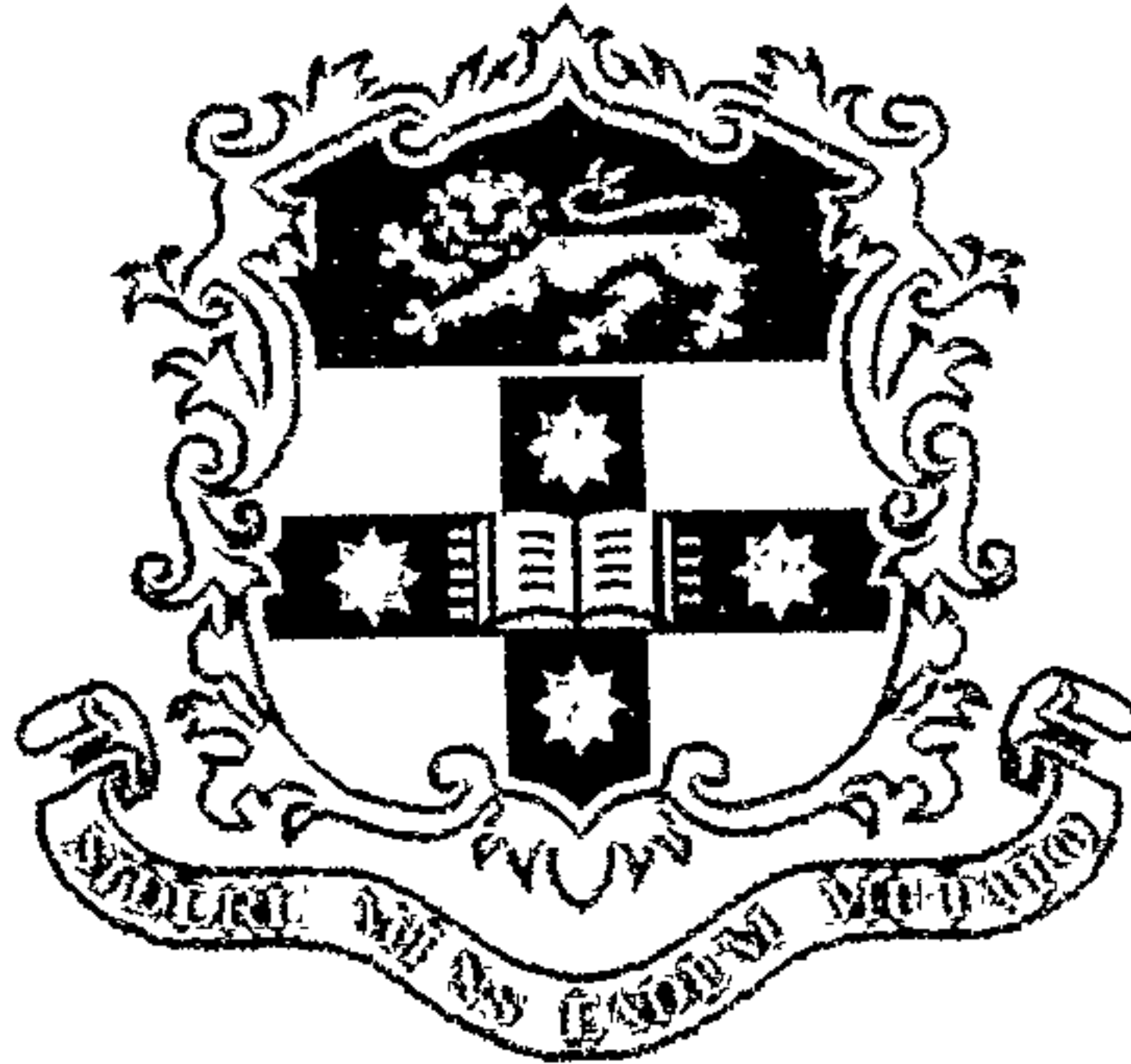


THE INTEGRITY OF THE PERIODONTAL LIGAMENT IN RESPONSE TO TRAUMA



MICHELE MAY-YEE FONG

BDS (HK)

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Dedication

To Wallace, my husband, for your endless encouragement, support and love when I felt frustrated, lost my sense of humour and reason. You have been the ultimate cheerleader, coach and partner.

