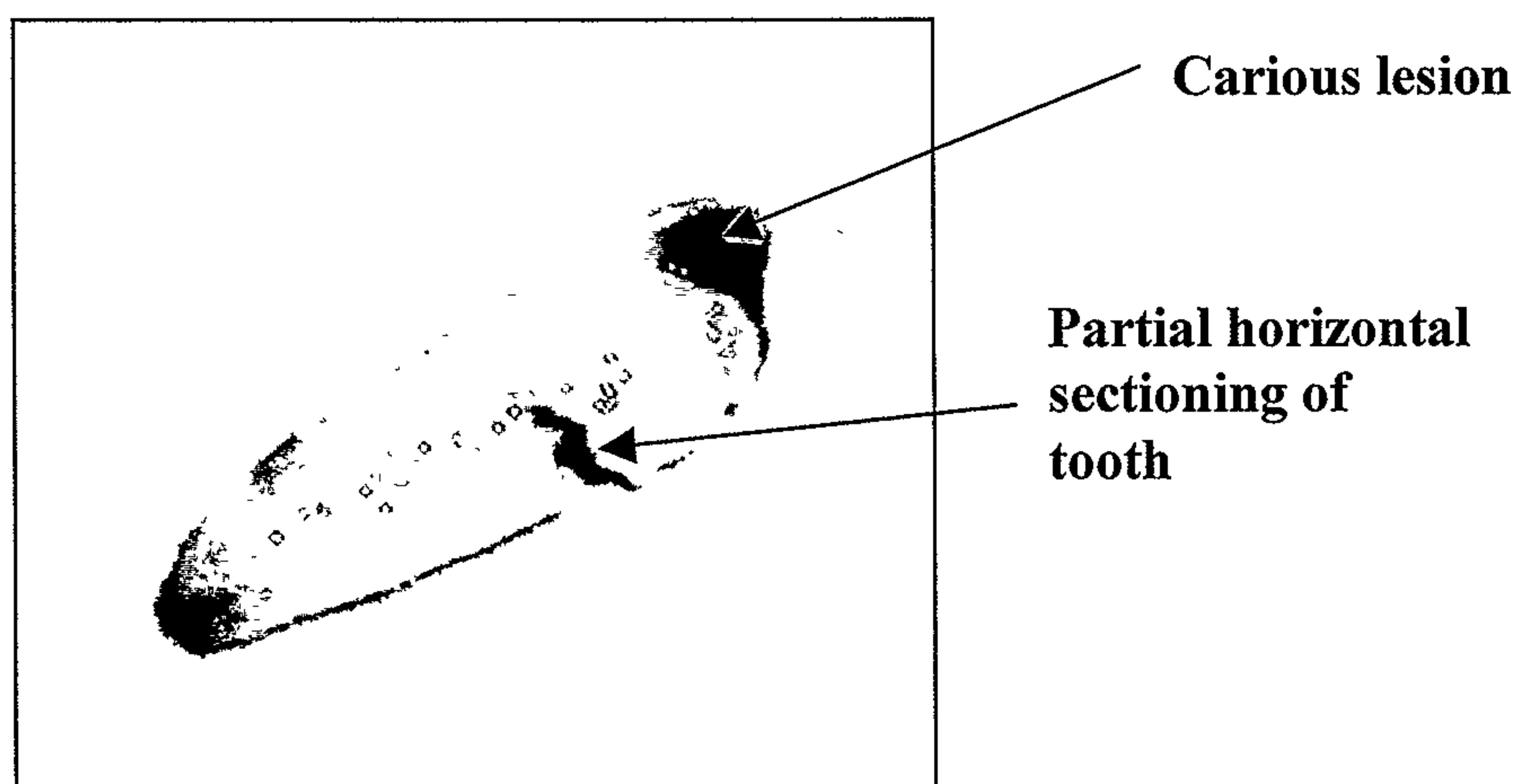


Figure II.5 Tooth partially sectioned



Figure II.6 Splitting tooth with chisel in sterile petri dish

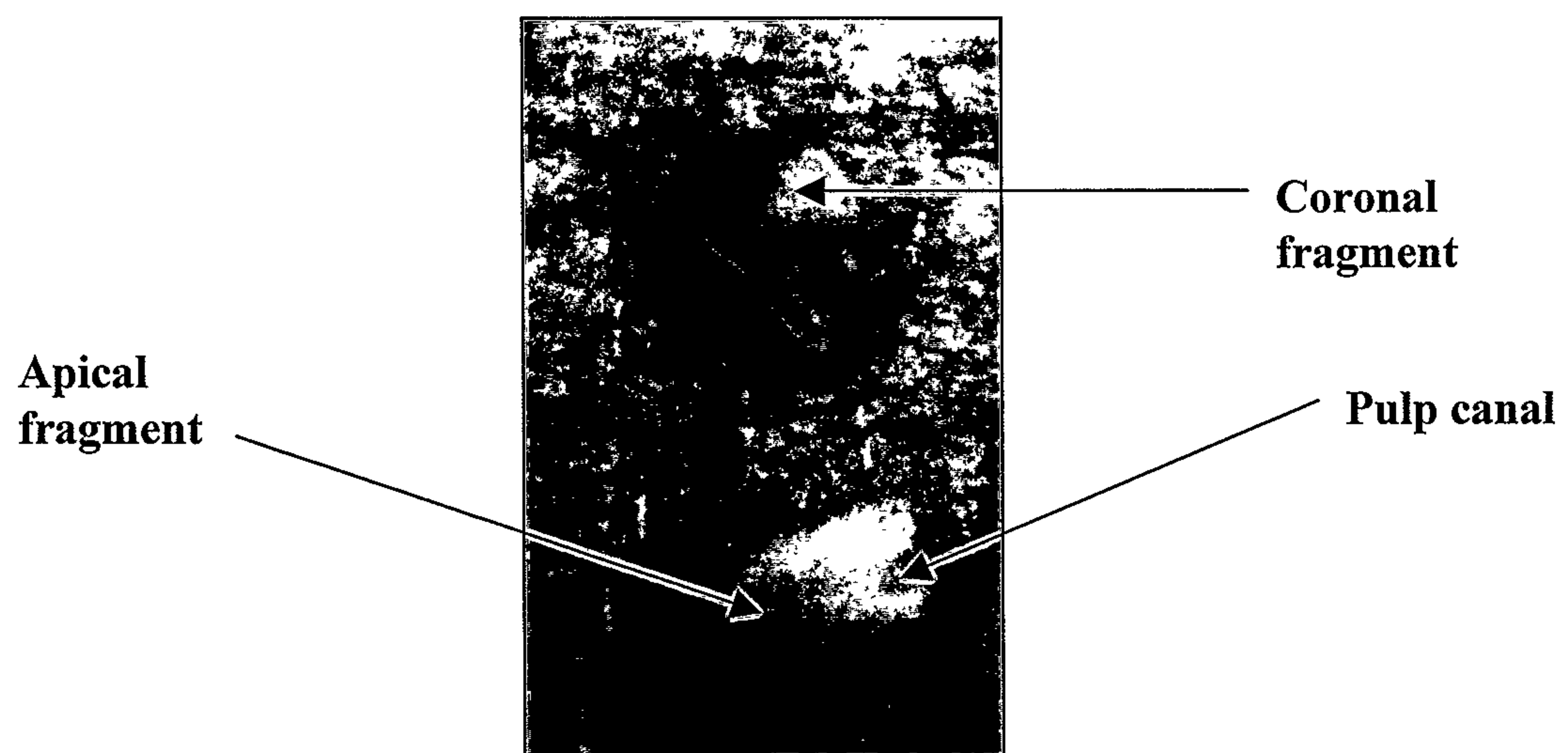


Figure II.7 Tooth split in two pieces

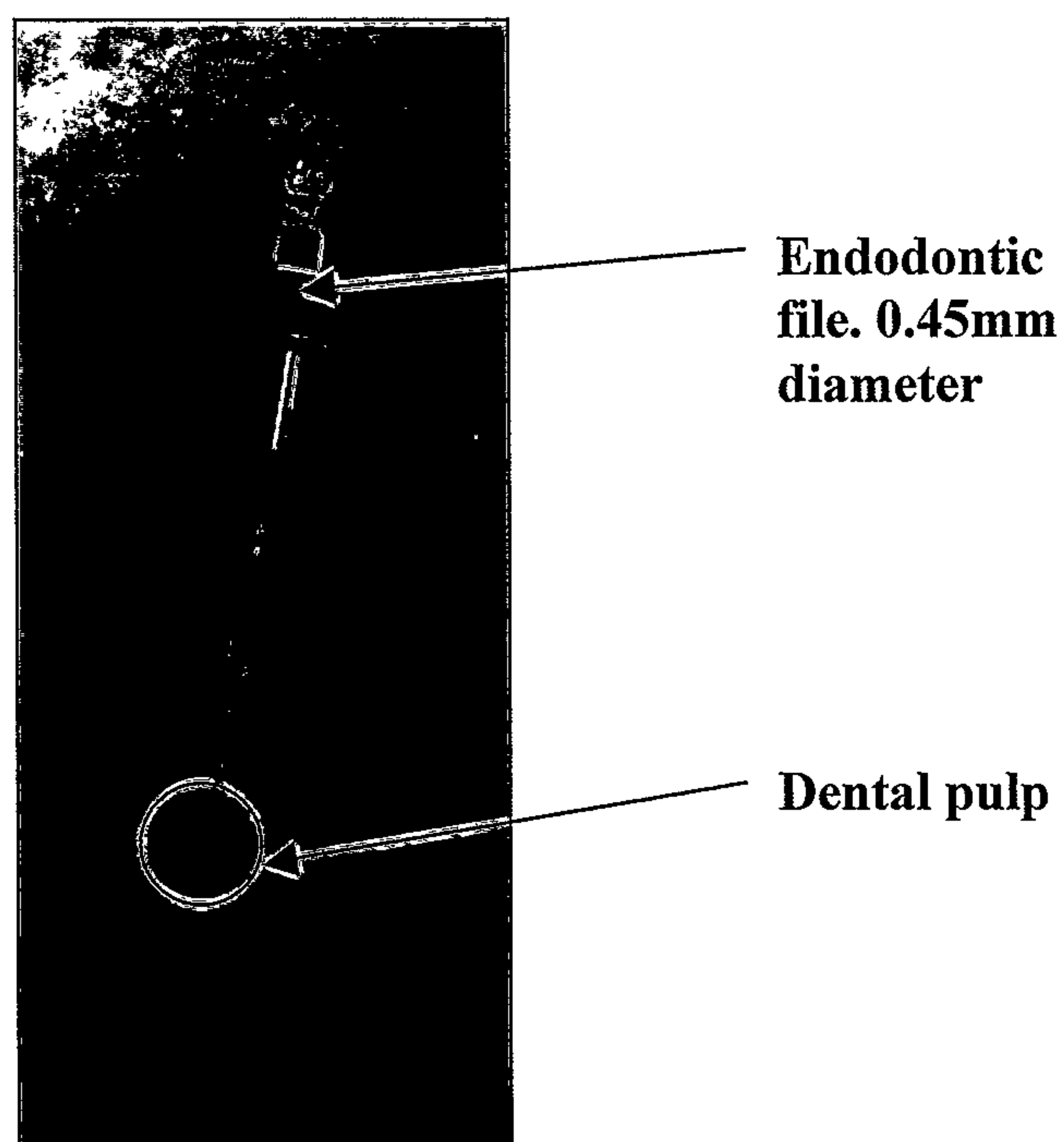


Figure II.8 Removing pulp with endodontic file

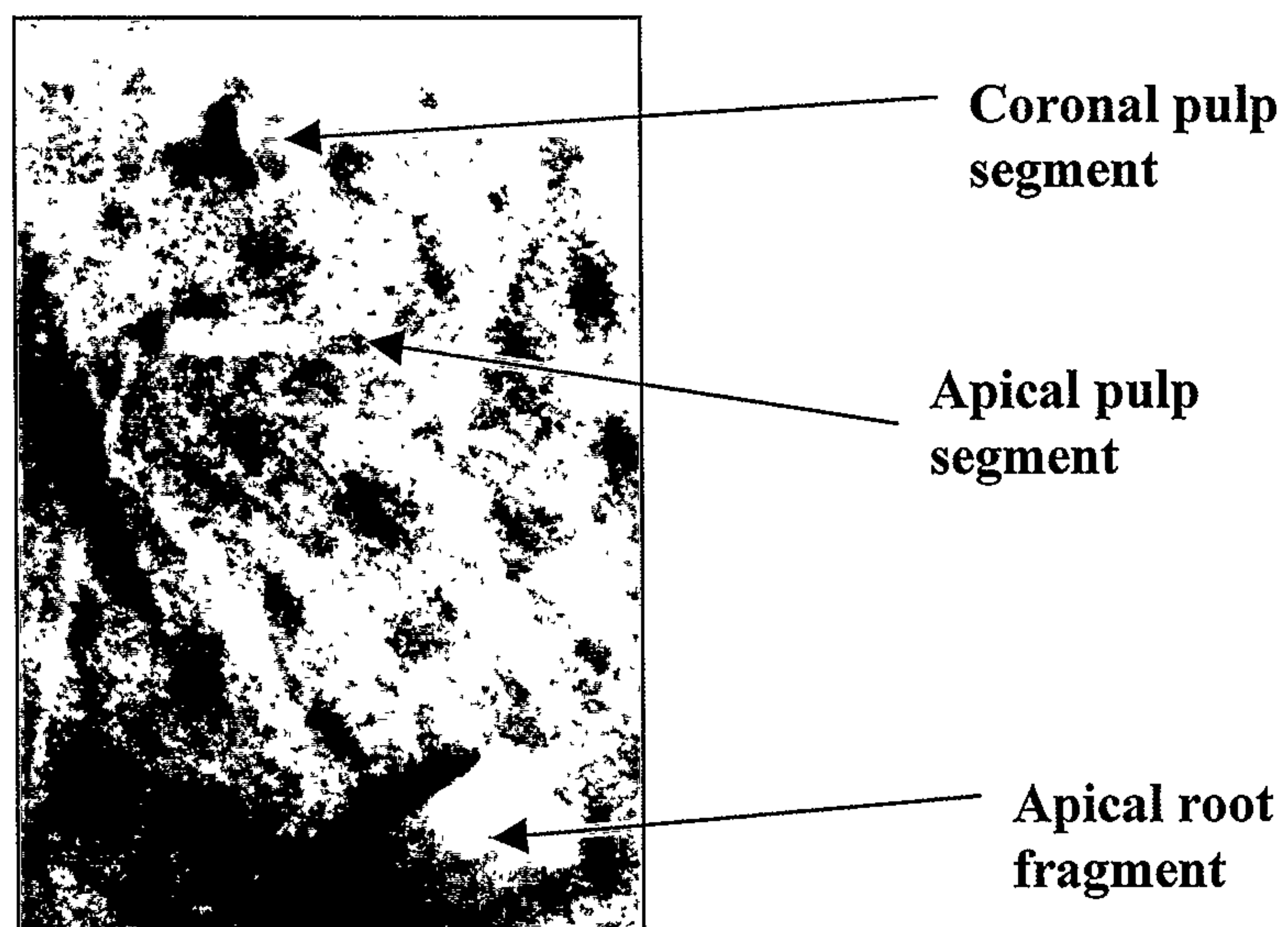


Figure II.9 Pulp removed in 2 pieces

Each pulp resulted in up to 2 explants. Explants were placed into 25cm² culture flasks and covered with 5 ml of dental pulp cell growth medium. Explants were left undisturbed for 48 hours, when extra media was added.

II.4.3 Establishment of a Culture System to Allow Proliferation of Harvested Tissue

All cultures were maintained in a water jacketed, humidified incubator with a 5% (v/v) carbon dioxide atmosphere mixed in air at 37°C. All cell culture materials and equipment were sterile and all cell culture procedures were performed aseptically to minimise bacterial, fungal and mycoplasmal contamination. Cell cultures were monitored for contaminations by visual examination with an inverted phase contrast microscope. Cultures found to be contaminated with micro-organisms were immediately discarded.

Dental pulp cell growth medium was used. The culture medium was changed every second day once the explants had attached well to the flask surface. IGF-1 was added at 500ng/ml to some cultures that were not growing strongly.

II.4.4 Subculture of Cells

Cells were not subcultured until strong outgrowth and confluence of cells was noted at approximately 5 weeks in culture. A number of methods were trialed. Trypsinisation was first attempted using the same protocol as for osteoblasts. 0.02% EDTA was added to poor effect. A final method was developed to detach the dental pulp cells using 5 ml of 0.25% trypsin incubated at 37°C for 10 minutes. The resulting cell suspension was neutralised with growth medium containing FBS and centrifuged at 2000 rpm for 5 minutes to obtain a cell pellet. The cells were resuspended in osteoblast growth medium and counted using a haemocytometer. Cell suspensions were subsequently diluted in growth medium and re-flasked in more than one culture flask. When cells had been subcultured for the first time, penicillin and streptomycin were removed from the growth medium. Cells were not plated for experiments until at least third passage.

II.4.5 Cryopreservation

Surplus cells not immediately required for experimentation were cryopreserved. Cells were detached from the culture flask and the pellet resuspended in a solution containing 10% DMSO (v/v). This solution was then frozen in a cryovial at -80°C. Cryovials were transferred to liquid nitrogen storage for long-term preservation at least 24 hours and not more than one week subsequent to freezing at -80°C. When

required for use, cells were thawed and transferred directly into a culture flask containing dental pulp cell growth medium. This medium was discarded and replaced on the following day to remove DMSO.

II.4.6 Characterisation of cells grown in culture

II.4.6.1 Immunohistochemistry

Cultured dental pulp cells from two pulp explants were characterised by detection of osteonectin expression. Fourth passage cells were processed for immunohistochemical examination after 14 days in culture on Thermanox coverslips. Each explant gave rise to five replicates which underwent immunohistochemical examination.

Cells were fixed with 3% paraformaldehyde. Media was removed, and the wells rinsed 3 times with filtered milliQ water. The cell multilayer was covered with 3% paraformaldehyde, and incubated for 18 minutes at room temperature. Paraformaldehyde was removed and wells rinsed 3 times for 5 minutes with milliQ water. For storage, wells were filled with 70% ETOH and stored in airtight conditions at 4°C. Cells were rehydrated immediately prior to immunohistochemistry.

Thermanox cover slips were removed from the wells and attached to slides using entellin adhesive. Primary antibodies were diluted in distilled water. The incubation with primary antibodies was performed for 1 hour at a 100µg/ml concentration. The staining was revealed using the labelled streptavidin-biotin kit (LSAB plus kit)

according to the manufacturer's instructions. Controls were performed by incubations with unrelated primary antibodies and skin fibroblasts cultured under similar conditions.

II.4.6.2 Growth Factors

Cultured dental pulp cells from three pulp explants were plated onto Thermanox coverslips in 96 well cell culture plates at 15×10^3 cells per well. Media was changed every second day. At 7 days after plating when cells were confluent treatments were added for a further 21 days. Each treatment had five replicates. Treatments consisted of dental pulp cell growth medium, 5mM β GP, TGF- β 1 at 4 μ g/ml (with reduced FBS in growth medium), 5mM β GP and TGF- β 1 (with reduced FBS in growth medium), IGF-1 at 500ng/ml (with reduced FBS in growth medium) and dental pulp cell growth medium with reduced FBS.

Parallel cultures of osteoblasts and fibroblasts were carried out to act as positive and negative controls under similar culture conditions.

II.4.6.3 Alkaline Phosphatase Activity

Measurement of alkaline phosphatase (ALP) activity was used to characterise the dental pulp cells, and to assess the effect of growth factors on cultured dental pulp cells. ALP activity was measured using a commercially available kit.

Cells were rinsed three times with PBS. 110 μ l of ALP reagent was added to each well at 10 second intervals. Cells were incubated for 12 minutes at 37°C from the time of

adding the first reagent, and checked regularly for colour change. When the first colour change was noted, the reaction was halted by the addition of 150µl of 1M NaOH to each well at 10 second intervals. 250µl of reagent was removed from each well at 10 second intervals and added to a fresh 96 well plate. The plate was gently agitated for two minutes, then absorbance read at 405nm on the spectrometer.

Osteoblasts were used as positive controls (reaction stopped at 13 minutes), and Fibroblasts as negative controls (reaction stopped at 15 minutes).

II.4.6.4 Scanning Electron Microscopy

The morphology and coverage of dental pulp cells were analysed using Scanning Electron Microscopy (SEM). Dental pulp cells were fixed on Thermanox cover slips after 14 days in culture. Osteoblasts and skin fibroblasts cultured under similar conditions were used as positive and negative controls.

Cells were fixed by 2% glutaraldehyde in PBS. Warmed (37°C) fixative was added to the culture medium in equal volumes for 5 minutes. The mixture was then replaced with pure fixative at room temperature under gentle agitation. The plates were left at room temperature for 1 hour, followed by storage at 4°C in the fixative for 24 hours. After 24 hours the cells were rinsed with PBS for three 3-minute washes, and then dehydrated in a graded ethanol (50%-70%-80%-95%-100%) with 10 minutes at each grade.

Prior to SEM observation the specimens were coated with gold-palladium. The specimens were examined under SEM (Philips, 505) operating at 10kV and at magnifications of 700 and 1400 times. The SEM was located in the Electron Microscopy Unit at the University of Sydney.

II.5 Statistical Analysis

Since in most cases a simple comparison between two treatment groups was made, the significance of results was analyzed by the Student's unpaired t-test (two-tailed). $P < 0.05$ was considered statistically significant. Where multiple comparisons were made the results were analyzed by 1-way ANOVA with Tukey's post test. All statistical analyses were performed using GraphPad InStat statistical analysis software.

Chapter III.Results

III.1Osteoblast Studies

III.1.1 Basic Cell Culture and Examination of Growth of Cells in a Matrix

Initial experiments were performed with human osteoblast cells, in order to examine a more readily available mineralising cell type. In biological systems, mineralising cells such as osteoblasts and odontoblasts are normally embedded in a three dimensional matrix. Gelfoam was used to mimic this condition in a culture system.

Osteoblasts in culture were seen to grow strongly in wells containing Gelfoam. This growth was examined by histological section. Prepared sections were examined under light microscopy. Osteoblasts were observed to have infiltrated the Gelfoam matrix, and proliferated well. H&E staining of the sections showed cellular infiltration of the matrix (Fig.III.1).