To our parents, Rosa, Alex, Grace and Calvin, thank you for giving me the freedom and confidence to realise my personal dreams and aspirations.

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Declaration of Authorship

This is to certify that the work presented in this thesis was carried out by the author, Michèle May-yee Fong, who was a post-graduate candidate for the Master of Dental Science, in the discipline of Orthodontics, School of Dental Science, Faculty of Dentistry, University of Sydney, and that the work has not been submitted to any other university or institution for a higher degree.

31st August 1999

A handwritten signature in black ink, appearing to read 'Michèle Fong', with a long horizontal stroke extending to the right.

Michèle May-yee Fong

Abbreviations

°C	Degrees Celcius
μm	Micrometre
BF	Bone Formation
BMP	Bone Morphogenic Proteins
DAB	Diaminobenzidine
DBM	Demineralised Bone Matrix
EC	Epithelial Cell
ECR	Epithelial Cell Rest
EDTA	Ethylene Diaminetetraacetic Acid
EMT	Epithelial-Mesenchymal Transition
FGF	Fibroblast Growth Factors
H & E	Haematoxylin and Eosin
HEBP	Hydroxyethylidene Bisphosphonate
Ig	Immunoglobulin
IGF	Insulin-Like Growth Factor
kD	Kilodalton
kg	Kilogram
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimole
OR	Odontoblastic Reaction
PDL	Periodontal Ligament
PGDF	Platelet Derived Growth Factor
RR	Root Resoption
TGF	Transforming Growth Factor
W	Watt
WF	Wistar-Furth

Abstract

The aim of this study was to determine the response of the residual epithelial network following traumatic disruption of the periodontal ligament (PDL). The hypothesis to be tested was that the presence of intact epithelial cells in the periodontal ligament prevents ankylosis, and contributes to the regeneration of a functional periodontal ligament, subsequent to traumatic disruption.

A time-course study was performed involving 4-6 week old donor and 10-12 week old recipient syngeneic Wistar-Furth (WF) rats. Mandibular incisors from donor rats were extracted, root discs 2mm in length obtained from the middle 1/3 of the root with viable periodontal ligament and control discs denuded of periodontal ligament were transplanted into prepared cavities in the frontal bone of recipient rats. At intervals, rats were perfused with paraformaldehyde fixative and the implant area was histologically examined.

Results indicate that 3 days after transplantation there is degeneration of tissue followed by proliferation of granulation tissue at 10 days. After 21 days, signs of bony fusion with dentine were evident in sites transplanted with denuded control discs. A fibrous capsule had developed around some test transplants. There was also a marked odontoblastic reaction with extensive deposition of regular dentine within the pulp chambers. By 60 days there was extensive ankylosis and bone replacement in control sites. Test sites showed mixed reactions with foci of ankylosis interspersed with fibrous tissue separating dentine/cementum from bone. Odontoblast activity was so extensive that the pulp chamber was obliterated in some cases.

The role epithelial cells play in the maintenance of the periodontal ligament was examined by labelling the epithelium with antibodies (AE1/AE3) specific to cytokeratins following antigen retrieval by heat treatment. Sample root discs used for transplantation provided a positive control for cytokeratin-containing epithelial cells. The pattern of regularly spaced epithelial cells lying in close approximation to the root surface was maintained at 3 and 10 days post-transplantation but epithelial cells remained consistently absent in 21-day and 60-day samples.

These results indicated that, related to the persistence of a fibrous capsule, epithelial cells had disappeared by 21 days post-transplantation. The mechanism could be an epithelial-mesenchymal transformation (EMT).

CHAPTER 1

INTRODUCTION

Situated between the roots of teeth and the alveolar wall, the periodontal ligament (PDL) is a unique connective tissue. This ligament has demonstrated remarkable capacity for repair and regeneration. When up to 1mm in diameter of the periodontal ligament is lost, through disease or trauma, it is still capable of replenishing itself via differentiation from adjacent cells.