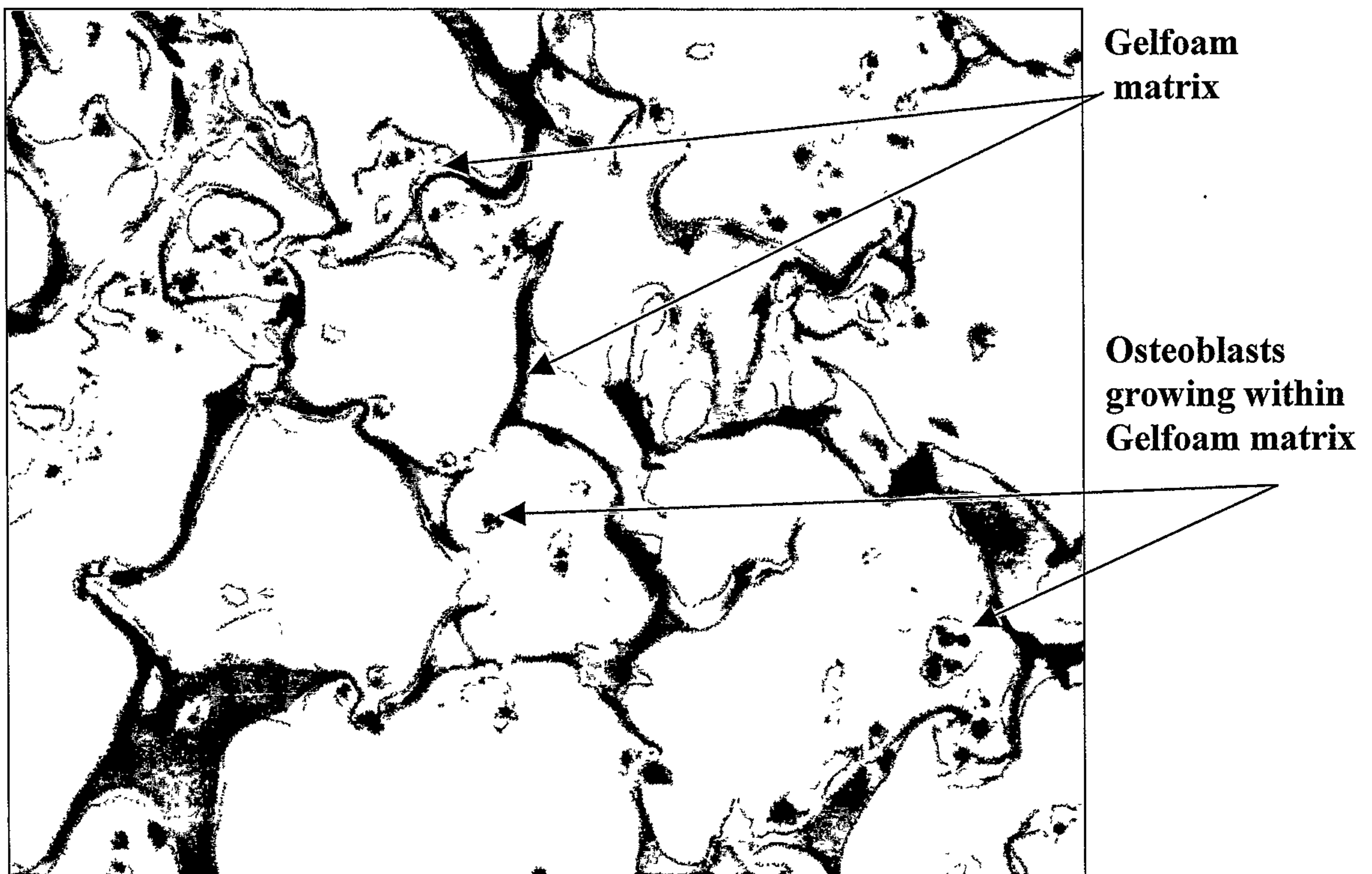


Figure III.1 H&E staining of untreated cells at 20X magnification

At higher magnification, the osteoblasts can be seen more clearly. The cells are closely related to the matrix material. Some active mitoses can be seen. (Fig.III.2)

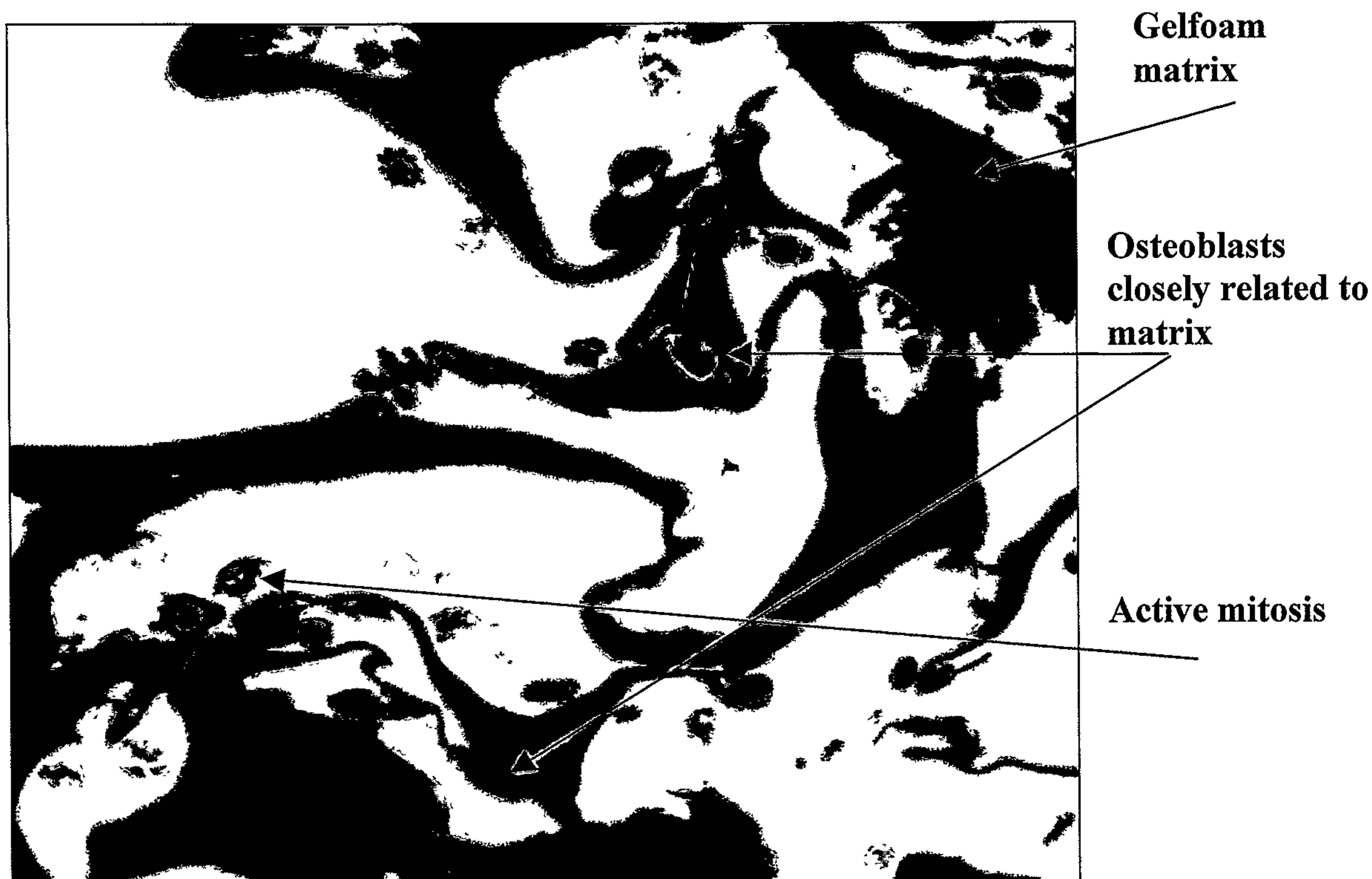


Figure III.2 H&E staining of untreated cells at 40X magnification

III.1.2 Influences on Mineralisation

Potential influences on mineralisation were examined. These factors were examined in the basic osteoblast culture and in the Gelfoam cultures. Mineralisation was assessed by alizarin red assay and [^3H]-tetracycline assay. Factors tested for modification of mineralisation in cell culture included calcium, a phosphate source (βGP), $1,25(\text{OH})_2\text{D}_3$ and dexamethasone.

III.1.2.1 Effect of $1,25(\text{OH})_2\text{D}_3$

The effect of $1,25(\text{OH})_2\text{D}_3$ was first examined in the Gelfoam cultures. When examined under light microscopy, H&E staining of cells treated with $1,25(\text{OH})_2\text{D}_3$ at

1×10^9 concentration showed denser infiltration and proliferation of cells into the matrix than the original untreated sections (Fig.III.3).

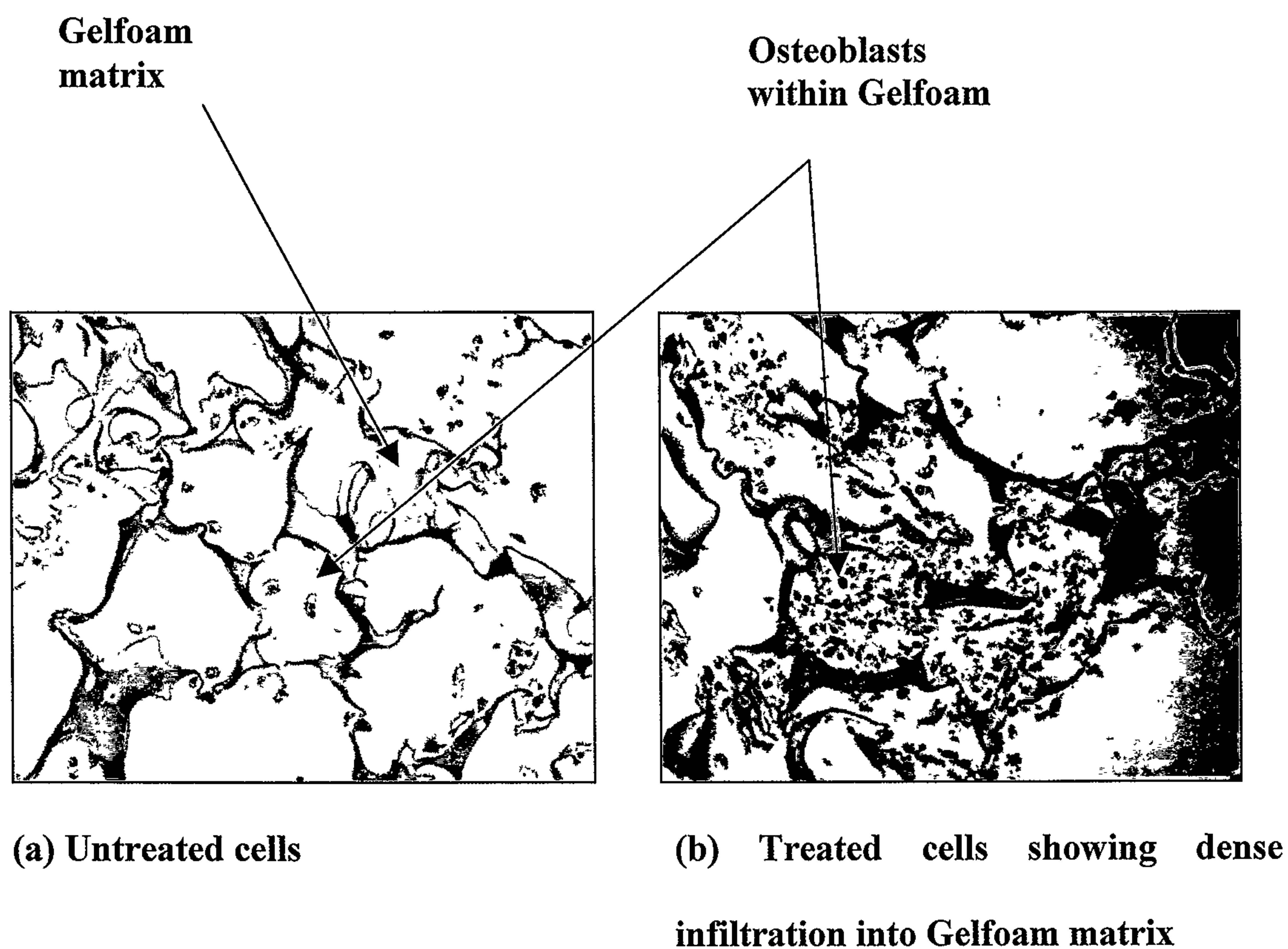


Figure III.3 H&E staining. Comparison of cellular infiltration and proliferation in Gelfoam matrix of untreated cells (a) and cells treated with $1,25(\text{OH})_2\text{D}_3$ (b) at 20x magnification.

When the treated cells are viewed at higher magnification, the extent of cellular infiltration and proliferation can be seen (Fig.III.4). Cells are multilayering on the external surface of the matrix and multiple active mitoses can be seen. $1,25(\text{OH})_2\text{D}_3$ was seen to increase infiltration and proliferation of osteoblastic cells within a matrix.

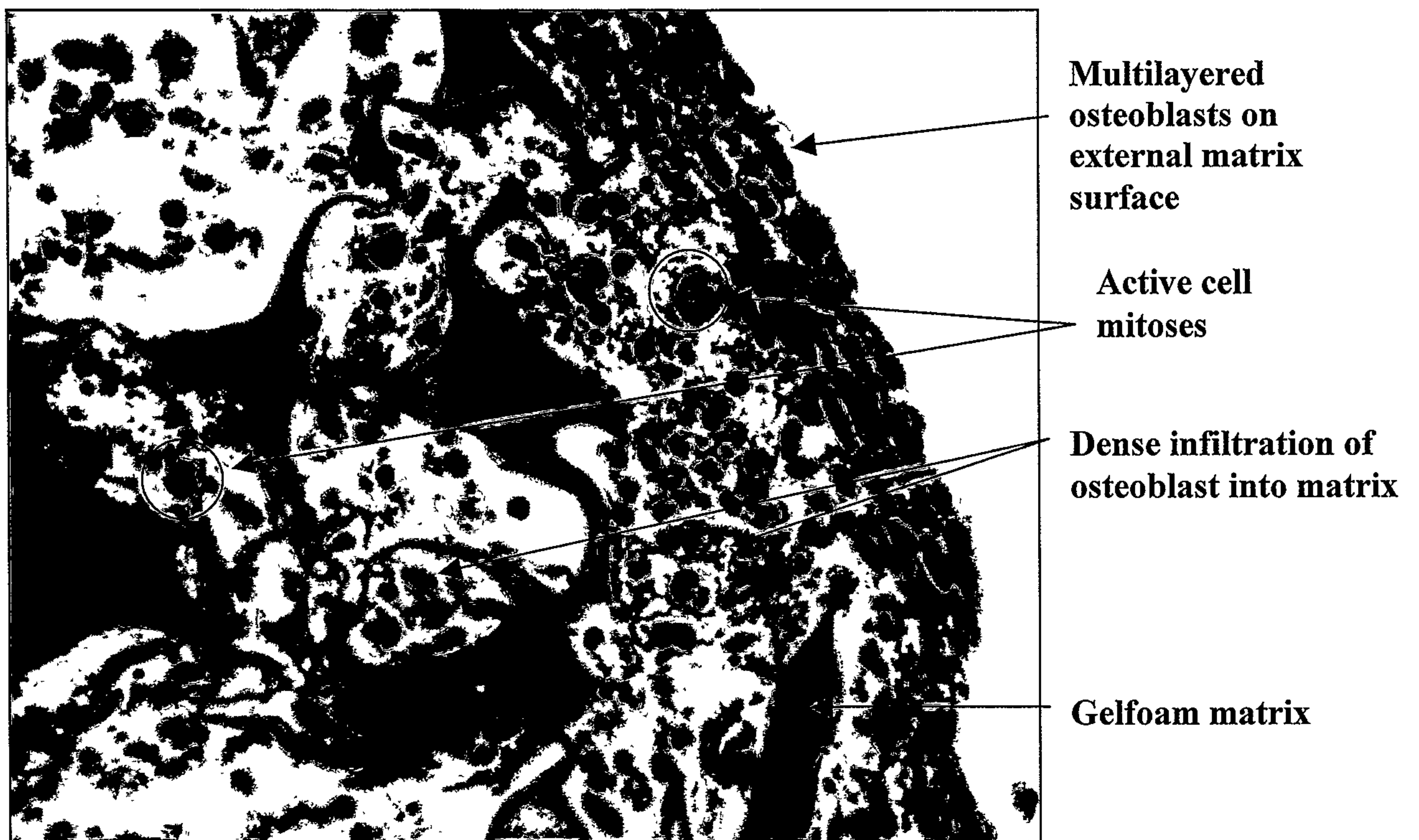


Figure III.4 H&E staining of treated cells at 40X magnification

The effect of $1,25(\text{OH})_2\text{D}_3$ on mineralisation of osteoblasts grown in a routine monolayer culture system was examined, and assessed by alizarin red assay and by $[^3\text{H}]$ -tetracycline assay. An increase in calcium accumulation as measured by alizarin red staining was noted between vehicle treated wells and those treated with $1,25(\text{OH})_2\text{D}_3$, but was not significant ($p>0.05$) (Fig.III.5). The addition of etidronate as a potential inhibitor of mineralisation had no effect (data not shown).

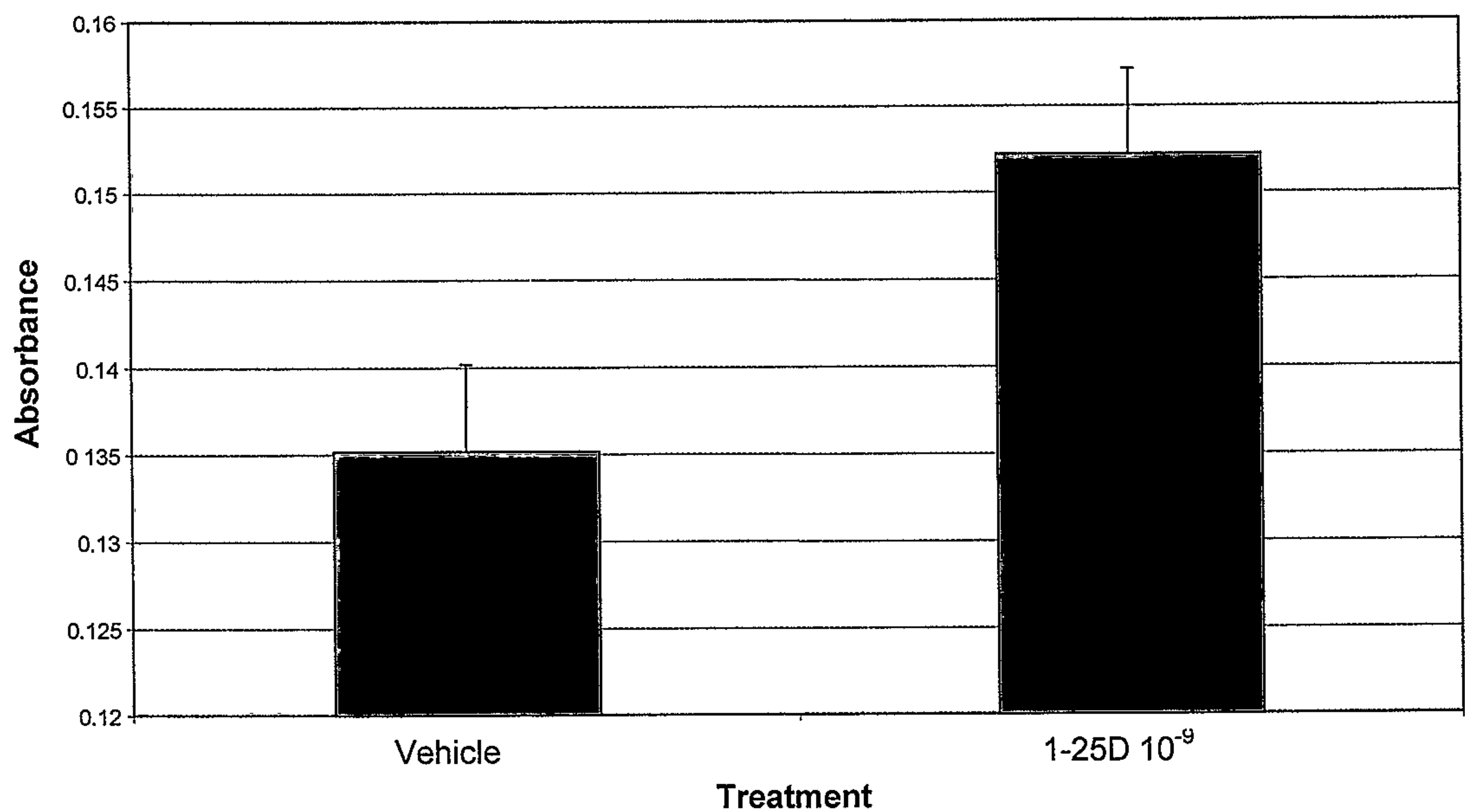


Figure III.5 Alizarin red assay. Effect of 1,25(OH)₂D₃ on osteoblasts in culture

Assessment of mineralisation as measured by [³H]-tetracycline incorporation showed an increase in wells treated with 1,25(OH)₂D₃ compared to no treatment, but again, this was not significant (p>0.05) (Fig. III.6)

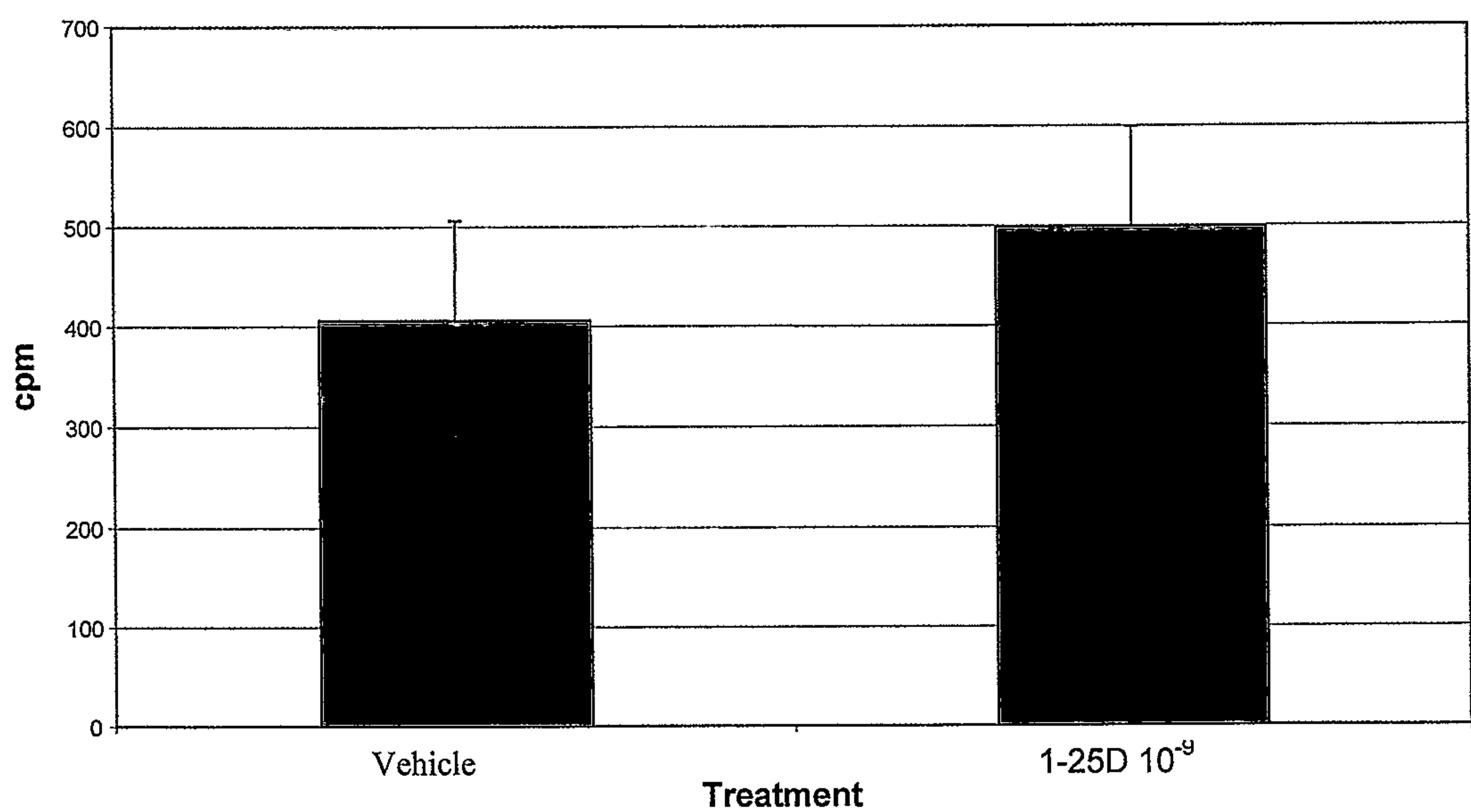


Figure III.6 Tetracycline incorporation. Effect of 1,25(OH)₂D₃ on osteoblasts in culture

III.1.2.2. Effect of Dexamethasone

The effect of dexamethasone on osteoblast mineralisation in culture was first assessed by alizarin red assay. There was no significant effect of treatment with Dexamethasone ($p>0.05$). (Fig.III.7).