It has been shown that ankylosis may occur subsequent to tooth injuries when periodontal ligament cells are mechanically removed, displaced or chemically altered. Bone cells invade the ligament space and establishes a direct connection between tooth and alveolar socket. This altered tooth support can result in a loss in function and root resorption (Andreasen 1980a, Nyman *et al.* 1982, Wesselink *et al.* 1994).

Localised ankylotic areas can at times be removed and the integrity of the ligament restored when periodontal ligament fibroblasts or their progenitors are allowed access to the area ahead of other cells (Andreasen and Skougard. 1972, Andreasen 1976a,b). Repopulation of the area can occur from fibroblasts purported to originate from adjacent periodontal ligament and endosteal spaces, forming new periodontal fibres, cementum and alveolar wall (Wesselink *et al.* 1994).

Data from clinical and biological studies have provided evidence that periodontal regeneration in formation of alveolar bone, cementum and periodontal ligament is achievable although clinical therapies have not been predictable (Graves and Cochran 1994, Howell *et al.* 1996, MacNeil *et al.* 1995, MacNeil and Thomas 1993, Pitaru *et al.* 1994). The origin of the components of the mature periodontal ligament after wounding is speculative. In particular, the role of the epithelial component in this event has been elusive. An enhanced understanding of the cellular and molecular mechanisms regulating periodontal tissue regeneration is required for further development of predictable clinical procedures.

It has been previously postulated that it is the presence of intact epithelial cell rests that prevents ankylosis and regenerates a functioning periodontal ligament.

However, most of the previous studies involved re-implantation into the original extraction socket where amongst other factors, results have been contaminated by the disrupted remnants of the periodontal fibres remaining in the socket. It is expected that transplantation in an ectopic or heterotopic site will eliminate this factor.

With the objective to improve the understanding of the biology within the periodontal ligament and, in particular, to examine the possible involvement of the epithelial component in periodontal ligament healing, a model has been developed for future studies. Ultimately, it is expected that the responses of the periodontal ligament to environmental changes will be predictable.

CHAPTER 2

REVIEW OF THE LITERATURE

The periodontium is the developmental, biological and functional unit of tissues which anchor teeth to bone, provide inter-dental linkage to a row of teeth and seal the oral mucosal opening created during eruption. Four different tissues are involved; gingiva, cementum, alveolar bone and the periodontal ligament. Experimental evidence has indicated that the cells of the dental follicle give rise to cementoblasts, osteoblasts and fibroblasts, which form the supporting periodontal tissues. The gingiva is not derived from the dental follicle, however it is tooth related and intimately follows the changes of the adjacent tooth and bone (Schroeder 1986).

THE PERIODONTAL LIGAMENT

The principal function of the periodontal ligament is to anchor the tooth root to alveolar bone and to distribute multidirectional mechanical stresses such as masticatory forces; it thus exhibits features of a shock absorbing system (Takano-Yamamoto *et al.* 1994). Some of these functional attributes are met by masses of collagen fibre bundles that span the space between bone and the cementum covering the root of the tooth and by an incompressible matrix comprised mostly of glycosaminoglycans, glycoproteins, and glycolipids. Besides being a highly vascular and specialised cellular connective tissue located between the tooth and alveolar bone providing attachment and support, the periodontal ligament also provides sensory functions.

Some of the most interesting features of the periodontal ligament are its adaptability to rapidly changing external mechanical stress levels and its remarkable capacity for renewal and repair. The cells of the normal periodontal ligament include osteoblasts and osteoclasts on the bone side; fibroblasts, epithelial cell rests (ECR), macrophages, undifferentiated mesenchymal cells, neural elements, endothelial cells in the body of the periodontal ligament, and cementoblasts on the root surface (Ten Cate 1994).

ORIGIN AND DEVELOPMENT OF THE PERIODONTAL LIGAMENT

As the periodontal ligament is derived from the inner layer of the dental follicle after root development has begun, development and maturation of the periodontal ligament is dependent on the formation of dentine and the synthesis of cementum (Ten Cate 1994). Odontoblasts differentiate from cells at the periphery of the dental papilla as Hertwig's epithelial root sheath encloses the expanding dental papilla. Fragmentation of the epithelial root sheath, first described by Provenza and Seibel (1986), and formation of dentine, allow migration of dental follicle cells toward dentine and subsequent differentiation into cementoblasts (Cho and Garant 1988).