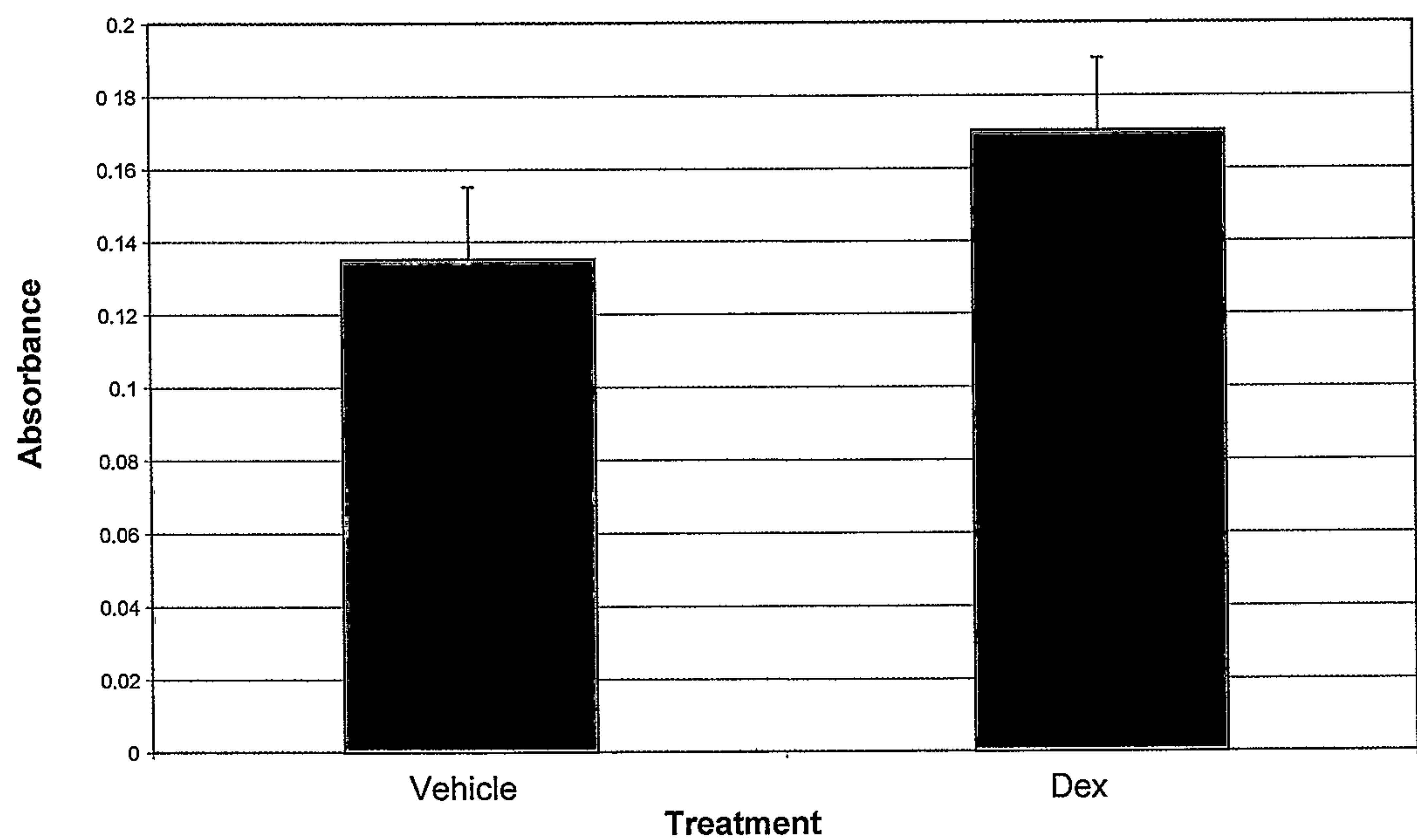


Figure III.7 Alizarin Red assay. Effects of Dexamethasone on osteoblasts in culture

When examined by [³H]-tetracycline assay, there was no significant effect of dexamethasone on cell mineralisation ($p>0.05$). No inhibitory effect of etidronate was seen (data not shown). (Fig.III.8)

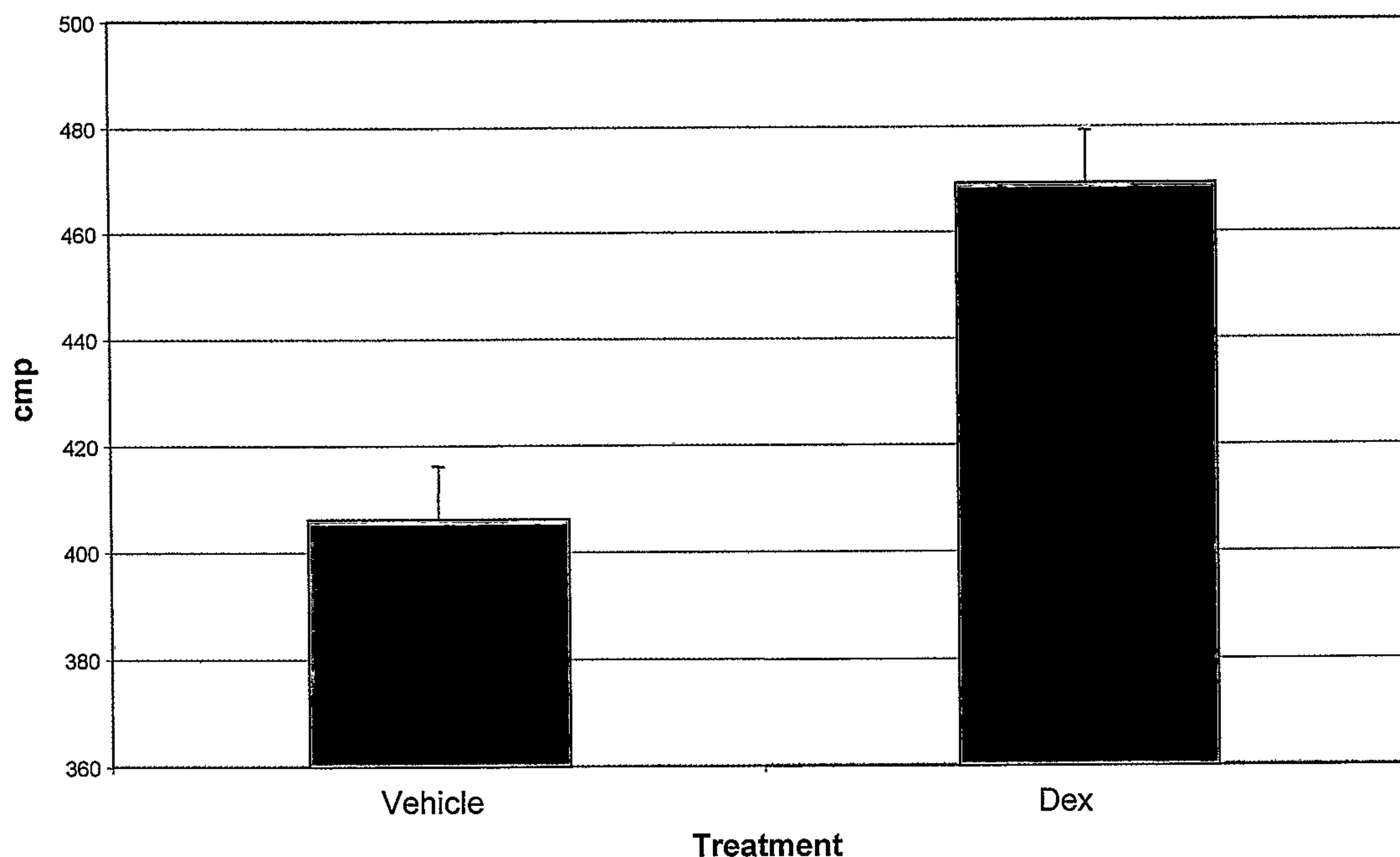


Figure III.8 Tetracycline incorporation. Effects of Dexamethasone on osteoblasts in culture

III.1.2.3. Effect of β GP and Calcium

The effect of β GP and calcium-containing media on osteoblasts in culture was examined at two separate time periods. Both a source of phosphate (β GP) and calcium were found to be required for optimal mineralisation of osteoblasts in culture. Measurement by alizarin red assay showed a clear increase (55%) in calcium accumulation in cells treated with β GP for 14 days. However the difference was not quite significant ($p=0.0508$, $p>0.05$) (Fig.III.9).

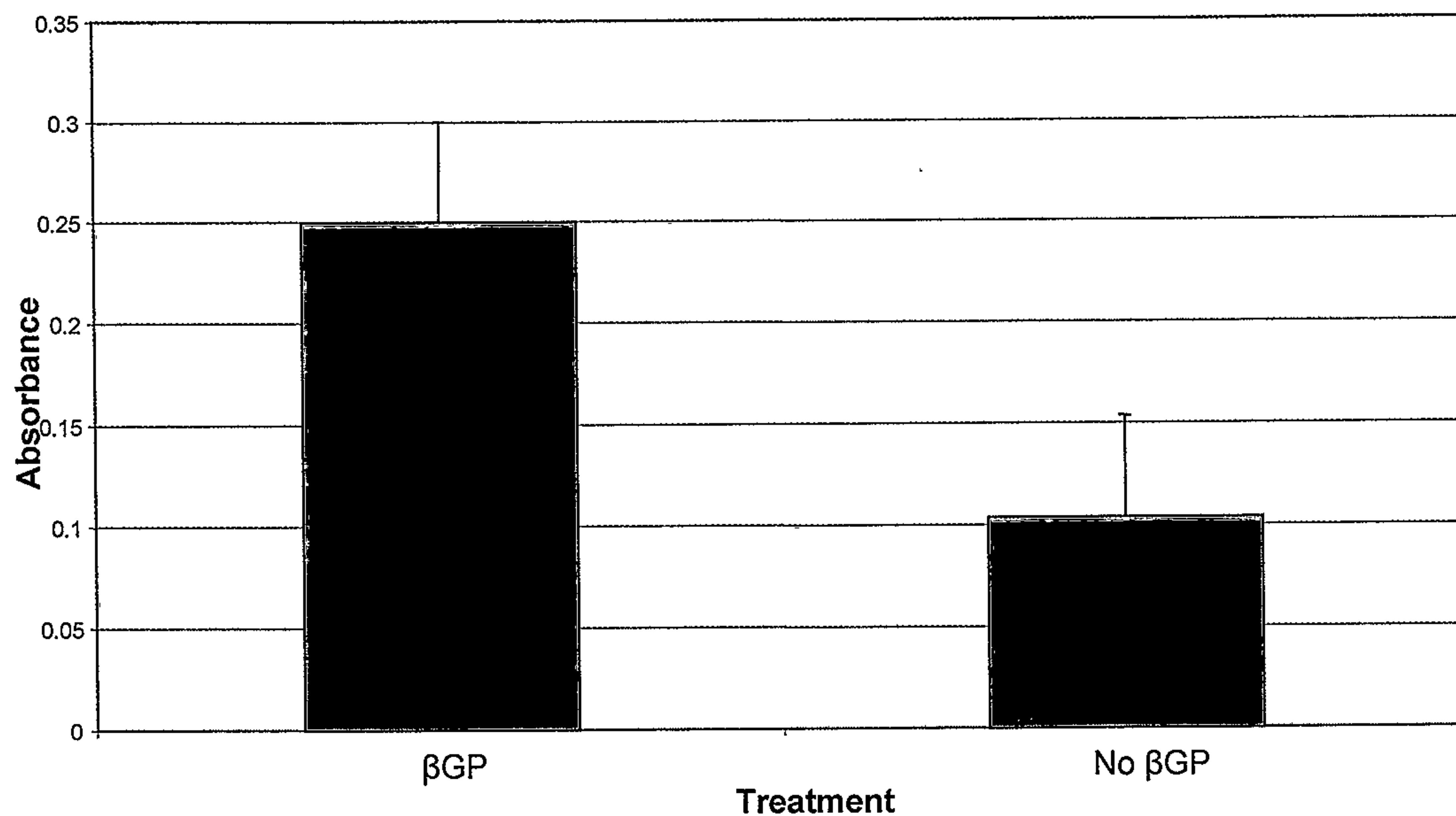


Figure III.9 Alizarin red assay. Effect of β GP on osteoblasts in culture after 14 days of treatment

The absence of added calcium (there is some calcium in FBS) did not affect mineral accumulation. Better mineralisation (although not significant, $p>0.05$) was seen in the presence of both added calcium and β GP. (Fig.III.10).

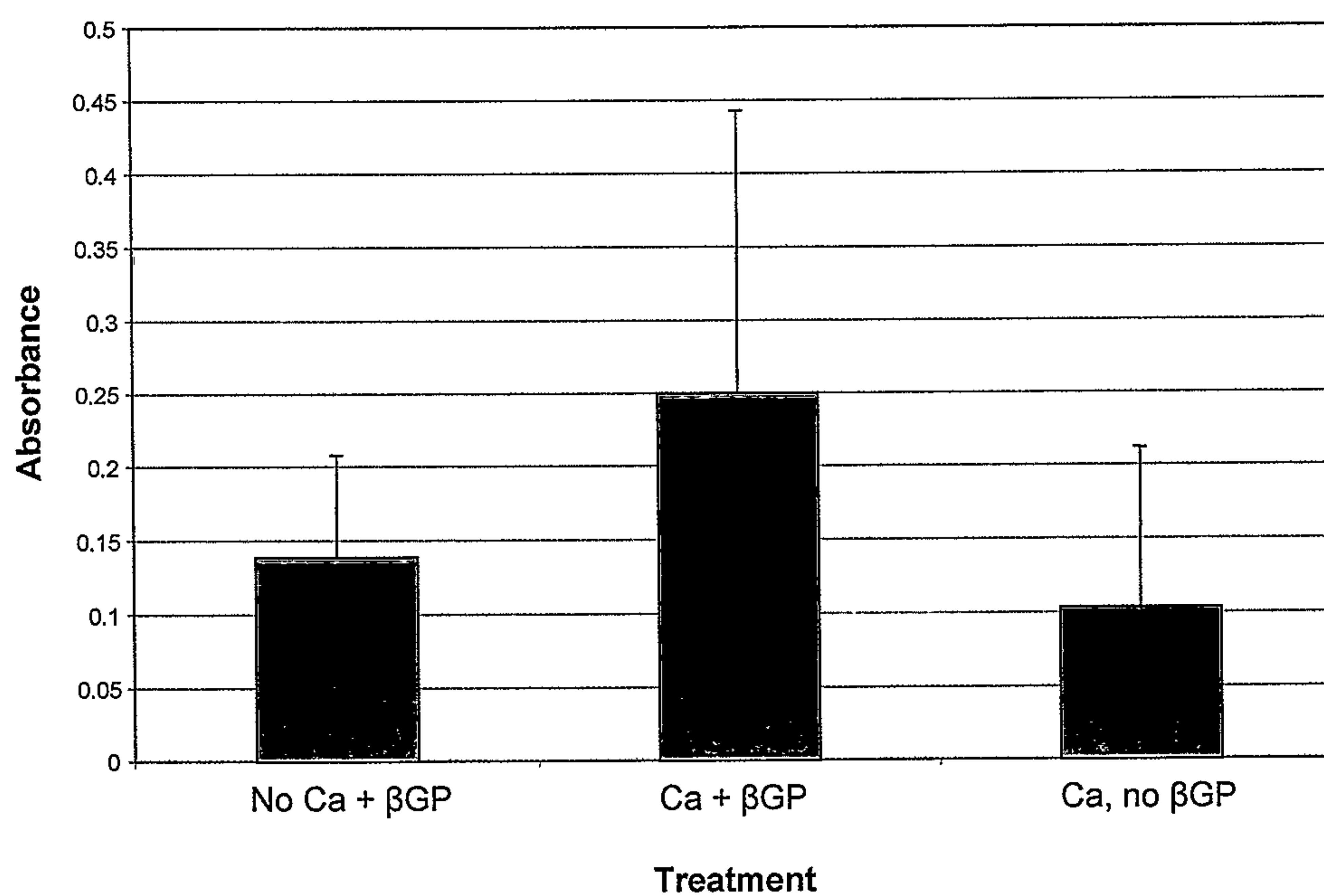


Figure III.10 Alizarin red assay. Effect of calcium and β GP on osteoblasts in culture after 14 days of treatment

After 21 days of treatment, the effect of the absence of β GP from the medium was more apparent, with the wells treated with β GP producing 2.5 times the amount of mineralisation when assessed by alizarin red assay, which was extremely significant ($p<0.0001$). (Fig.III.11).

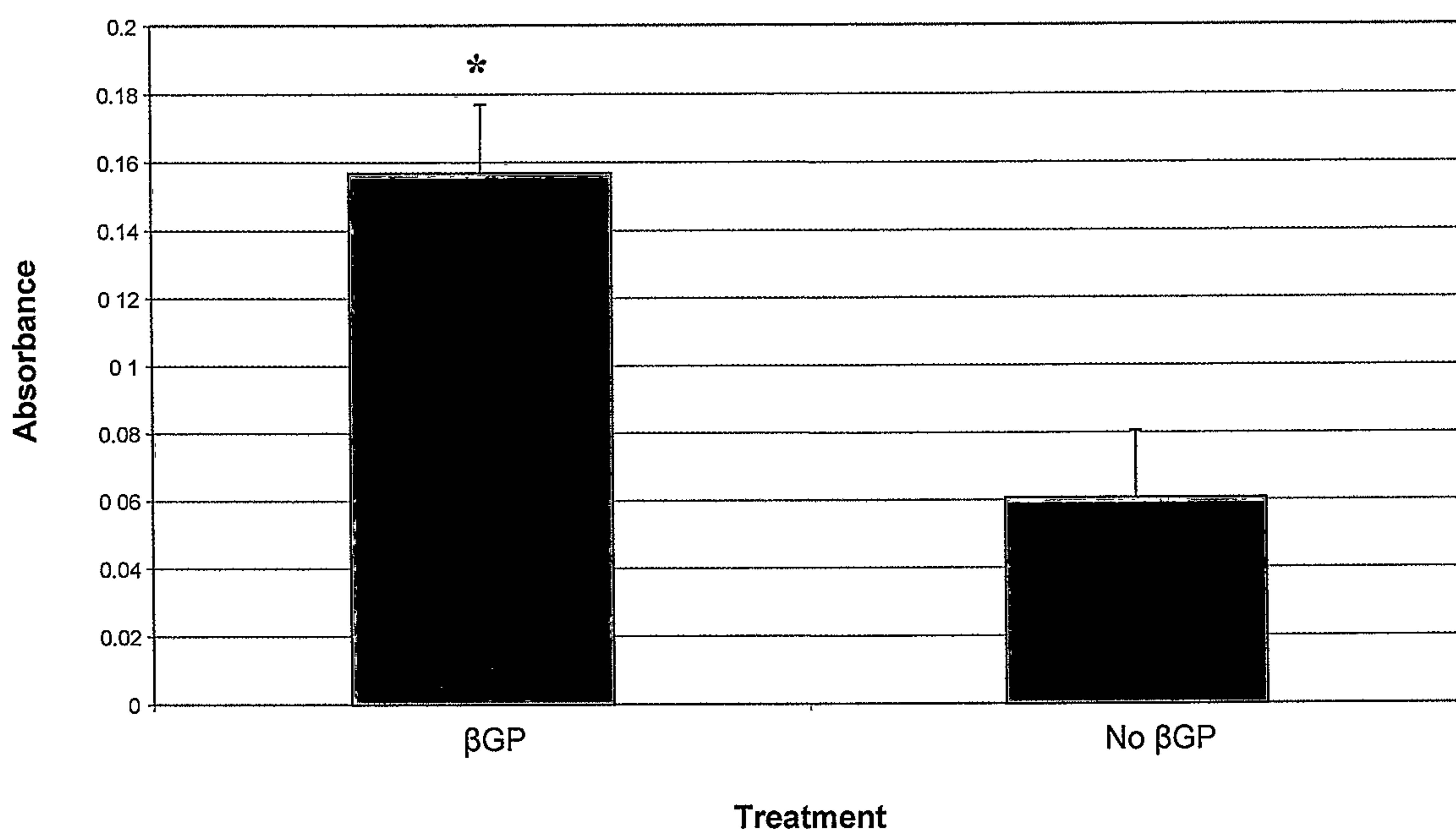


Figure III.11 Alizarin red assay. Effect of β GP on osteoblasts in culture after 21 days of treatment

* Treatment with β GP significantly increased mineralisation compared to treatment without β GP; $p<0.0001$.

When assessed by [^{3} H]-tetracycline incorporation, little mineral was deposited in the absence of either added calcium or β GP. (Fig.III.12). The addition of calcium and β GP caused a significant increase in mineralisation ($p<0.01$).

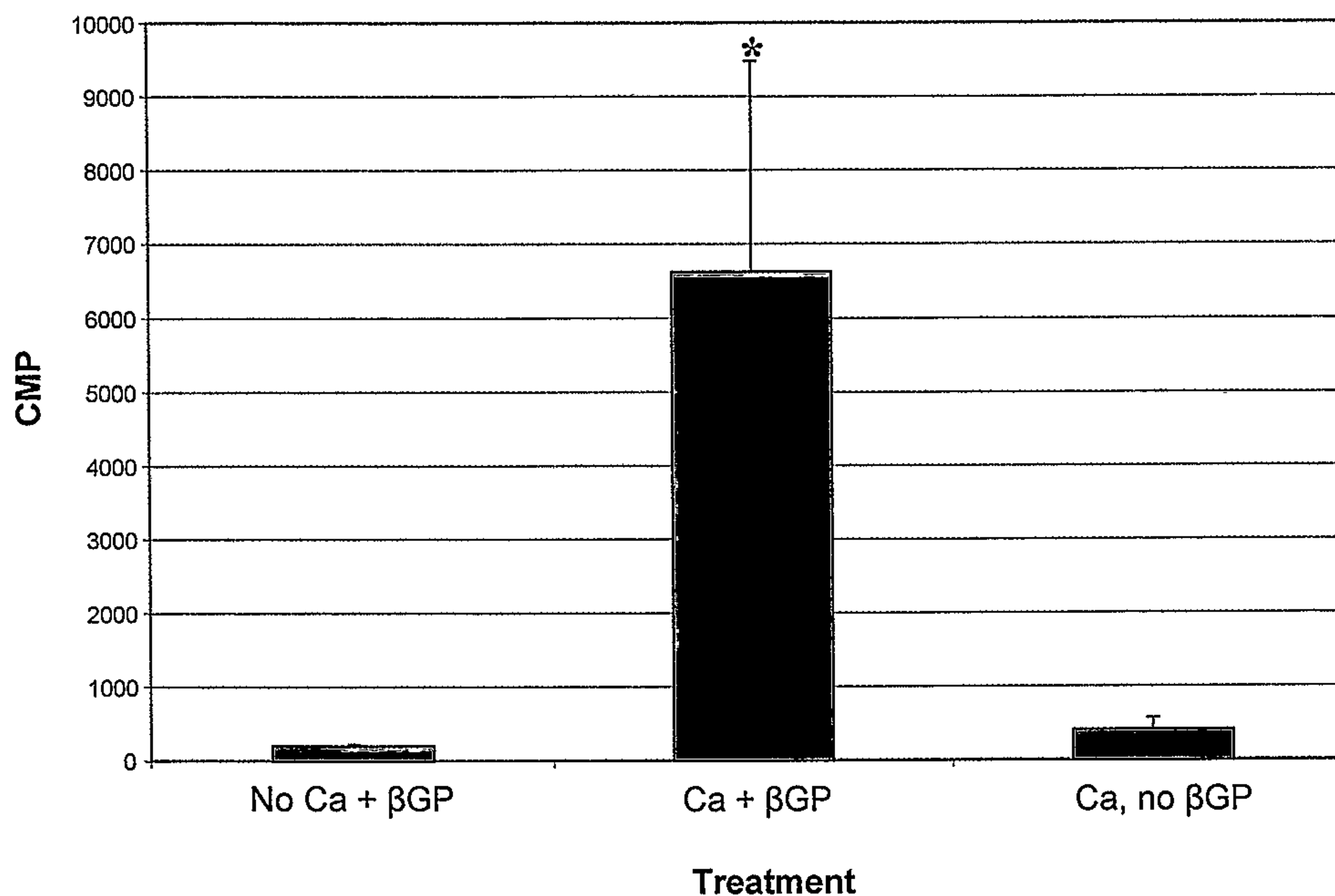


Figure III.12 Tetracycline incorporation. Effect of calcium and βGP at 21 days of treatment

* Treatment with calcium and βGP very significantly increased mineralisation compared to treatment with βGP and no added calcium; $p < 0.01$. Compared to treatment with added calcium and no βGP, the increase in mineralisation was also significant ; $p < 0.05$.

III.1 Odontoblast Studies

III.2.1 Methods for Obtaining Odontoblastic Cells from Available

Material

A total of 47 teeth were collected from 17 patients. A number of methods were trialled to determine the most efficient and aseptic procedure to obtain dental pulp cells for culturing. The most effective method has been described in chapter 2. The most important noted aspects of this protocol were:

- Efficient sectioning of teeth to enable atraumatic collection of dental pulp
- Maintenance of dental pulp cell viability through communication with transport medium
- Prevention of infection through maintenance of high levels of cleanliness and asepsis

The time period between collection of teeth and placement of pulp explants into culture flasks varied between 3 and 8 hours. The time period did not appear to affect the growth of the pulp explants.

Dental pulp explants were noted to take 5 to 7 days to attach firmly to the base of the culture flask, and were easily disturbed by movement. In order to encourage attachment, only a small amount of media was placed initially into the flask, with extra media added after 3 days. If the explant detached from the flask surface, it was found to be possible to encourage re-attachment by draining most of the media from the flask, and allowing the explant to settle in a minimal volume of media for 20 minutes. This process did not appear to have a deleterious effect on the growth of cells.

Infection of cultures was a significant problem in the early collection of dental pulps. The first method trialled resulted in fungal infections within 2 days of collection on all samples. As the collection method was refined, contamination became less, but was still a significant problem. Up to 50% of samples collected were lost to contamination.

The first dental pulp successfully harvested was via the second method trialled (splitting the tooth with a surgical chisel). This first pulp explant obtained showed signs of cell proliferation and adhesion after 10 days in culture (Fig.III.13). A small number of long, spindle-shaped cells were noted to extend from the periphery of the explant. Growth of this first explant was slow, and at 17 days no further growth was noted, however in some areas the cells appeared to be attempting to form a more organised pattern (Fig.III.14).



Figure III.13 Sample 1. Early cellular outgrowth 10 days after collection (Inverted phase-contrast micrograph, 40x magnification)

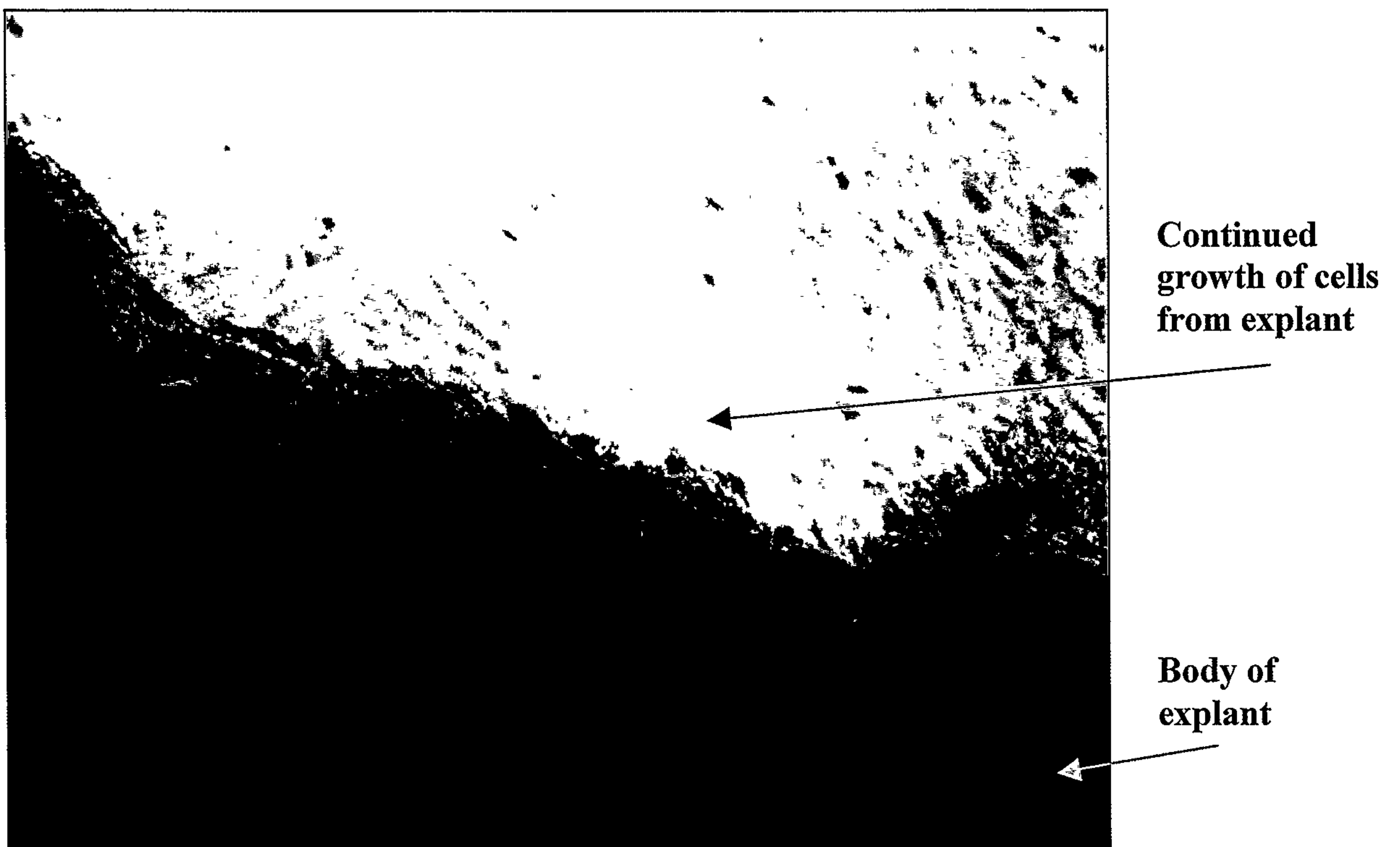


Figure III.14 Sample 1. Early cellular outgrowth at 17 days with organisation of cells (Inverted phase-contrast micrograph, 40x magnification)

The next group of dental pulps obtained was via method three, which became the final protocol. The pulp explants underwent less traumatic handling, and appeared to attach more readily to the flask surface. Good cellular adhesion and very early cellular proliferation was noted at 3 days (Fig.III.15)

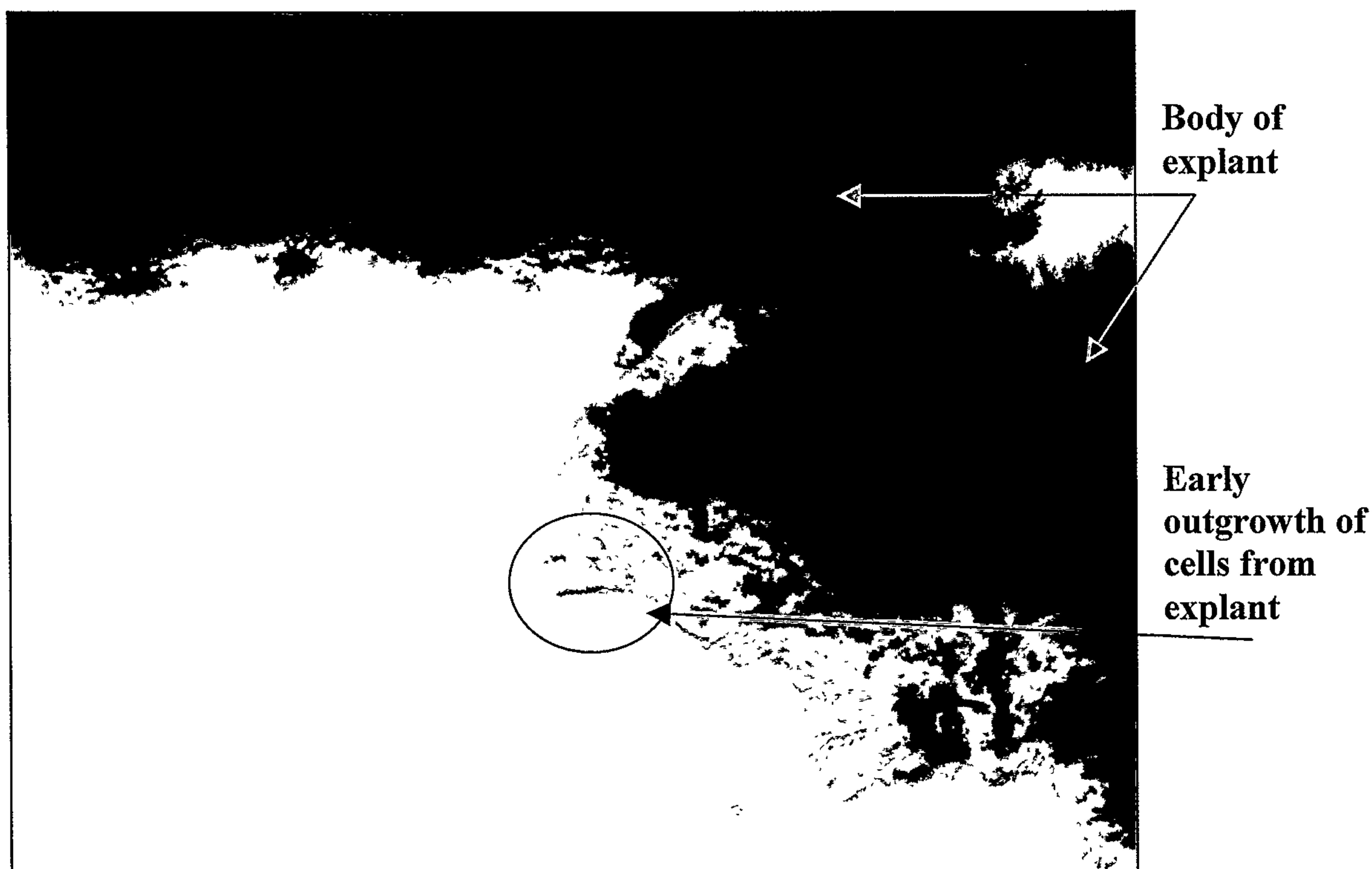


Figure III.15 Sample 2. Early cellular outgrowth at 3 days after collection (Inverted phase-contrast micrograph, 20x magnification)

III.2.2 Establishment of a Culture System to Allow Proliferation of Harvested Tissue

III.2.2.1 Progression of Dental Pulp Explant Cultures

All samples were harvested by method 3, with the exception of the first sample (sample 1) which was harvested by method 2. Cells generally grew well in culture. In the case of a sample which did not grow strongly, treatment with a growth factor (IGF-1) was helpful in stimulating cellular proliferation and growth. IGF-1 was added to the culture medium for sample 1, which was noted to have slow growth. IGF-1 was added at 17 days. At 24 days, the explant was noted to have an increased strong outgrowth of cells. IGF-1 continued to be added to the medium, and the cells had

continued good growth, with progressive organisation and consolidation into a densely packed network (Fig.III.16)

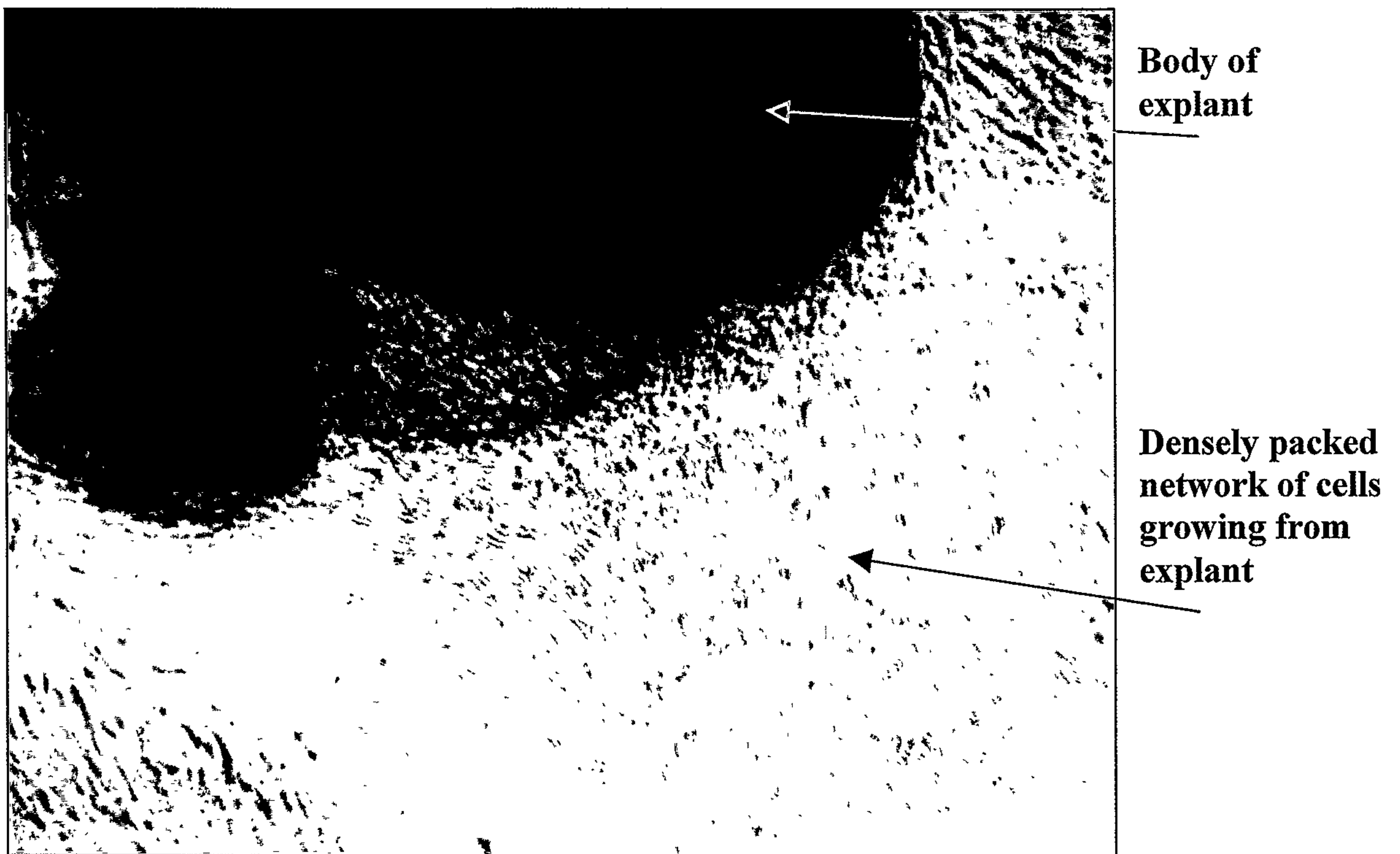


Figure III.16 Sample 1. Cellular outgrowth at 38 days following treatment with IGF-1 (Inverted phase-contrast micrograph, 5x magnification)

At 10 days after harvesting, the dental pulp explants collected via method three were noted to be growing well, with strong proliferation of cells. Regular and fibre-like structures started to appear from the explant border, which extended toward the peripheral parts. The cells were noted to be long, spindle-shaped cells, which formed branching networks (Fig.III.17). This network was maintained even at outgrowing boundary of the cells (Fig.III.18).

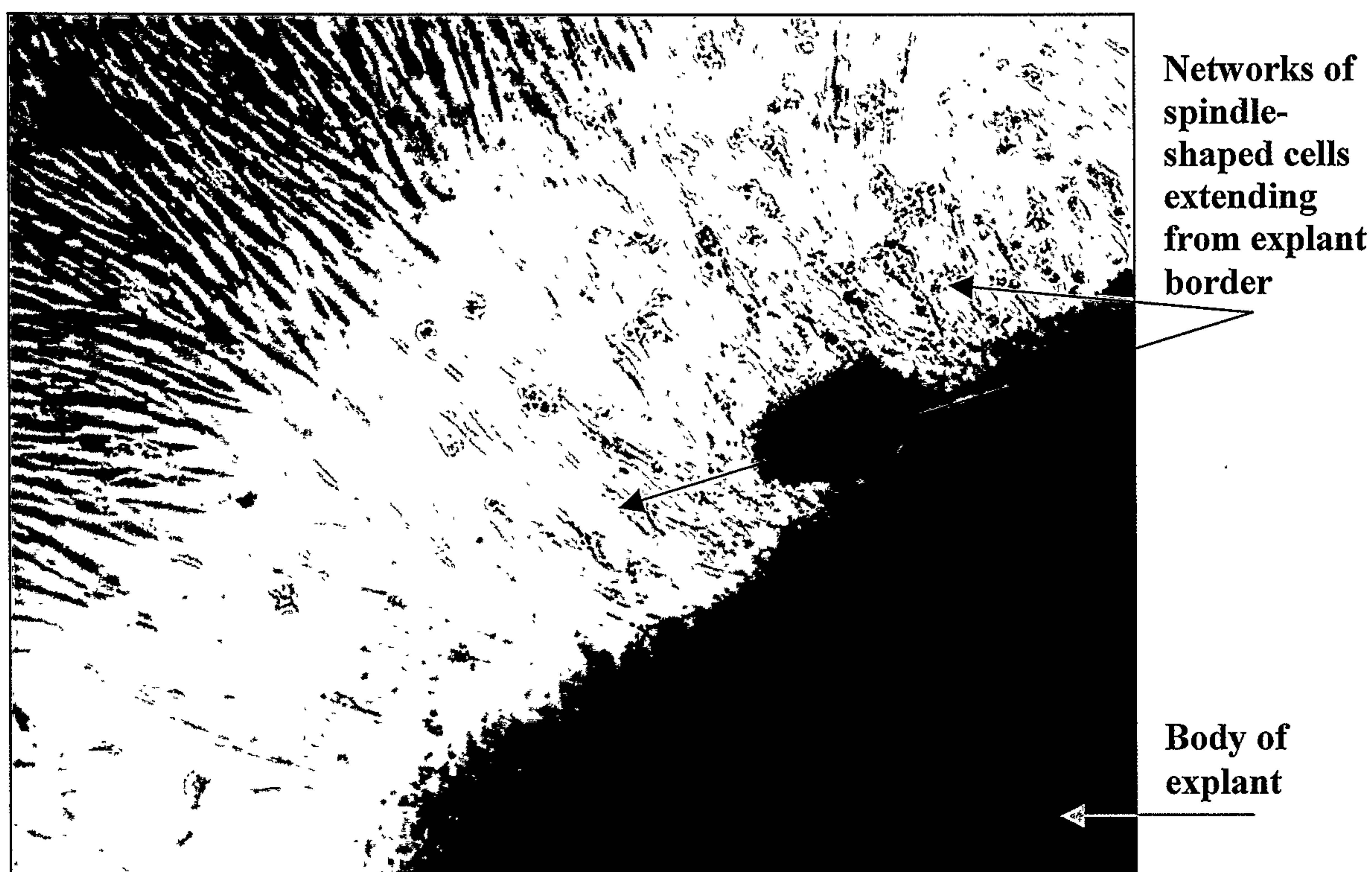


Figure III.17 Sample 2. Cellular outgrowth from explant surface at 10 days (Inverted phase-contrast micrograph, 40x magnification)

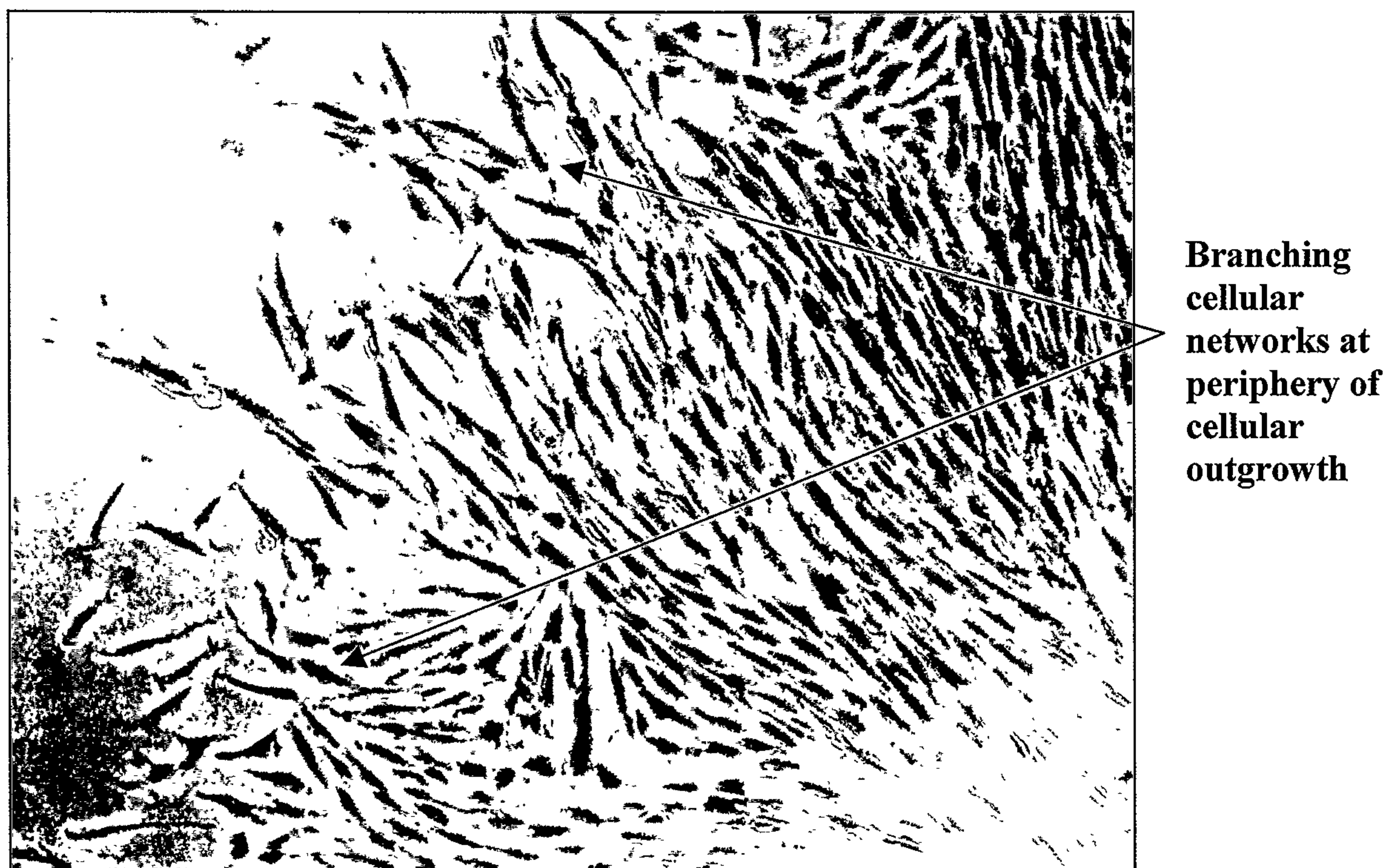


Figure III.18 Sample 2. Boundary of cellular outgrowth at 10 days showing organisation and branching cell network (Inverted phase-contrast micrograph, 40x magnification)

These cells collected via method three grew strongly without the addition of any growth factors. By 24 days in culture, the cell outgrowth was profuse and proceeding rapidly. The cells formed a densely packed network that was ordered and well-organised (Fig.III.19). The cellular outgrowths had a similar appearance to those from trabecular long bone explants (Patava 1996). No nodule or mineral formation was noted in the cultures.