During the formation of cellular or acellular cementum, there is a high rate of cell division (Perera and Tonge 1981) and expansion of cell populations forming periodontal ligament collagen fibrils (Schroeder 1986) and cementum. It is still not known whether these heterogeneous cell populations maintain their diversity into adult life and are preserved in the periodontal ligament.

Previous studies have also suggested the requirement of epithelial cells in replanted tissues for generation of a functional periodontal ligament (Löe and Waerhaug 1961, Lindskog *et al.* 1988). In recombination experiments, MacNeil and Thomas (1993) found periodontal ligament resulted only when epithelium was included with the tooth germ, suggesting an interaction between epithelial and mesenchymal tissues.

This led to the proposal that periodontal ligament formation is determined by interaction of dentine, extracellular matrix, epithelium and mesenchyme, sometimes described as an epithelial-mesenchymal interaction (Hall 1991). The rationale for involvement of epithelial tissues is based on the established principle that these interactions are crucial for development of several tissues, including heart, hair follicles, limb buds and dentine and enamel in the crown portion of teeth.

COMPOSITION OF THE PERIODONTAL LIGAMENT

As with all soft fibrous connective tissue, the periodontal ligament consists of cells and a fibrous matrix in a gel of ground substances containing blood vessels and

nerves. The matrix consists primarily of collagen and the cells are mainly fibroblasts (Berkovitz 1990).

Table 1. Proportions of structural elements found in mouse molar periodontal ligament

Elements	Proportion (%)
Collagen	51
Fibroblasts	35
Vessels	10
Other cells	2.55
Nerves	1
Oxytalan fibres	0.45

(Freezer and Sims 1987)

Matrix

Type I and III collagen form the bulk of the fibres in the periodontal ligament and are usually found in bundles of cross-banded fibrils. The fibre bundles are anchored at each end in cementum and bone by Sharpey's fibres, which can extend great distances into the appositional side of the socket wall (Beertsen *et al.* 1997).

The majority of the bundles range from 1-4 μ m in diameter and are generally arranged in distinct orientation (crestal, horizontal, oblique, apical interradicular) to enable adaptation to functional stress in different parts of the ligament (Berkovitz 1990). These principal collagen fibres do not necessarily run a straight course from the tooth to the alveolar bone but may appear wavy due to a complex network of overlapping bundles in layers. It is believed that this arrangement ensures that some collagen bundles are always in tension (Sloan 1978a,b, 1979, 1982).

Two immature forms of elastin fibres; oxytalan and eluanin exist within the ligament. Oxytalan fibres are bundles of microfibrils running vertically apically along the cementum surface forming a network surrounding the root which terminates in the apical complex. It is thought that they act to regulate vascular flow in relation to tooth function. Oxytalan fibres and eluanin, which also consist of bundles of

microfibrils, form a network extending from the cementum to bone and run parallel to the collagen fibre bundles (Ten Cate 1994).

Ground substances

There have been few studies on the exact composition of the ground substance. Most research indicated a similarity to other connective tissues in components and ratios (Ten Cate 1994). The principal glycosaminoglycan in bovine periodontal ligament appears to be dermatan sulfate (Pearson 1982). However, it has been reported that there is more ground substances within collagen fibre bundles in areas subjected to compression than tension (Berkovitz *et al.* 1981). In contrast, other data indicate that the types of glycosaminoglycans change in response to compressive forces (Gillard *et al.* 1977, Pearson 1982). It is estimated that up to 70% of the periodontal ligament ground substance consists of water, which has a significant effect on its ability to withstand compression.

Cells of the periodontal ligament

The principal cells of the periodontal ligament are fibroblasts, which are large cells with an extensive cytoplasm containing all the organelles associated with protein synthesis and secretion necessary to sustain the high rate of collagen turnover in the ligament. The presence of a well-developed cytoskeleton with a prominent actin network indicates the functional demands required of the cells (Ten Cate 1994).