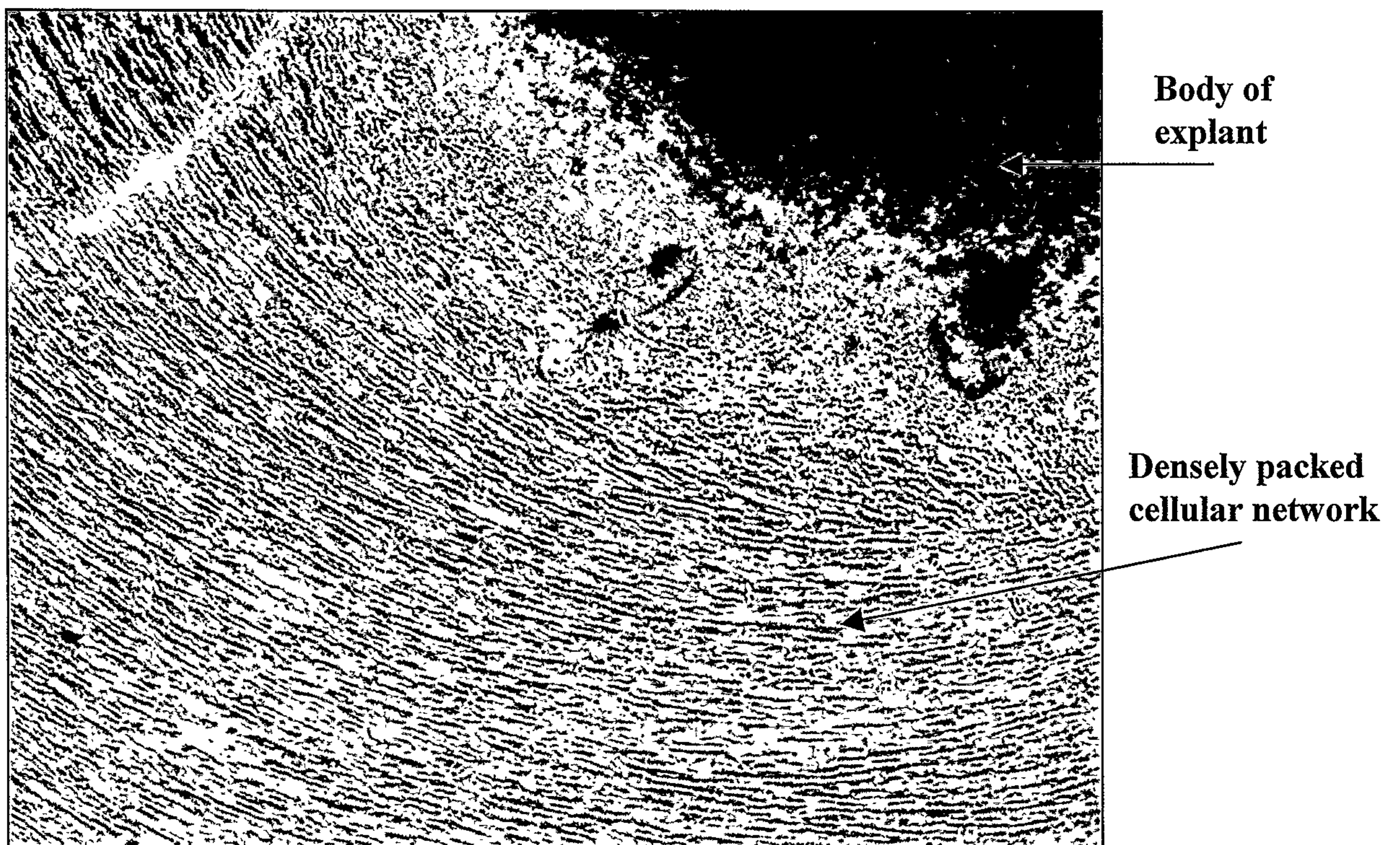


Figure III.19 Sample 2. Cellular outgrowth at 24 days showing strong cellular proliferation and organisation (Inverted phase-contrast micrograph, 10x magnification)

Cells were passaged when the cellular network was seen to be spreading over the bulk of the flask. This was at approximately 34 days in culture.

III.2.2.2 Subculture of Cells

Initially cell cultures were subcultured according to the method reported by About et al (2000) (About et al 2000a). This was via 0.2% trypsin and 0.02% EDTA. This had the effect of completely destroying the cells. The next trypsinisation protocol tested was the standard laboratory method for trypsinisation of bone cells, using 0.25% trypsin. The standard 7 minute trypsinisation period did not produce adequate detachment of cells from the flask. Various time frames were tested for trypsinisation periods, with 10 minutes found to give adequate detachment of cells whilst maintaining cellular viability. Cells were found to subculture well, and quickly adhered to the culture surface. The subcultured cells were seen to proliferate at a far greater rate than the initial cultures, and acquired a spindle shaped morphology, with some polygonal cells. In some areas the cells were noted to multi-layer. Subcultures were ready for passaging after 14 to 21 days.

III.2.2.3 Storage of Cells

Surplus cells not immediately required for experimentation were stored under liquid nitrogen, and brought up when required. It was noted that the majority of cultures did not survive this process. For this reason, all characterization experiments were carried out on freshly subcultured cells, not ones that had been in frozen storage.

III.2.3 Characterisation of Cells Grown in Culture

III.2.3.1 Immunohistochemistry

Dental pulp cells were characterised by immunohistochemistry. Polyclonal antibody to osteonectin was used to differentiate between odontoblast-like cells and fibroblasts. Dental pulp cell cultures from 2 differing samples were tested. All samples showed uniformly strong staining for osteonectin (Fig.III.20). In contrast, fibroblasts that were cultured under similar conditions showed no reaction to osteonectin staining (figure III.21). From the strong, uniform expression of ON, it appears that the dental pulp cells in culture are expressing an odontoblast-like phenotype.

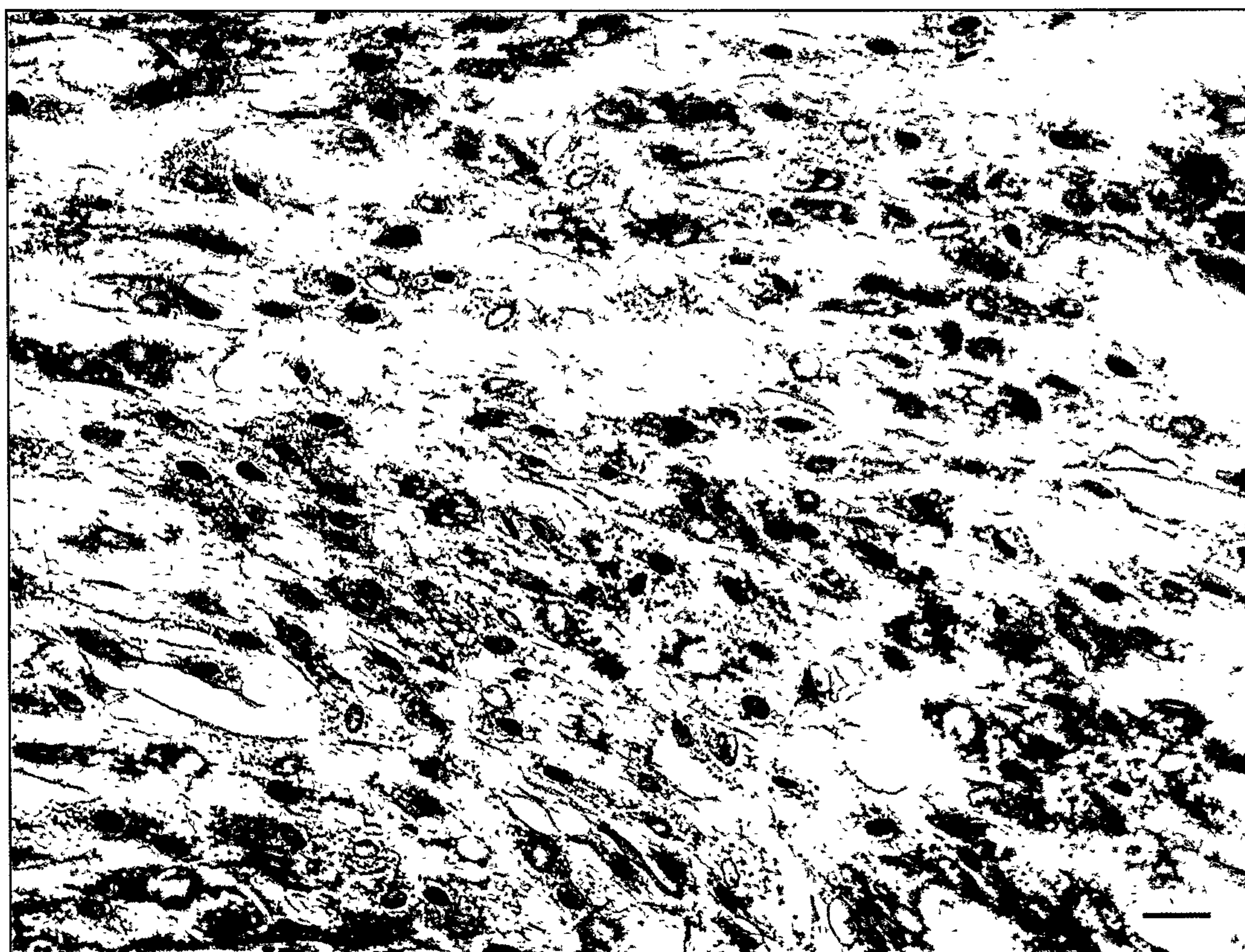


Figure III.20 Pulp cell culture stained for osteonectin (Light micrograph, 10x magnification)

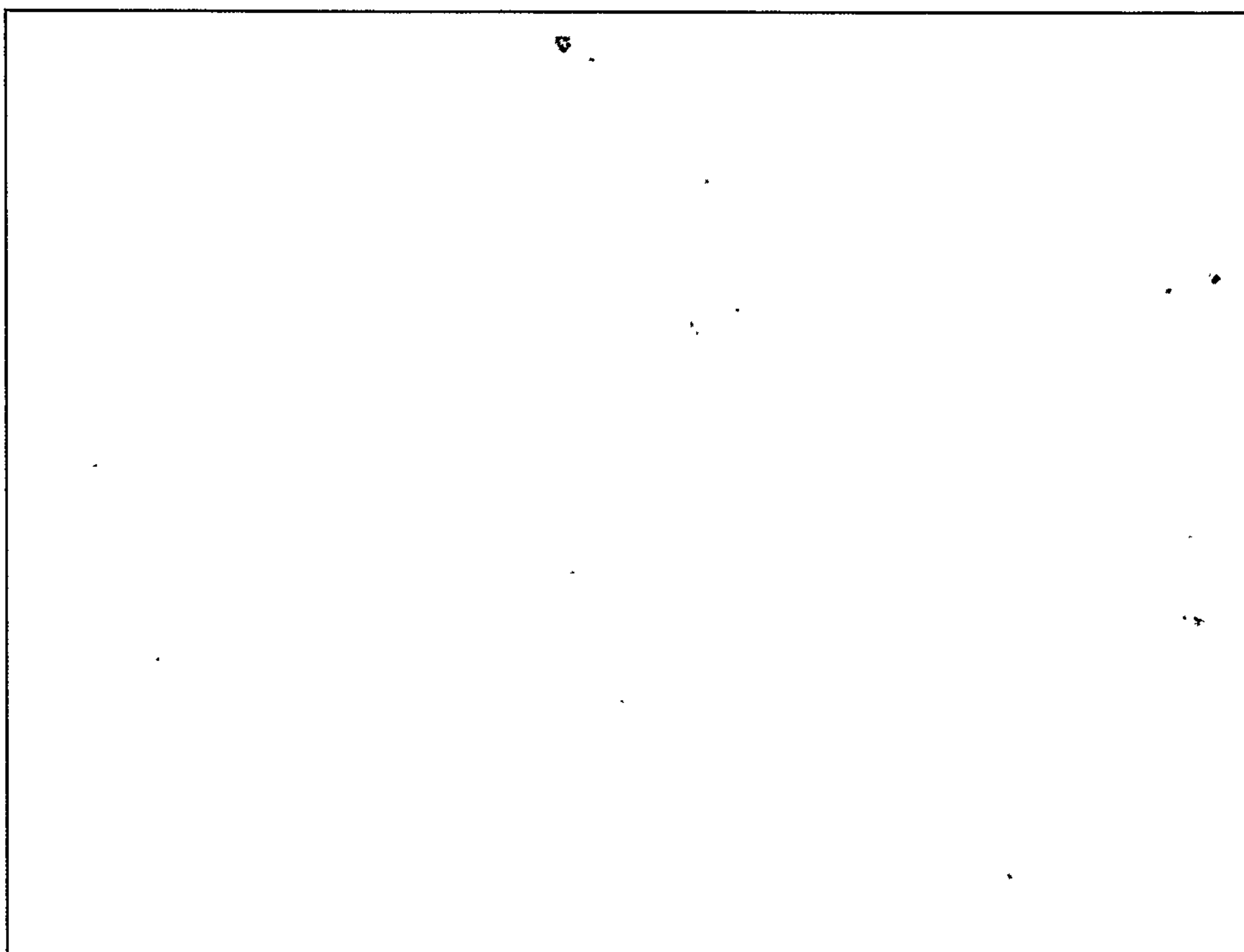


Figure III.21 Fibroblast control stained for osteonectin (Light micrograph, 10x magnification)

III.2.3.2 Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity was used as a marker of mineralising potential in the cultures, and to examine the effect of growth factors on the cell cultures.

Firstly, the ALP activity was compared between a dental pulp cell culture, an osteoblast culture (positive control) and a fibroblast culture (negative control) which had all been maintained in similar conditions (Fig.III.22). The osteoblast cultures showed strong ALP activity compared to the pulp cell culture (2x increased activity), and significantly increased activity compared to the fibroblast cultures ($p < 0.01$). The dental pulp cells showed strong ALP activity (that was not significant) that was 4 times that of the fibroblasts, which showed negligible ALP activity.

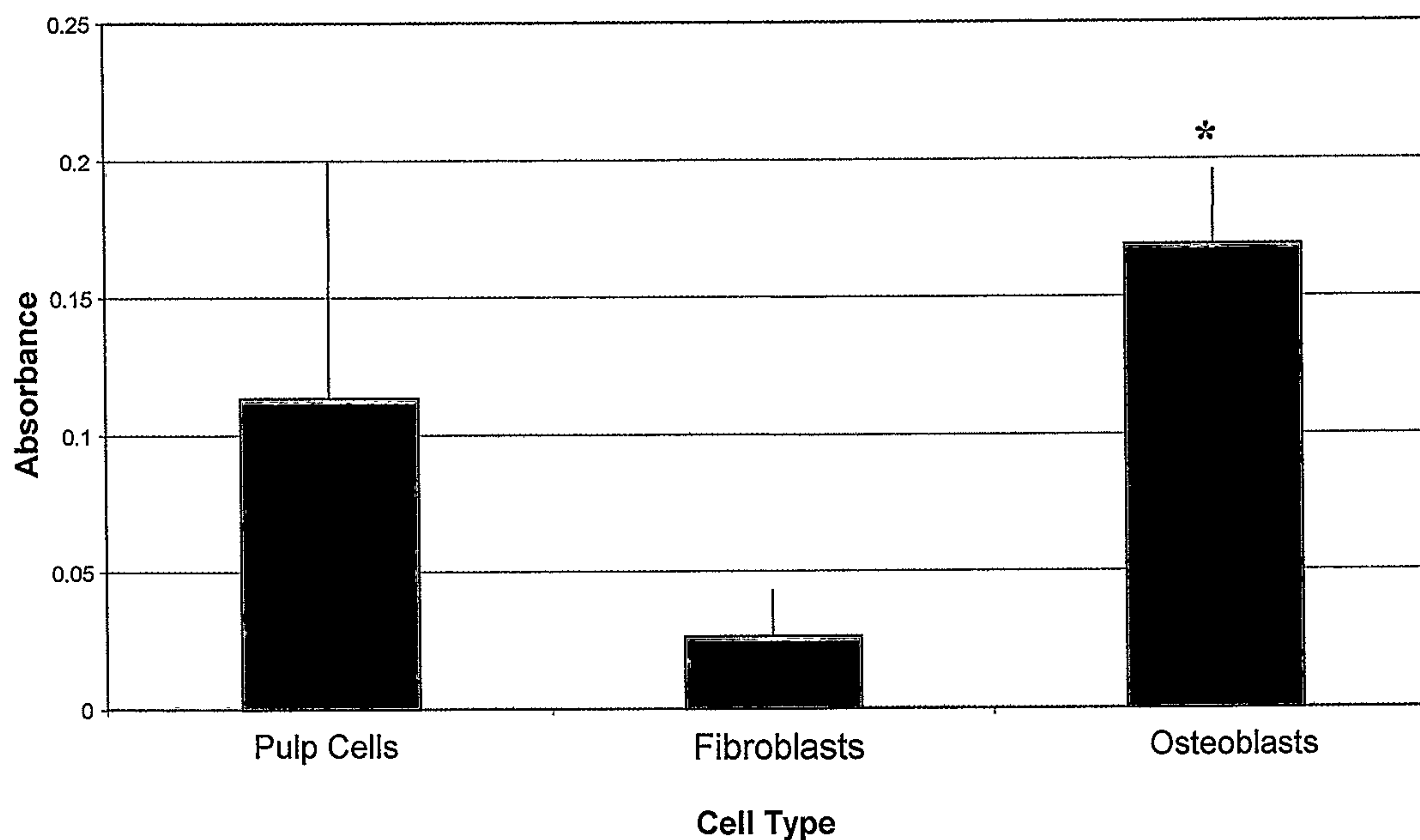


Figure III.22 Alkaline Phosphatase activity of dental pulp cells and controls in basic growth medium at 21 days in culture

* ALP activity of osteoblasts was significant compared to ALP activity of fibroblasts ($p < 0.01$), but not to dental pulp cells.

The effect of β GP in the culture medium was then examined (Fig.III.23). The ALP activity of the mineralising cells (osteoblasts and dental pulp cells) was seen to increase. The ALP activity of the dental pulp cells increased very significantly four-fold ($p < 0.01$), and the ALP activity of the osteoblast increased 1.5 times ($p < 0.05$). The ALP activity of the dental pulp cells was greater than that of the osteoblasts by a factor of 1.3, which was not significant. Again the ALP activity of the fibroblasts was negligible, and not affected by the addition of β GP to the culture medium. The ALP activity of the dental pulp cells treated with β GP was significantly higher ($p < 0.001$) than that of fibroblasts cultured in plain medium and fibroblasts treated with β GP.

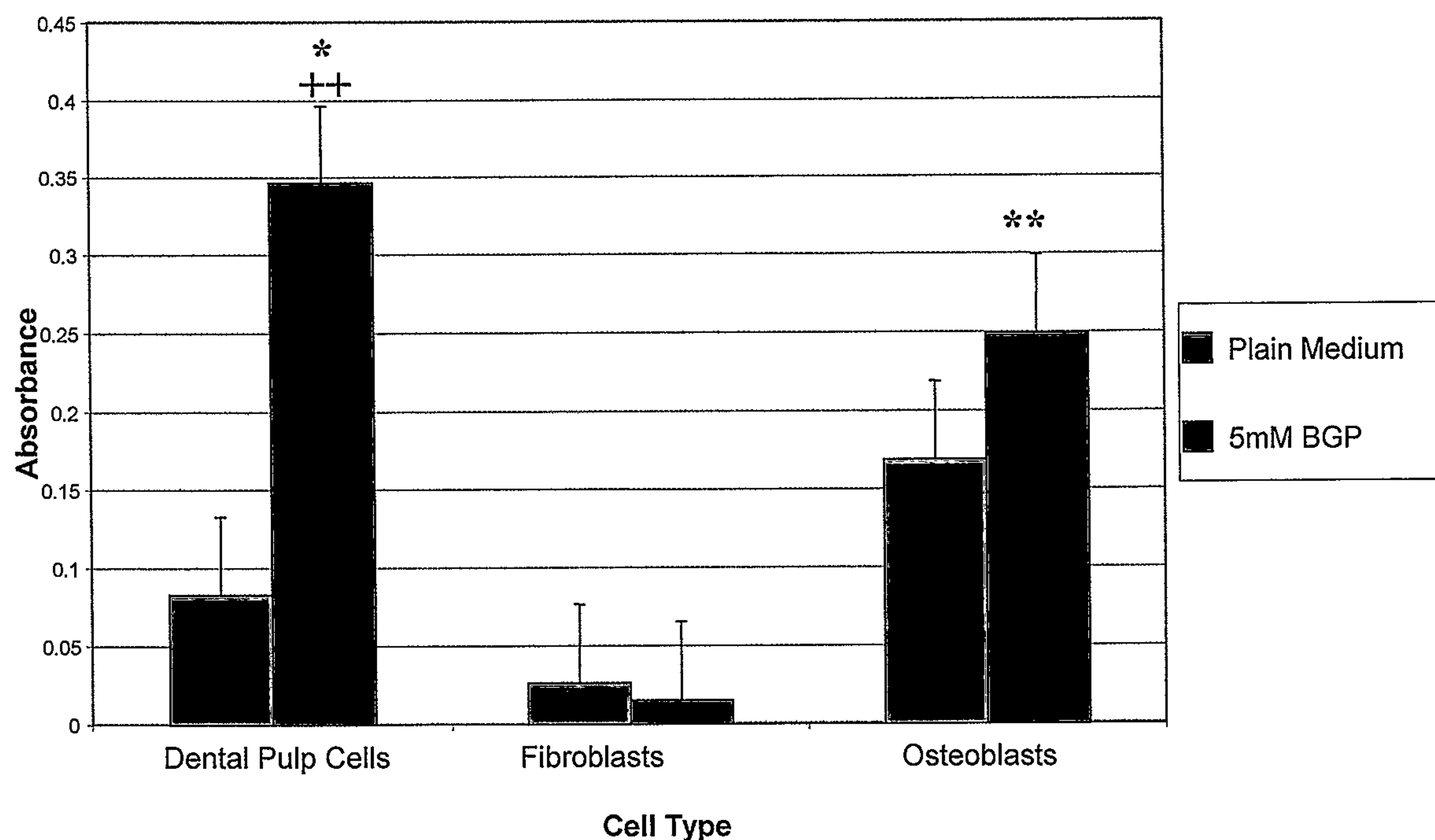


Figure III.23 Effect of β GP on Alkaline Phosphatase activity of dental pulp cells and controls after 21 days of treatment

* Dental pulp cells treated with β GP showed a very significant increase in ALP activity compared to pulp cells in normal growth medium; $p < 0.01$

++ Dental pulp cells treated with β GP showed an extremely significant increase in ALP activity compared to fibroblasts cultured in plain growth medium and treated with β GP; $p < 0.001$

** Osteoblasts treated with β GP showed a significant increase in ALP activity compared to fibroblasts cultured in plain medium and treated with β GP; $p < 0.05$

III.2.3.3 Growth Factors

Alkaline phosphatase activity was further used to examine the effect of differing growth factors on the dental pulp cell cultures and controls. The addition of TGF- β 1 to the culture medium produced a significant 2.5 times increase in ALP activity in the dental pulp cell cultures ($p < 0.05$) (Fig.III.24). The addition of β GP to the culture medium produced a greater significant increase in ALP activity ($p < 0.05$). However,

when TGF- β 1 and β GP were added to the culture medium, a very significant increase in ALP activity was noted ($p < 0.01$) (Fig.III.25). This increase was twice that of TGF- β 1 alone, and 1.3 times that of β GP alone.

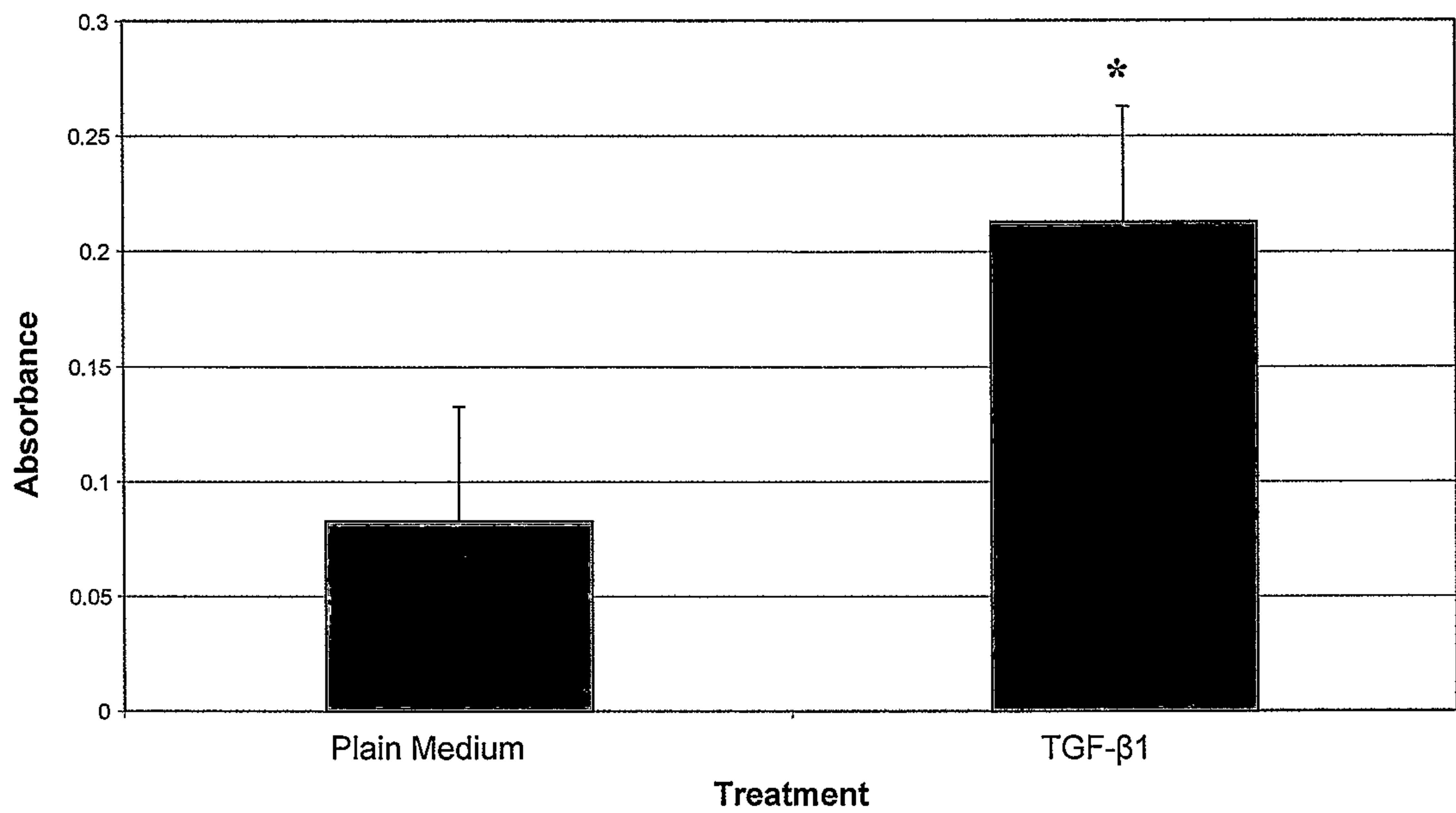


Figure III.24 Effect of TGF- β 1 on Alkaline Phosphatase activity of dental pulp cells after 21 days of treatment

* Dental pulp cells treated with TGF- β 1 showed significantly increased ALP activity compared to cells in normal growth medium; $p < 0.05$

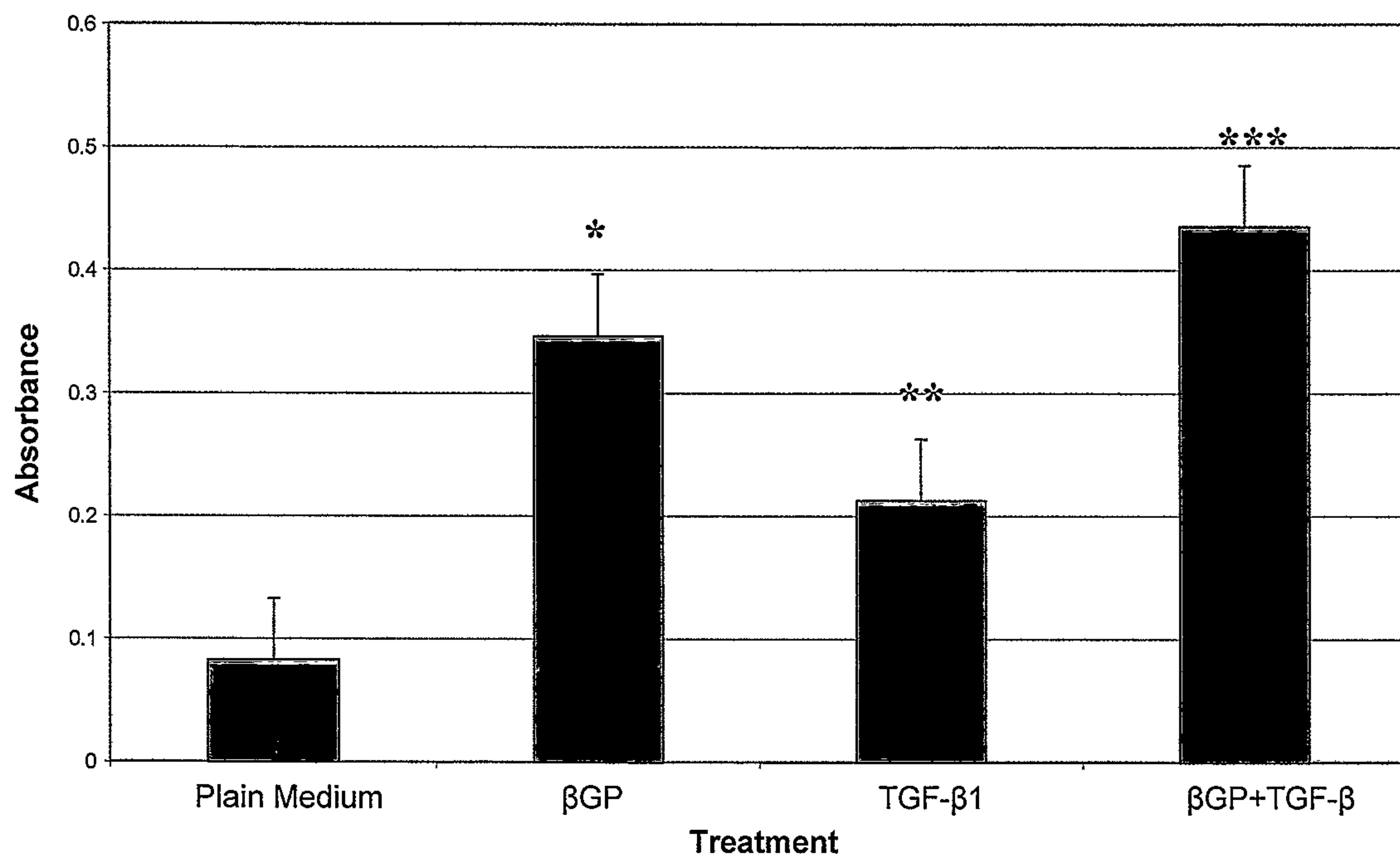


Figure III.25 Effect of β GP and TGF- β 1 on Alkaline Phosphatase activity of dental pulp cells after 21 days of treatment

* Dental pulp cells treated with β GP showed significantly increased ALP activity compared to cells in normal growth medium; $p < 0.05$

** Dental pulp cells treated with TGF- β 1 showed significantly increased ALP activity compared to cells in normal growth medium; $p < 0.05$

*** Dental pulp cells treated with β GP and TGF- β 1 showed very significantly increased ALP activity compared to cells in normal growth medium; $p < 0.01$

The addition of IGF-I to the culture medium had no noticeable effect on the ALP activity of the dental pulp cells (Fig.III.26). There was in fact a slight (though not significant) decrease in ALP activity in the cultures treated with IGF-I.

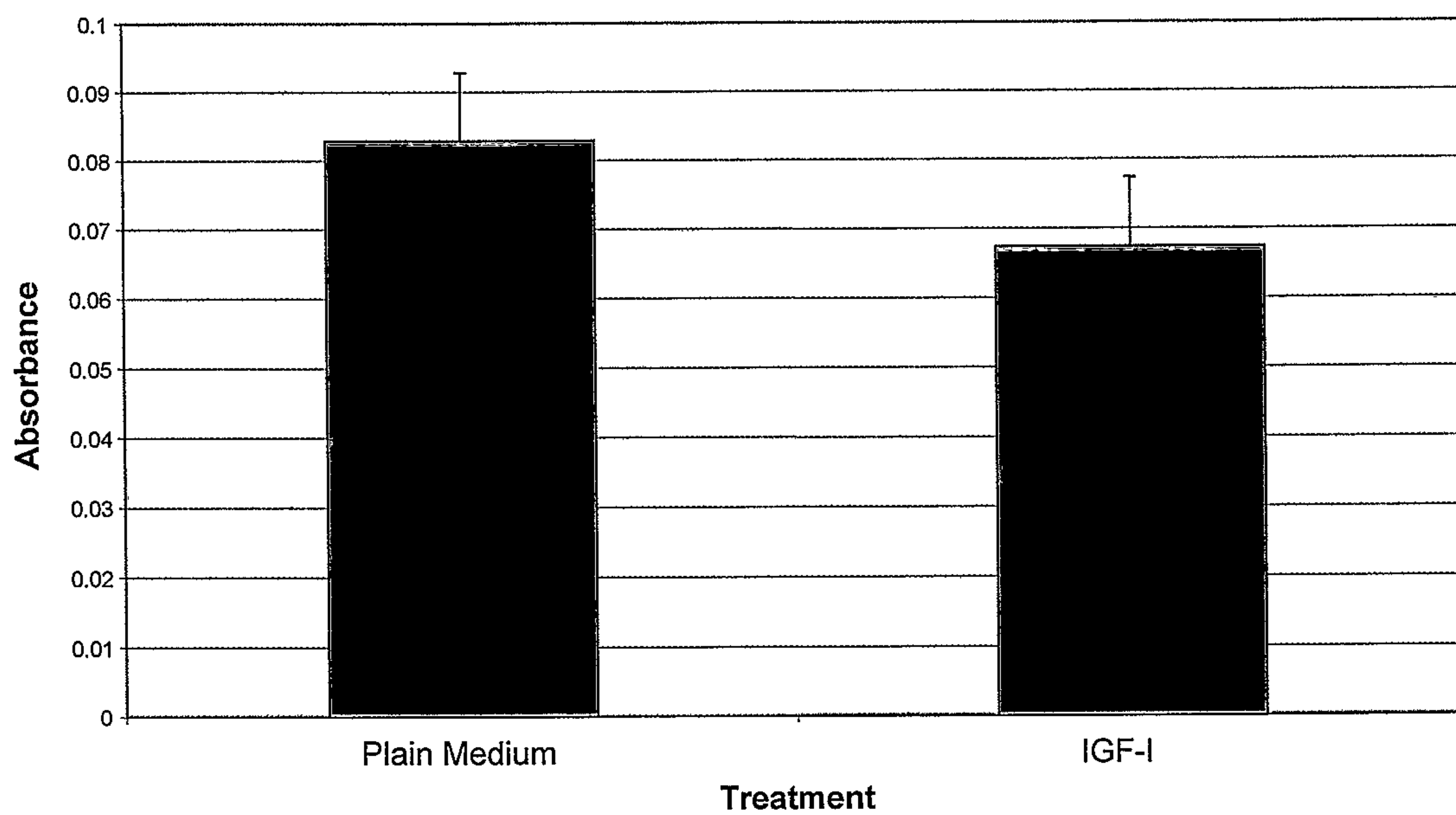


Figure III.26 Effect of IGF-I on Alkaline Phosphatase activity of dental pulp cells

The addition of growth factors to the culture medium had no effect on the ALP activity of the fibroblast cell cultures (Fig.III.27).

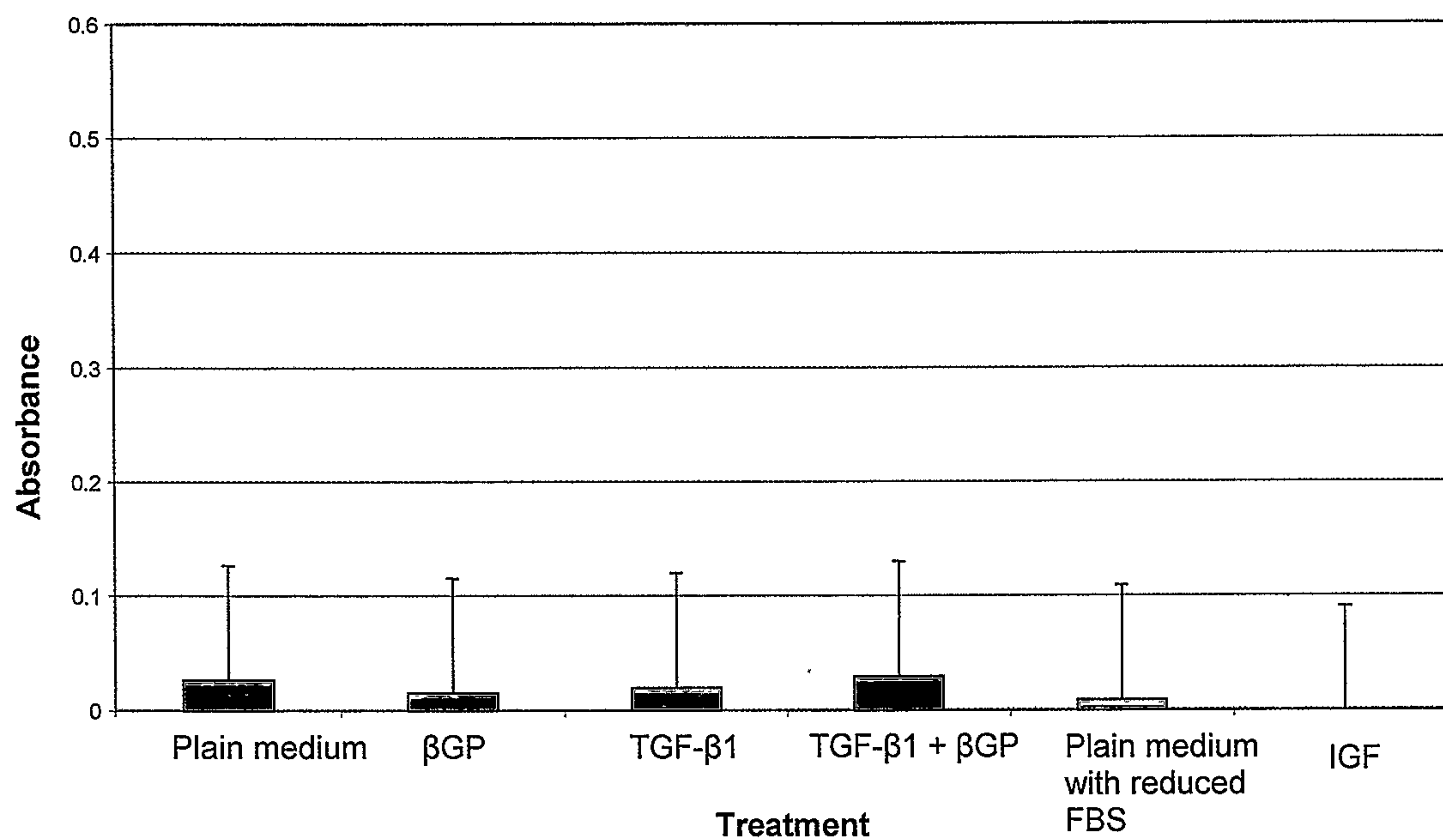


Figure III.27 Effect of βGP and growth factors on Alkaline Phosphatase activity of fibroblasts

The osteoblast cell cultures showed strong ALP activity overall (Fig.III.28). The addition of TGF-β1 had some effect on increasing the ALP activity, but overall the effect of the growth factors was not significant or as dramatic as the effect in the dental pulp cell cultures (Fig.III.29).

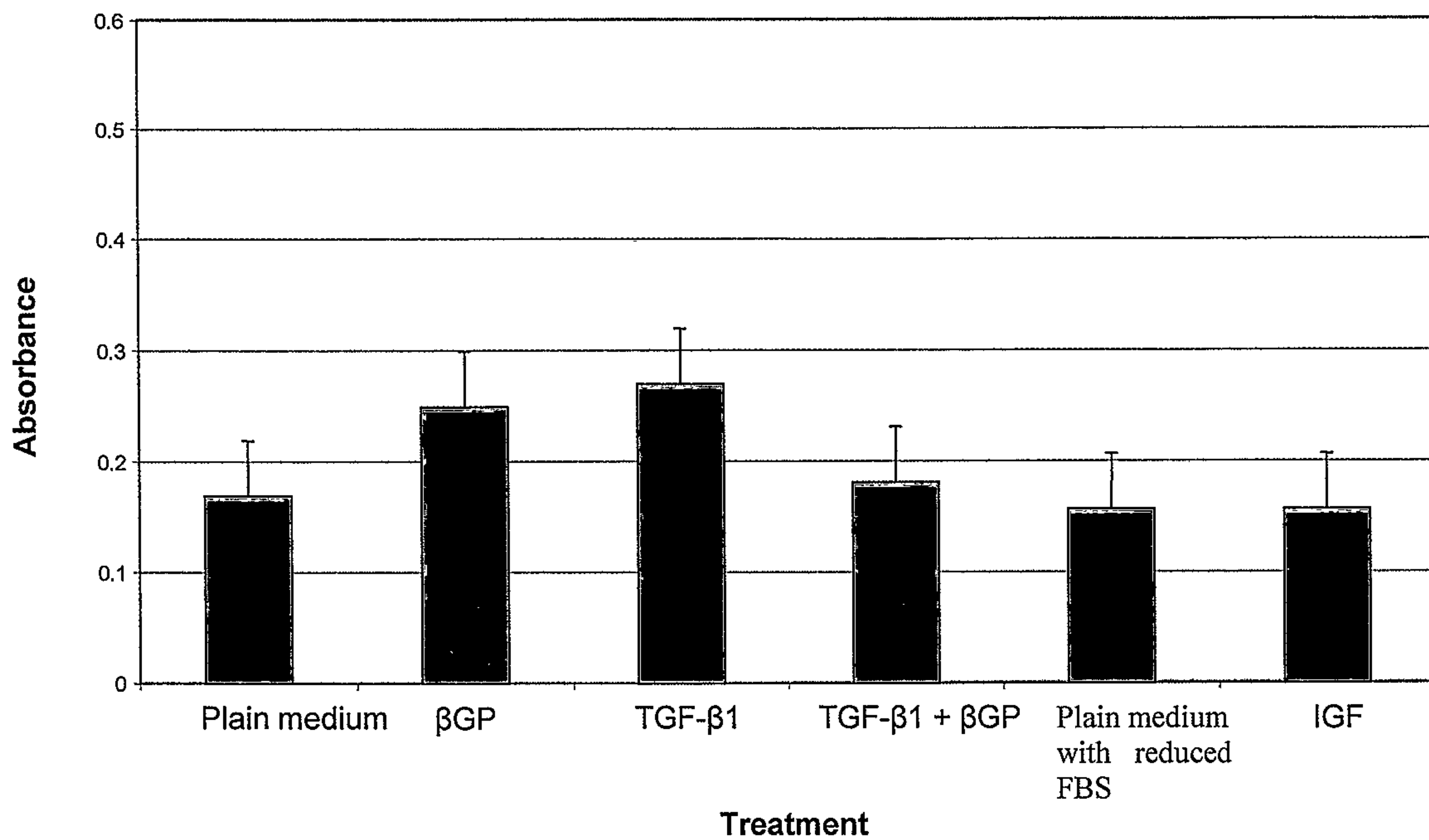


Figure III.28 The effect of β GP and growth factors on the Alkaline Phosphatase activity of osteoblasts

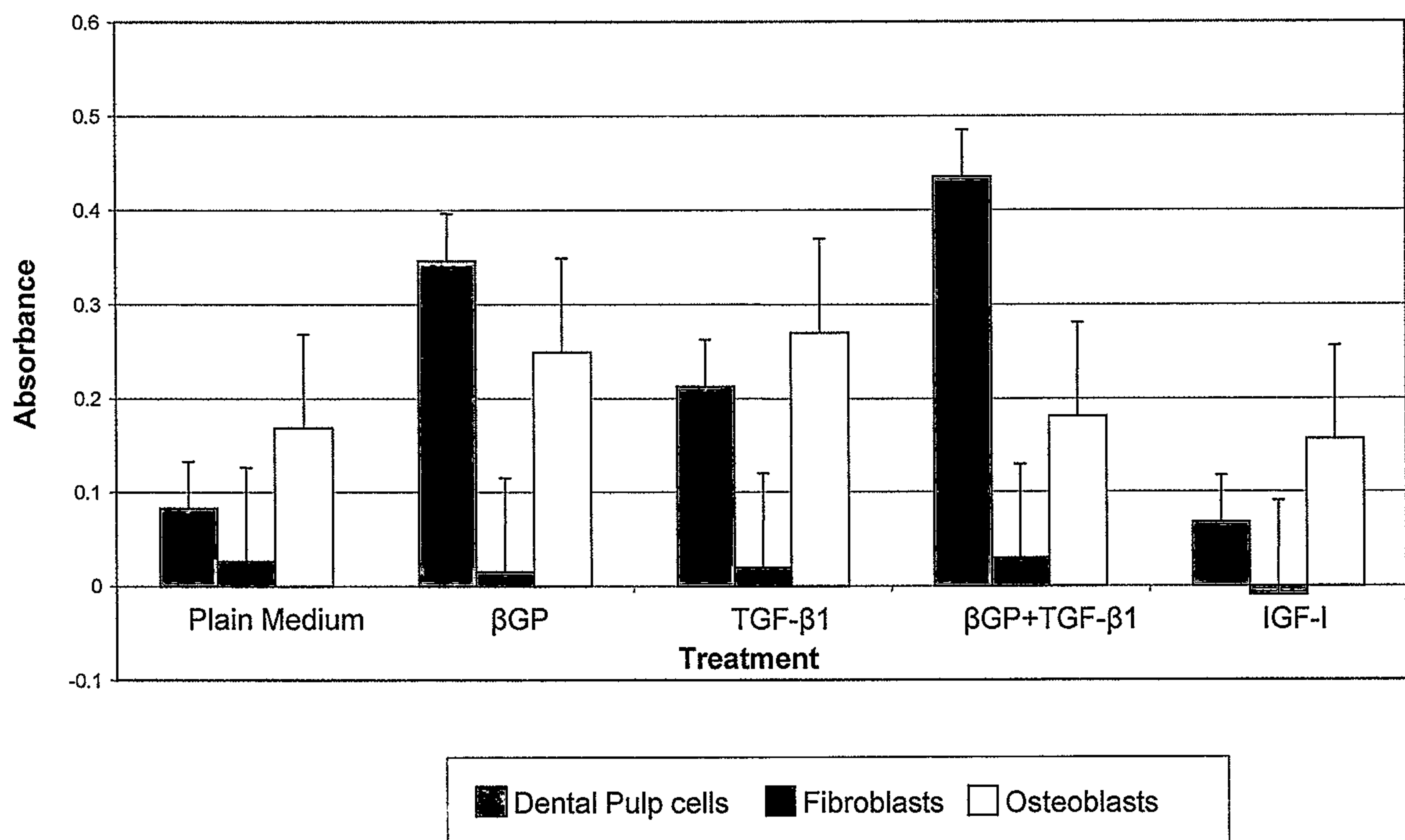


Figure III.29 Comparison of the effect of β GP and growth factors on Alkaline Phosphatase activity of all cell types

III.2.3.4 Scanning Electron Microscopy

Dental pulp cells, fibroblasts and osteoblasts were examined under scanning electron microscopy (SEM) to examine cell morphology.

The dental pulp cells were noted to have a generally elongated morphology (Fig.III.30).

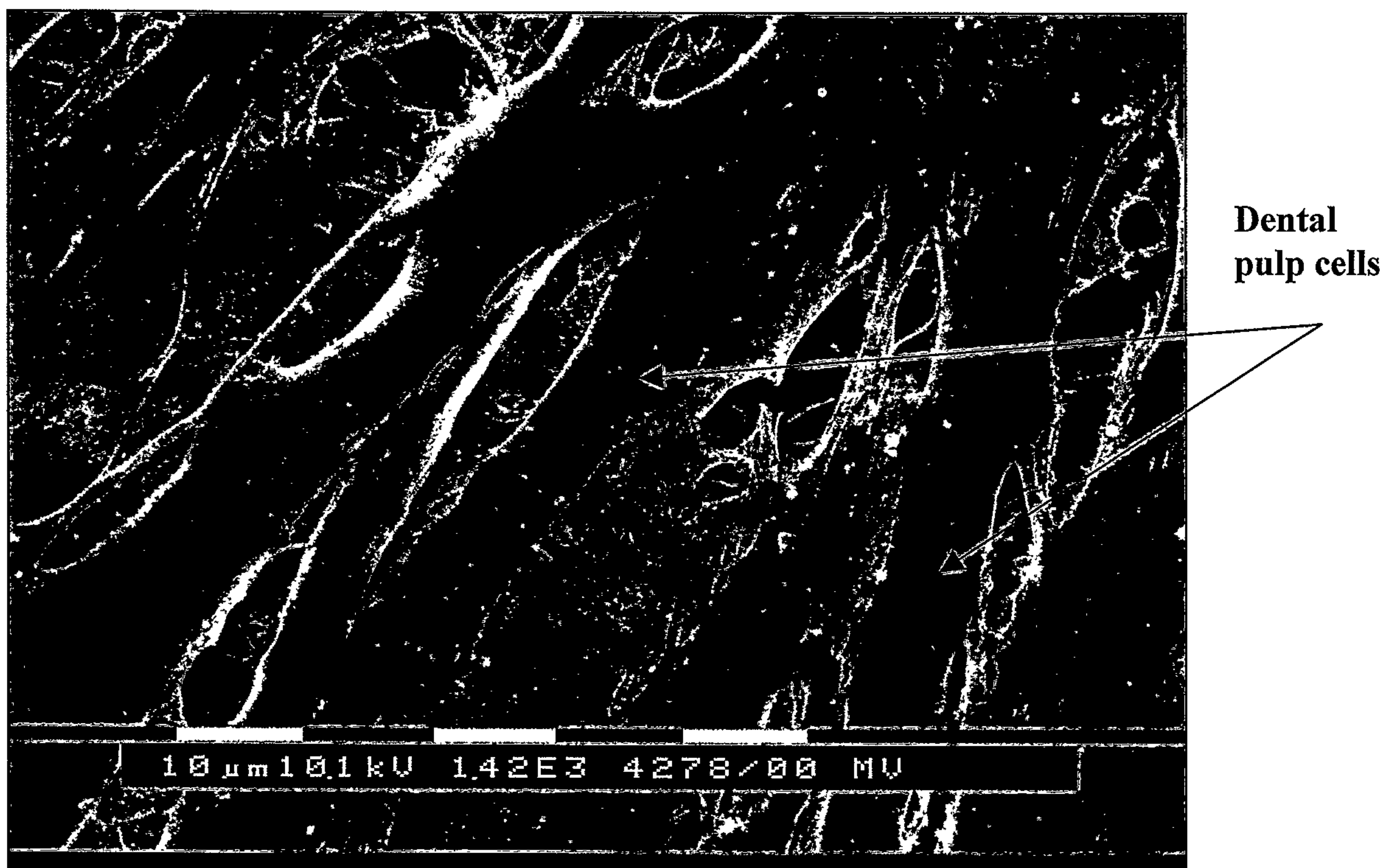


Figure III.30 SEM of Dental Pulp cells showing elongated morphology

The cells formed broad, branching networks of densely packed cells (Fig.III.31).



Figure III.31 SEM of Dental Pulp cells showing broad, branching networks of cells

In appearance, the dental pulp cells were similar to the fibroblast control (Fig.III.32).

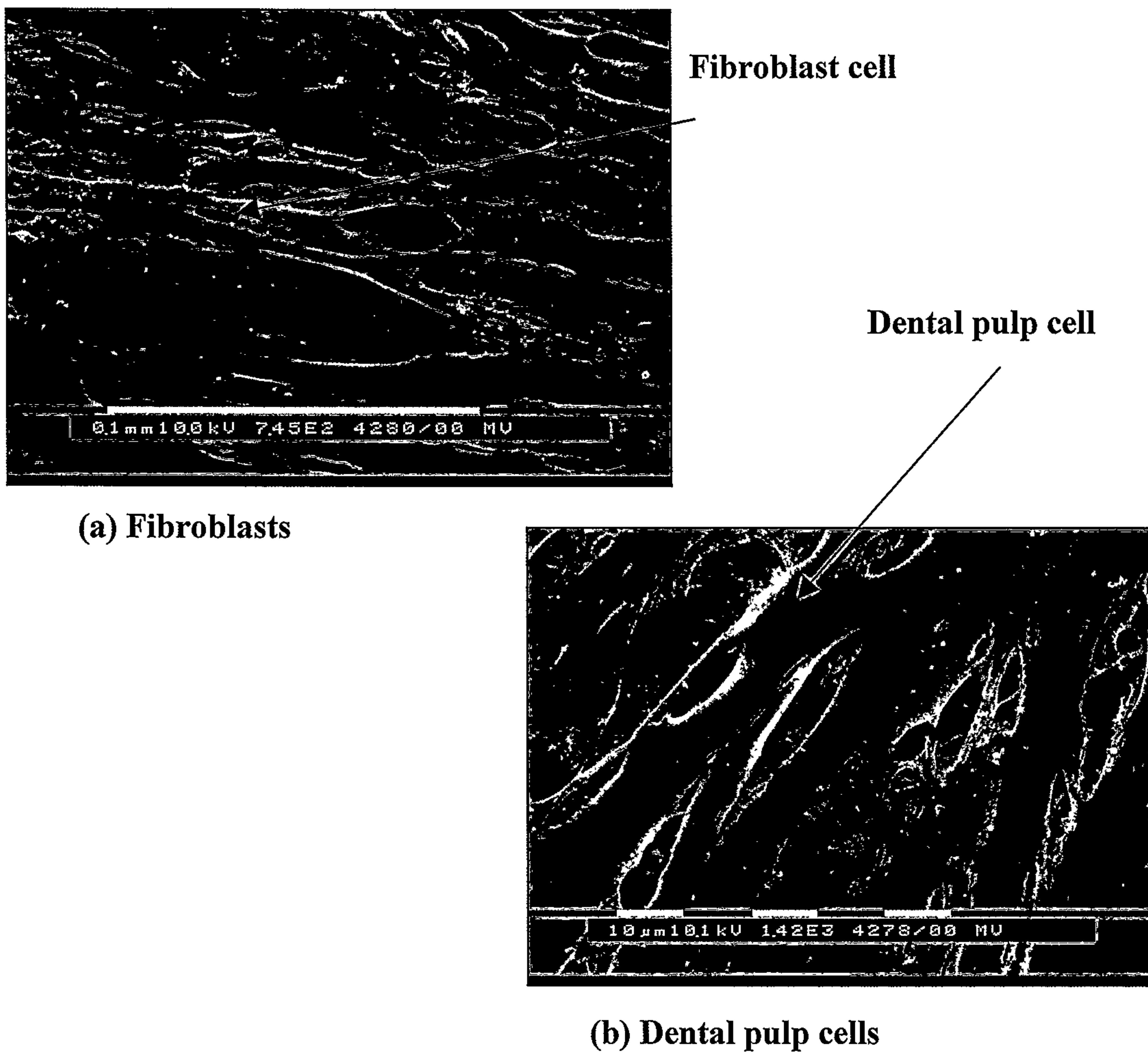


Figure III.32 SEM of (a) fibroblasts and (b) dental pulp cells showing similar morphology

However, in some areas the dental pulp cells were noted to have a broader, polygonal-shaped morphology (Fig.III.33).

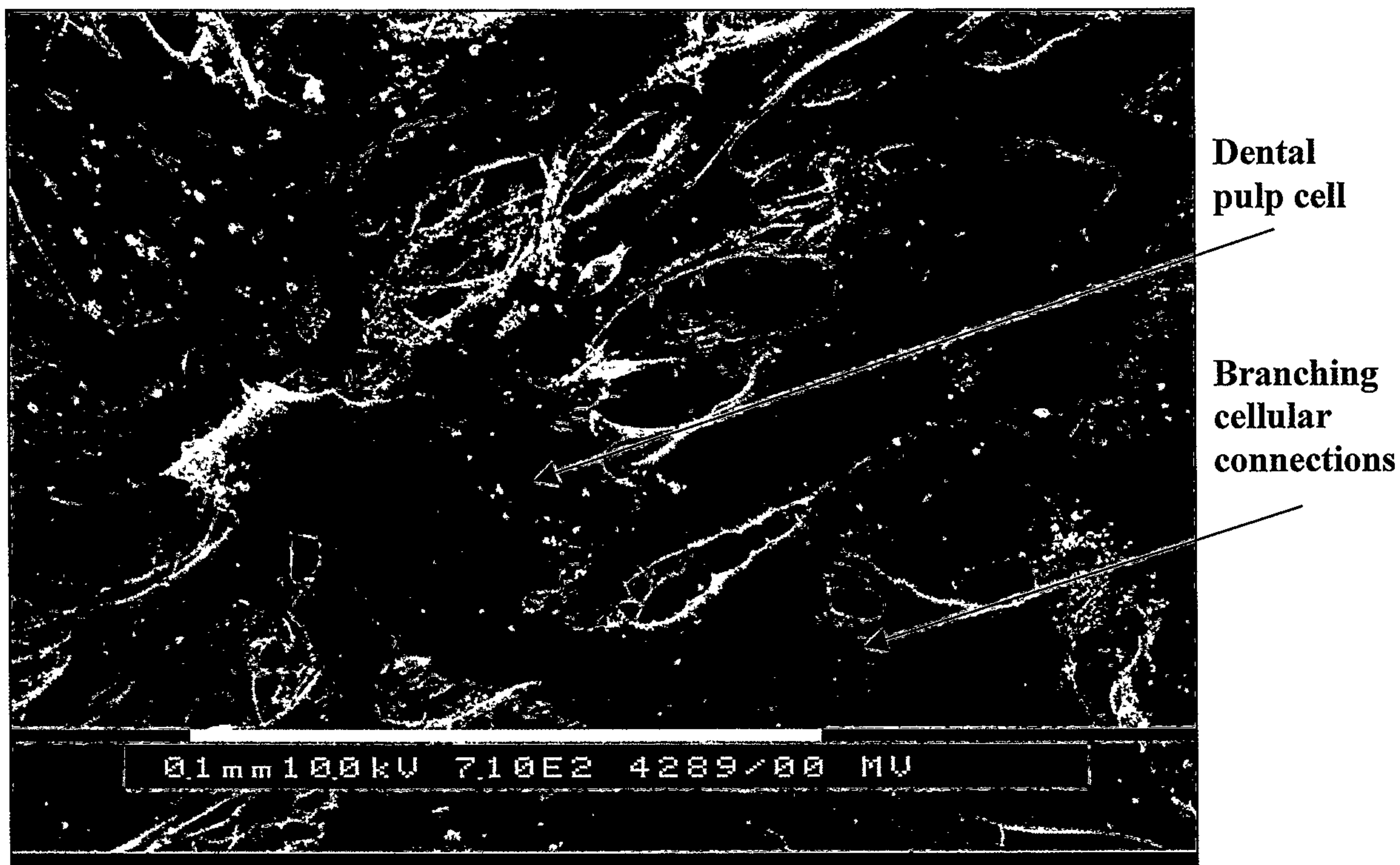


Figure III.33 SEM of dental pulp cells showing polygonal morphology

These cells were similar to the osteoblast controls, which were generally large, polygonal multilayered cells (Fig.III.34).



Figure III.34 SEM of osteoblast control showing large, polygonal multilayered cells

Chapter IV. Discussion

IV.1 Osteoblast Studies

Initial experiments were performed with human osteoblast cells, in order to examine a more readily available mineralising cell type. The studies using osteoblasts were designed primarily to provide the opportunity to learn cell culture techniques and examine the behaviour of mineralising cells in culture systems.

IV.1.1 Basic Cell Culture and Examination of Growth of Cells in a Matrix

In biological systems, mineralising cells such as osteoblasts and odontoblasts are normally embedded in a three dimensional matrix. Gelfoam was used to mimic this condition in a culture system.

An established line of osteoblast cells was used for initial studies. These cells were initially derived from mesenchymal stem cells in human trabecular bone that was harvested during surgery. When cultured under conditions that provide ascorbate and phosphate, they have been shown to differentiate into well characterised osteoblasts (Slater et al 1994a; Slater et al 1994b). Ascorbic acid has been shown to induce differentiation of mouse bone marrow cells into osteoblast-like cells (Otsuka et al. 1999). Ascorbic acid induces alkaline phosphatase activity, the deposition of calcium and the formation of mineralised nodules by bone marrow stromal cells.

When seeded into a matrix, the osteoblastic cells were observed to grow strongly. Scaffolds are increasingly playing an essential role in tissue engineering by guiding new tissue growth *in vivo* and *in vitro* (Griffith 2002). Gelfoam is a sterile, absorbable gelatin sponge used as a haemostatic agent in surgical procedures. It is readily available for commercial purchase, and is relatively inexpensive. The Gelfoam acts as an extracellular matrix, with the aim of providing a cell-adhesion substrate for the osteoblastic cells.

When histological sections were examined microscopically, the Gelfoam was seen to consist of a highly interconnected network of open pore cells. This type of structure has been shown to enhance superior bone cell colonisation and mineralisation throughout a scaffold, rather than just a superficial mineralisation (Holy et al. 2000). The osteoblast cells could be seen to line the walls of the Gelfoam pores and in some areas were multilayering. The cells that had been treated with $1,25(\text{OH})_2\text{D}_3$ showed a higher rate of proliferation and infiltration, with increased areas of multilayering. The enhanced proliferation and pattern of infiltration of cells treated with $1,25(\text{OH})_2\text{D}_3$ is in accordance with the findings of Holy et al (2000). The improved cellular growth of treated samples demonstrates the importance of $1,25(\text{OH})_2\text{D}_3$ supplementation for osteoblast growth and differentiation. The provision of a matrix to direct and support cellular growth, and the provision of non-specific mineral stimulation in the form of ascorbic acid and βGP may be more important for osteoblastic growth.

IV.1.2 Influences on Mineralisation

Potential influences on mineralisation of osteoblasts in culture were examined in a basic cell culture system. Mineralisation was assessed by alizarin red assay and [^3H]-tetracycline assay.

IV.1.2.1 Effect of $1,25(\text{OH})_2\text{D}_3$ and Dexamethasone

Dexamethasone is a glucocorticoid hormone, with a range of biological roles. These include inhibition of osteoblast proliferation, but stimulation of chemotaxis, type I collagen formation and fibronectin-metabolism of bone stromal cells (Kim et al. 1999). $1,25(\text{OH})_2\text{D}_3$ has been demonstrated to increase mineralisation activity of osteoblastic cells in culture (Marie et al. 1989), although high doses have been shown to inhibit mineralisation (Hock et al. 1986).

The addition of $1,25(\text{OH})_2\text{D}_3$ to the osteoblast cultures as a treatment resulted in a slight increase in mineral accumulation as observed by alizarin red staining. When mineralisation was assessed by [^3H]-tetracycline incorporation, there was a more pronounced increase in mineralisation in cultures containing $1,25(\text{OH})_2\text{D}_3$ compared to no treatment. Dexamethasone had little effect in this system. It appears that the effect of specific mineralisation modifiers is not as important as non-specific modifiers such as the availability of phosphate and calcium.

IV.1.2.2 Effect of Calcium and β GP in Media on Mineralisation

β GP is known to promote mineral deposition when added to the culture media of cells with the potential to produce mineral, such as chick limb-bud mesenchymal cells (Boskey et al 1996), human and rat bone marrow cells (Yao et al. 1994) and human odontoblastic cells (Camps et al. 2002). Various concentrations of β GP have been trialed. A level of 5mM has been shown to enhance and bring forward mineralisation of osteoblasts in culture (Slater et al 1994a).

In the initial studies, it was postulated that the effect of β GP may be the most important factor in mineralisation of short term osteoblast cultures. To further examine this effect, cells were studied in a low calcium medium. Alizarin red staining clearly showed that the presence of β GP was the determining factor for increased calcium accumulation. When examined by [^{3} H]-tetracycline incorporation, the effect of β GP was not so apparent, with low overall mineral accumulation. However, the effect of β GP was seen to increase over time, with the β GP having a more significant effect on mineralisation at 21 days than at 14 days in culture. [^{3} H]-tetracycline assay showed a dramatic increase in the effect of β GP and calcium in the culture medium. This is in accordance with other observations (Holy et al 2000; Slater et al 1994b). Clearly calcium and β GP are required for mineralisation, with other agents having a lesser effect.

IV.2 Odontoblast Studies

IV.2.1 Methods for Obtaining Odontoblastic Cells from Available

Material

There are no reported studies in the literature which pertain to harvesting dental pulp cells from extracted primary teeth. There are very limited studies concerning the collection and culturing of human dental pulp cells, yet it is acknowledged that human studies are essential for understanding the processes involved in the disease dynamics of the pulp-dentine complex (Tjäderhane et al 1998). The limited numbers of reports on obtaining human odontoblasts use extracted third molar teeth from young people. The most promising method appeared to be that of About et al (2000), which reported that the apices of the third molars collected were cut off with a scalpel, and the pulps easily removed. The age of the patients was given as 16 years old (About et al 2000a). This method was trialled initially, but was immediately found to be less than ideal. The teeth used in this experiment were fully mature primary teeth – that is, they had full root formation with an intact apex and no evidence of root resorption. It was found to be impossible to remove the apex with a scalpel. Following this attempt, a communication was made with the team who had produced the papers of About et al (2000). It was determined that the teeth used in their studies were in fact very immature third molar teeth, that were removed from patients aged 12 to 16 years old. The apices of these teeth were very immature, and the pulps apparently very easy to remove (M-L Couble, personal communication, 2003). The step that involved removal of the apex with a scalpel can be assumed to involve only removal of apical fibrous tissue.

At the Westmead Centre for Oral Health (NSW, Australia), third molar teeth are not routinely extracted on young patients. It is seen to be preferable to remove these teeth when root formation is more complete, as it makes removal easier (Personal communication, Department of Maxillo-Facial surgery, Westmead Centre for Oral Health, 2003). Hence there is not a ready supply of immature third molar teeth to use for dental pulp cell culture. In addition, it must be questioned whether the use of teeth that are still developing give a true representation of the “real-life” clinical situation involving disease processes in mature teeth. It is well-recognised that developing teeth and primary dentinogenesis involve a complex interplay of interactions between mesenchymal and epithelial components, hormones and growth factors (Thesleff & Sahlberg 1996). In the *in vivo* situation, the interplay of factors is very different (Tziafas 2004). Although the work by About et al represents a significant advance in the understanding of pulp dynamics in humans, it must be considered whether the use of immature teeth provided the cell cultures with growth factors and conditions that may not be present *in vivo* (About et al 2000a).

The present investigation aimed to make use of readily available resources. In the department of Paediatric Dentistry at the Westmead Centre for Oral Health, New South Wales, many children present with carious primary teeth that require extraction under general anaesthesia as part of complete dental treatment and rehabilitation (Alcaino et al 2000). These teeth would normally be disposed of as biological waste. It was considered that the extracted teeth may be used to as a readily available source of dental pulp cells. In addition, as the teeth were fully formed, only the dental pulps would be cultured. This would give a more realistic representation of an *in vivo*

situation, as there was no possibility of extra growth factors being present. It was not known whether the dental pulps of primary teeth have a differing pulpal response to permanent teeth. It is known that primary teeth do have the capability to form reparative types of dentine, as evidenced by the success of indirect pulp treatment in primary teeth, and the clinical signs of intra-pulpal obliteration following trauma (Diab & ElBadrawy 2000;Farooq et al. 2000;Ranly & Garcia-Godoy 2000).

A number of methods were trialled to find the most efficient and effective way of obtaining dental pulps from extracted primary teeth. The initial method trialled was based on two studies that reported partially sectioning teeth with saws or drills to obtain the dental pulps (Guo et al 2000;Tjäderhane et al 1998). A laboratory motor was used in the sterile biohazard hood. It was found to be extremely difficult to manage the amount of heat produced by the motor. The use of frequent saline washing was not effective for heat control, and made the samples very difficult to handle. The majority of the dental pulps were destroyed by heat production. The small number of pulp explants that were obtained through this method developed severe fungal infections within days of harvesting. It was thought that this was due to the fact that the technique was not absolutely sterile. In addition, some dental pulps appeared to be dead on arrival at the laboratory, despite a clinical appearance of vitality. It was considered that these pulps may have been clinically necrotic, or that the transport medium could not penetrate through the root apex to the cells of the dental pulp.

Ideally all scoring or sectioning of teeth would be done in a sterile biohazard hood. However, the limited availability of portable, sterile dental drills or sectioning equipment made this an impractical measure. Hence alternatives were sought that

would be as sterile as possible, but still efficient and practical. A second method was trialled, again based on Tjäderhane et al (1998) in which the tooth had a groove cut into the surface with a high-speed dental drill using a sterile diamond bur and sterile coolant. This initially was done in the dental clinic for the sake of convenience. The tooth was then transported to the laboratory biohazard hood, where final sectioning was completed using surgical chisels. This method was more successful, but again it was difficult to hold the tooth steady and maintain sterility.

These difficulties led to the development of the final protocol. The teeth were sectioned in the operating theatre, which represented a “clean”, if not absolutely sterile environment. The teeth were scrubbed with Betadine prior to sectioning, to reduce the bacterial load as much as practicable. All drills and coolants were also sterile. The teeth were almost completely sectioned, and placed into cold sterile media. There was communication between the dental pulps and the transport medium. The dental pulps were noted to survive in transport medium for up to 8 hours. It was considered that the communication created between the pulp and the medium allowed for maintenance of cellular viability. Without a deliberately created communication, the opening of the root apex would most likely have been insufficient to allow ingress of media to the dental pulp. On arrival at the laboratory, it was found that the teeth were easily sectioned with a surgical chisel, as they had been almost sectioned by the drill. The most efficient plane of sectioning was horizontally, as the apical and coronal pulpal fragments could be accessed more easily. With a vertical sectioning, there was increased trauma to the pulp during sectioning. The pulp fragments were removed with endodontic files or simply small tissue forceps. This method seemed to allow atraumatic retrieval of the dental pulps in as aseptic a manner as possible.

IV.2.2 Establishment of a Culture System to Allow Proliferation of Harvested Tissue

Once the mode of harvesting for the dental pulps was established, the culture system was optimised. The basic culture medium was based on that used for other mineralising cells (osteoblasts) and those reported in the literature (About et al 2000a; Couble et al 2000). Initial problems with excessive bacterial and fungal contamination seemed to be related more to the initial harvesting technique rather than the culture system. The rate of infections slowed dramatically as the sampling technique was refined, and the investigator became more proficient at extracting the dental pulps efficiently.

Antibiotics have been used in most reported cultures of human dental pulps (Couble et al 2000; Tjäderhane et al 1998), and some investigators have added antifungal medicaments as well (Tjäderhane et al 1998). About et al (2000) did not use antibiotic supplementation (About et al 2000a). Antibiotics are a useful tool in cell culture, but are not recommended for routine use. They are of particular use in primary cell cultures, where their use is effective, non-toxic and stable (Perlman 1979). For this reason, antibiotics were added to the initial primary cultures to manage contamination, but were not added once the cultures were growing strongly.

Couble et al (2000) did not state how long the explant cultures took to develop strong cellular outgrowth in culture. The cultures were maintained for 4 to 8 weeks before further characterisation (Couble et al 2000). No mention was made of whether cells

were subcultured during this time. About et al (2000) noted cell proliferation and adhesion of dental pulp cells after 3 days of explant culture (About et al 2000a). After 3 weeks in culture, regular and fibre-like structures started to appear from the explant border and extend toward the peripheral parts. In the presence of β GP, the deposition of mineral crystals was noted. The dental pulp cell cultures in this experiment adhered in a similar fashion to that of About et al (2000). In healthy cultures, initial adhesion and proliferation was noted within 3 days, and regular structures appeared between 10 and 21 days. This is in accordance with other published observations regarding more immature pulps (About et al 2000a). It was noted that the explants were very susceptible to movement. At one point early in the culture period, the flasks were moved due to a fault in the incubator. All samples detached from the culture surface and were floating. Although it was possible to encourage reattachment, and the cell proliferation continued, it is possible that this disturbance lengthened the time frame required to reach strong proliferation.

When the cells reached confluence, they were subcultured. Although About et al (2000) reported using trypsin and EDTA, when this was tried all the cells were destroyed. It was decided to follow the protocol used for osteoblasts, which proved to be too short for the dental pulp cell cultures. A final subculture protocol was achieved through trialing different time periods of trypsinisation. The final protocol seemed to give good detachment of cells without cellular destruction. As seen with cells from more immature pulps, the subcultured cells adhered to the culture surface more quickly, and proliferated faster than the original explants (About et al 2000a).

The morphology of the proliferating cells as seen by light microscopy appeared to be similar to that reported by other investigators (About et al 2000a; Onishi et al 1999). The cells were predominantly spindle-shaped, and appeared from the explant border. They proliferated towards the peripheral parts as regular, fiber-like structures. The appearance of the cultures was noted to be similar to osteoblasts grown from the trabecular ends of long bones (Patava 1996; Slater et al 1994a). Although no mineral nodule formation was seen, there were areas of concentration of fibres in a circular pattern.

About et al (2000), noted deposition of mineral crystals along and within the fibrous structures, and the formation of mineralized nodules (About et al 2000a). This was observed in cultures treated with β GP for 8 weeks. No mineralisation was noted in cultures that were not treated with β GP. Surprisingly, the culture medium of About et al (2000) was not supplemented with ascorbic acid required for collagen synthesis. β GP is used in cell culture systems to induce and/or increase mineralisation of collagenous matrices produced by mineral-forming cells *in vitro* (Boskey et al 1996). The investigators speculated that the mineralisation process may have been accelerated if ascorbic acid had been added to the culture system. They did not comment on the possibility of using ascorbic acid without β GP.

Couple et al (2000) did not observe the formation of mineral in the culture system, even when β GP was added (Couple et al 2000). However, they did note the cells grown in the presence of β GP showed a greater proportion of polygonal-shaped cells, as opposed to fibroblastic cells. When the cultures were examined under transmission electron microscopy, areas of mineralisation were noted in the β GP treated cultures. It

was suggested that β GP may have a role in pulp cell differentiation *in vitro*, but this function remains to be proven.

Gronthos et al (2000) did not report the use of β GP in their methodology of culturing human dental pulp stem cells (Gronthos et al 2000), apparently relying on ascorbic acid in the culture medium to promote growth and differentiation. Dexamethasone was added to stimulate the formation of mineral. However, in their discussion the use of non-organic phosphate in addition to ascorbic acid and dexamethasone was mentioned. Yokose et al (2000) supplemented their media with ascorbic acid and 1mM β GP (Yokose et al 2000). This was based on concerns that in the presence of high ALP and β GP, calcium may deposit even in a cell-free system and be interpreted as mineral formation (Chung et al. 1992).

In this study, the media was supplemented with ascorbic acid, to promote the growth of mineralising cells. β GP was used as a treatment in further investigation of the character of subcultured cells. The absence of visible mineral formation in the explant cultures may be in part due to the absence of β GP in that culture system. Alternatively, the explant cultures may not have differentiated as quickly, and may not have produced observable mineral even with the addition of β GP.

The first explant obtained did not display as strong cellular proliferation as subsequent explants. A decision was made to add a growth factor (IGF-I) to the culture medium in an attempt to stimulate growth. IGF-I was chosen for convenience, as it was readily available at the time it was required. In addition, IGF-I has been considered to be an useful general growth factor, as insulin is an important hormone for the growth and

proliferation of a variety of somatic cells in culture (Straus 1984). Some studies have indicated that it may be involved in the regulation of cell growth in dental pulp explants (Nagata et al 1989). As the biological effects of insulin and IGF-I and IGF-II are similar to each other (LeRoith & Roberts Jr 1993), it has been suggested that the insulin-like growth factors may play an important part in the growth and differentiation of dental pulp cells (Onishi et al 1999). However, the conclusion that IGF-I might have a primary role in the growth and differentiation of pulp cells has not been supported by other investigators. The effect of IGF-I on the first dental pulp cell culture may be is most likely to be a general stimulation of cellular growth, rather than a specific effect on differentiating dental pulp cells. The effects of IGF-I were further investigated in subcultures of dental pulp cells.

Other dental pulp explants displayed strong adhesion and cellular proliferation without the addition of stimulatory factors to the culture medium. This was in accordance with the reports in the literature (About et al 2000a;Couble et al 2000). In further investigations, only the pulp cultures grown without extra stimulation were used.

IV.2.3 Characterisation of Cells Grown in Culture

IV.2.3.1 Protein Expression

The detection of dentinal proteins (type I collagen and non-collagenous dentine proteins) has been used by multiple investigators to characterise cells grown in mixed dental pulp explant cultures. As dental pulp cells differentiate into odontoblast-like cells they begin to express gene products and proteins that form the organic

component of dentine. Two proteins have been shown to be only present in dentine - dentine phosphoprotein (DPP) and dentine sialoprotein (DSP) (Butler & Ritchie 1995). Antibodies are not commercially available to these dentine-specific proteins.

In this investigation, dental pulp cells were characterised by immunohistochemistry. Specifically, the expression of osteonectin (ON) was examined. ON is one of the non-collagenous proteins in dentine, and is also present in bone (Butler & Ritchie 1995). It is a phosphorylated glycoprotein which inhibits hydroxyapatite growth, but promotes calcium and phosphate binding to denatured collagen. In erupted porcine teeth, anti-ON staining in dentine has been shown to be concentrated around the dentinal tubules, weaker in the cells of the pulp, and intense in odontoblasts (Tung et al 1985). In human teeth, the predentine, odontoblasts and their cell processes show a strong staining reaction (Reichert et al 1992). In situ hybridisation studies with ON mRNA show an intense reaction over the odontoblasts. ON has been considered to be a positive marker of odontoblast phenotype (Bègue-Kirn et al 1994). Anti-ON is commercially available for purchase and use, making it an efficient method of characterising dental pulp cells.

Polyclonal antibody to ON was used to differentiate between odontoblast-like cells and a fibroblast control. Dental pulp cell cultures from 2 differing samples were tested. All samples showed uniformly strong staining for ON. In contrast, fibroblasts that were cultured under similar conditions showed no reaction to osteonectin staining. The use of immunohistochemistry allowed the demonstration of a strong protein expression throughout the sample. Protein expression was uniformly strong throughout the sample, and concentration of expression was not noted. It appears that

in these dental pulp cell samples, the majority of cells are expressing an odontoblast-like phenotype.

About et al (2000) used immunohistochemistry to demonstrate the expression of osteonectin in dental pulp cells (About et al 2000a). ON was found to be expressed by all cells treated with β GP, and the staining intensity was higher in cells contacting the mineralisation nodules. It was not stated whether the expression of ON was examined in cells not treated with β GP.

Other investigators have made use of in situ hybridization to characterize dental pulp cells. Couble et al (2000) used this technique to demonstrate the presence of dentine sialophosphoprotein (which codes for DSP and DPP) (Couble et al 2000). The gene was seen to be weakly expressed in cells before mineralisation of the extracellular matrix, and more strongly by cells located in mineralising areas. Whilst the expression of DPP and DSP is absolute evidence of the development of an odontoblastic phenotype, their expression appears to be more pronounced during dentinogenesis and mineralisation (Butler 1998). Hence the use of DPP/DSP expression in young cultures of differentiating cells that are not yet producing mineral may not fully recognize the cellular phenotype.

IV.2.3.2 Alkaline Phosphatase Activity

It has been proposed that the acquisition of alkaline-phosphatase (ALP) activity may be a requisite for the differentiation and possible specialisation of pulp cells *in vivo* (Miller et al 1976). Alkaline phosphatase is a key enzyme which is important for mineralisation. The ALP activity was highest in the pulp cells just before their

differentiation into odontoblasts. This enzyme can be used as a method of identifying early odontoblast differentiation in dental pulp cell culture. The cultures in the current investigation appear to have early cellular differentiation (as seen by the lack of overt mineral formation). Hence detection of ALP activity was seen to be a useful characterisation tool.

In basic media, supplemented with ascorbic acid but not β GP, the dental pulp cells showed ALP activity than was considerably greater than that of the fibroblast control. Throughout all ALP activity investigations, the activity of the fibroblast control remained negligible. The ALP activity of the osteoblast control was greater than that of the dental pulp cells. The fact that the dental pulp cells demonstrated clear ALP activity was an encouraging sign that some cells were showing early differentiation into odontoblast-like cells without the addition of growth factors or inorganic phosphate.

When β GP was added to the dental pulp cell cultures, the effect on ALP activity was dramatic. The increase of ALP activity of the dental pulp cells was greater overall than that of the osteoblasts, with a far greater proportional increase. This effect of β GP is in keeping with the observations of other investigators. β GP was added at 5mM concentration to our cultures for 21 days. This concentration has been shown to enhance and bring forward mineralisation in osteoblast cultures (Slater et al 1994a). About et al (2000) did not state the concentration of β GP that was used, and cultures were maintained for 8 weeks (About et al 2000a). Couble et al (2000) used β GP at a concentration of 10mM for 4 to 8 weeks (Couble et al 2000). Yokose et al (2000) used a concentration of only 1mM β GP in addition to ascorbic acid, due to concerns

about spontaneous calcium deposition (Yokose et al 2000), and showed strong ALP activity in the dental pulp cell cultures. The significant effect of β GP on the ALP activity of the dental pulp cells gives further evidence of an odontoblast-like nature.

IV.2.3.3 Growth Factors

Alkaline phosphatase activity was further used to examine the effect of differing growth factors on the dental pulp cell cultures and controls. TGF- β 1 appears to play an integral role not only in the developing tooth, but also in the differentiation of mesenchymal cells into odontoblasts during reparative dentinogenesis (Bègue-Kirn et al 1992; Bègue-Kirn et al 1994; Martin et al 1998; Unda et al 2000). The dentinogenic activity of soluble dentine components has been primarily attributed to TGF- β 1 (Tziafas & Papadimitriou 1998). TGF- β 1 has also been suggested to play an essential role in regulating the pulp-dentine complexes' response to caries (Smith et al 1995). Palosaari et al (2001) have suggested that the collagen of mature human odontoblasts is not affected by TGF- β 1, and that the effect of TGF- β 1 is directed to the differentiation of primary and replacement odontoblasts (Palosaari et al 2001). It appears that TGF- β 1 has a wide range of activity, and is certainly implicated in the differentiation of dental pulp cells and preodontoblasts.

The addition of TGF- β 1 to the culture medium produced an increase in ALP activity. This increase was not as great as the increase produced by the addition of β GP. However, when TGF- β 1 and β GP were added, the increase of ALP activity was markedly increased. This indicates that the dental pulp cells are responsive to TGF- β 1, as would be expected if they are, as thought, in the early stages of differentiation. The effect of TGF- β 1 on dental pulp explants has not been extensively investigated.

The marked increase in activity when TGF- β 1 and β GP were added to the culture may reflect a synergistic effect of stimulating differentiation and mineralisation.

The addition of IGF-I to the culture medium had no noticeable effect on the ALP activity of the dental pulp cells. There was in fact a slight decrease in ALP activity in the cultures treated with IGF-I. The effects of IGF-I on differentiating dental pulp cells has been equivocal. In developing dental papillae, Bègue-Kirn et al (1992, 1994) found that certain combinations of growth factors could mimic the action of the inner enamel epithelium and induce the *in vitro* differentiation of odontoblasts. However, the effects of IGF-I were insignificant compared with those of TGF- β 1 and the BMP group. The odontoblast-like cells obtained in the presence of IGF-1 did not show strong odontoblastic traits.(Bègue-Kirn et al 1992;Bègue-Kirn et al 1994). These results again suggest that the dental pulp cells are differentiating toward an odontoblast phenotype.

IV.2.3.4 Scanning Electron Microscopy

Dental pulp cells and controls were examined under scanning electron microscopy (SEM), in an attempt to determine whether a difference in cellular morphology could be seen. Under SEM, the dental pulp cells had a similar appearance to the fibroblast controls. In some areas, however, the cells did display a more polygonal, broader morphology with some multilayering of cells. In general, SEM did not allow differentiation between a dental pulp cell and fibroblast phenotype. Most studies on dental pulp explants have not concentrated on the morphology of the cells. Some investigators have used transmission electron microscopy to examine ultrastructural

characteristics of the cells, and have found this to be useful, but not as significant as protein expression or mineralisation (Couble et al 2000; Unda et al 2000). Cell polarisation and the appearance of a cellular process (the main morphological features of odontoblast terminal differentiation) have not been convincingly reported in human studies (Couble et al 2000). This may be due to the absence of an appropriate ECM milieu. It is also unclear whether the cells obtained in *in vitro* studies are not true odontoblasts, or whether odontoblast-like cells do not attain the same morphology as primary odontoblasts. It may be that morphology overall is not going to be an accurate indicator of cellular phenotype.

IV.2.4 Stem Cells

When dental pulp tissue is injured, reparative dentine is formed by newly differentiated odontoblasts that arise either from mesenchymal cells situated perivascularly or from mesenchymal cells that form the subodontoblast layer in the dental pulp tissue (Torneck 1994). It has been suggested that these cells may originate from fibroblasts in the dental pulp (Fitzgerald et al 1990). The mechanisms affecting the secretory activity of mature odontoblasts as a response to external stimuli are not well-understood. The debate on the origin of odontoblast-like cells and the associated signalling mechanisms controlling migration, orientation, attachment and cytodifferentiation of pulpal cells remains to be resolved (Tziafas 2004).

The isolation of human dental pulp stem cells has been described (Gronthos et al 2000; Miura et al 2003). These cells were initially characterized as “stem cells” based on the fact that they could induce the formation of a dentine-like structure when

transplanted into mice (Gronthos et al 2000). Dental pulp stem cells from deciduous teeth were found to be able to produce not only dentine, but also bone and neural markers (Miura et al 2003). The cells were also shown to express cell surface molecules that are early mesenchymal stem-cell markers.

It has long been recognised that cells in the dental pulp have the ability to differentiate into functional odontoblasts or odontoblast-like cells, but, as discussed, the origin and control of this process is still subject to debate. It could be considered that the cellular populations produced by investigators such as Couble et al (2000) and About et al (2000) are in fact “dental pulp stem cells” that have been named “odontoblast-like cells” (About et al 2000a; Couble et al 2000). Certainly their cell populations display many of the same characteristics as those of Gronthos et al (2000) and Miura et al (2000). It is also likely that the population of odontoblast-like cells which grew from the dental pulp explants described in the current study are derived from dental pulp stem cells, which retain their proliferative capacity as well as their ability to differentiate into more mature odontoblastic cells. Perhaps with the advancement of research in stem cells, previous investigations into the nature and behaviour of the pulp-dentine complex should be re-examined.

Miura et al (2000) state that “deciduous teeth are significantly different from permanent teeth with regards to their developmental processes, tissue structure and function... (These cells) appear to represent a population of multipotent stem cells that are perhaps more immature than previously examined postnatal stromal stem-cell populations” (Miura et al 2003). It is clear that primary teeth differ in their behaviour compared to permanent teeth – after all, they are designed to be physiologically

resorbed to make way for the permanent dentition, however part of Miura's statement must be questioned.

Primary teeth undergo the same developmental processes as permanent teeth – they develop from the dental lamina as a tooth bud, and undergo the same developmental stages to lead to a mature tooth consisting of enamel, dentine, cementum and a dental pulp (Ten Cate 1994b). Obviously primary teeth have a limited life span, so it is possible that their pulp-dentine complex is not as developed, and the potential of the pulpal cells to differentiate may not be as great as in permanent teeth. However, primary teeth have been observed clinically to undergo production of reparative dentine as evidenced by the success of indirect pulp treatment in primary teeth, and the clinical signs of intra-pulpal obliteration following trauma (Diab & ElBadrawy 2000;Farooq et al 2000;Ranly & Garcia-Godoy 2000).

The behaviour of the pulp-dentine complex in primary teeth has not been investigated as thoroughly as it has been in permanent teeth. It is not known whether the dental pulp cells in primary teeth have the same potential to differentiate into odontoblast-like cells, but it is more probable that they would, rather than not. **It can be seen that even with early results from this study, dental pulp cell explants cultured from primary teeth display characteristics of odontoblast-like cells.**

Chapter V. Conclusion and Further Studies

At the beginning of this study there were 5 objectives to be addressed.

- 1. To optimise cell culture techniques using mineralising cells (osteoblasts initially).**

Cell cultures of mineralising cells were examined, and results obtained in accordance with reported studies, showing the importance of calcium and phosphate for mineral deposition. Evidence of the enhanced growth of cells in a matrix was obtained, with implications for further development of culture systems for mineralising cells. In particular, the growth of dental pulp cells could be tested in a three-dimensional system such as Gelfoam (or a similar material).

- 2. To establish methods for obtaining dental pulps from primary teeth.**

A number of methods were trialled to obtain human dental pulps from extracted teeth. An effective and efficient method was developed to obtain dental pulp explants that were suitable for culture. This involved sectioning of

freshly extracted human primary teeth under conditions of low contamination, which has not been reported previously. As these mature teeth are more generally available, this represents a significant advance.

3. To optimise cell culture conditions for the development of odontoblast-like cells from human dental pulp explants.

A cell culture system was optimised for human dental pulp explants. Culture of pulp explants in standard media containing ascorbate produced proliferation of mesenchymal cells that were able to be subcultured. It is likely, but remains to be tested, that omission of added β GP from this media favoured proliferation over differentiation.

4. To develop and characterise the odontoblast-like cells from human dental pulp explants.

The mesenchymal cells grown in culture were seen to have strong positive staining for a dentine matrix protein, and strong alkaline phosphatase activity. Addition of β GP further enhanced ALP activity. This is consistent with phenotypic odontoblastic stem cells. It would be helpful to test for dentine specific proteins or mRNA using RT-PCR. Studies of mineralisation by these cells in monolayer or three-dimensional culture would also be informative.

To facilitate further research with these cells, immortalisation by transfection with SV40 large T antigen and telomerase, as has been performed in bone cells, would be helpful (Darimont et al. 2002).

5. To examine the effects of growth factors on the odontoblast-like cells.

The addition of TGF β -1 to the culture medium was seen to enhance the functional activity of the dental pulp cells, while the addition of IGF-1 had no effect on those parameters, although it did assist cellular proliferation in one of the cultures. The effect of culture conditions and growth factors was seen to be consistent with phenotypic odontoblastic stem cells. A more extended study with known tooth-associated growth factors including BMPs may further delineate dental pulp cell responsiveness.

It can be concluded from the results of this study that it is possible to obtain phenotypic odontoblastic stem cells from human pulp explants originating from extracted, mature primary teeth. These cells will be able to be used to further study the cellular differentiation processes involved in tooth remineralisation, and may have further implications in wider stem cell research.

Chapter VI. References

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