The shape of the cell has been described as ovoid or flattened, deduced mainly from the nucleus, which stains more intensely than the cytoplasm (Fullmer 1967). Fibroblasts connect to each other via gap and adherence type junctions. Observations in rodent molar ligament revealed that each fibroblast is in direct contact and communication with neighbouring cells by 20 or more junctions (Beertsen and Everts 1977).

Within the periodontal ligament, fibroblasts as well as osteoblasts and cementoblasts are generated throughout life. It is not known whether they arise from a common precursor or specific progenitor cells. Undifferentiated mesenchymal cells are located within 5 μ m of the blood vessels and provide a source of progenitor cells (Ten Cate 1994). Little is known about the origin and life

cycle of these progenitor cells, although they can be identified by the capacity to incorporate tritiated thymidine (Berkovitz 1990). A state of homeostasis exists between cells of the periodontal ligament, thus production of new cells is balanced by migration of cells out of the ligament or apoptosis (Ten Cate 1994).

EPITHELIAL CELLS

There exists within the periodontal ligament, epithelial cell rests (ECR), which due to their persistence and potential for activity have led to speculations of a functional role (Hamner *et al.* 1970, Loe and Waerhaug 1961, Spouge 1980, Boyko *et al.* 1981, Lindskog *et al.* 1988). Epithelium is never seen in direct contact with bone; a thin buffer zone of fibrous connective tissue separates the two tissues, thus the interspersed cell rests could possibly act as an ankylosis inhibitor. Therefore, in health, a number of roles have been postulated for these cells from protection of root surface from resorption, preventing ankylosis, to maintaining the width of the periodontal ligament (Ten Cate 1996, Loe and Waerhaug 1961, Spouge, 1980, Lindskog *et al.* 1983).

After forming the roots, it is thought that the Hertwig's epithelial root sheath separates from the dentine surface and disperses. Malassez first described the remaining groups of cells in 1885 from sections of human teeth, known at times as epithelial cell rests of Malassez. Immediately after dispersion, there appears to be only one or two cells but increasing cellularity is noted with time (Brunette *et al.* 1979). The remnants are found grouped together and completely surrounded by connective tissue (Spouge 1980). ECRs have been described histologically as clusters of cells when seen in a cross section of the root (Provenza 1988). They appear more deeply stained and cuboidal when compared with the fibroblasts, and are enclosed by a complete basal lamina. These cells may also appear as a network in tangential sections (Orban 1952). Indeed Spouge in 1980 compared the residual network to a basketball hoop lying around the neck of the tooth. Recent 3-D reconstructions indicate the network may present as series of interconnected helical threads running from the epithelial attachment to the root apex (Barry 1998).

ECRs have a pentose shunt and contain glycogen and enzymes associated with glycolysis under anaerobic conditions (Ten Cate 1965, 1967, Grupe *et al.* 1967). The cells are characterized by a partly invaginated, round to ovoid nucleus with

mixtures of hetero- and euchromatin and the cytoplasm contains the usual organelles, glycogen storage granules, tonofilaments, lipid droplets, relatively abundant mitochondria and poorly developed rough endoplasmic reticulum (Csiba and Rohlich 1971, Hamamoto *et al.* 1989).

Berkovitz *et al.* (1997) found that the overall pattern of cytokeratins in ECR characterises cells derived from the basal layer of a stratified squamous epithelium rather than from simple epithelial cells. The types of cytokeratins found in normal human ECR have been determined using immunohistochemistry and these may include types 5, 7, 8, 14, 15, 17, 18, 19 varying in molecular weight from 40-70kD. The use of antibodies against specific keratins allows differentiation of epithelial tissue from others (Ragnarsson 1985).

The distribution of these cells appears to alter along the axis of the root lying closer to cementum at the apex than at the alveolar crest and ranging from 27 to 41 μm from the root surface (Valderhaug and Zander 1967, Wesselink and Beertsen 1993). With age there appears to be decreased cellularity (Reitan 1961, Simpson 1964, Wesselink and Beertsen 1993). The greatest abundance of cell rests is found at the apex in humans between the ages of 10 and 20 (Reeve and Wentz 1962) and later (up to 27 years of age) at the cervical region (Vanderhaug and Zander 1967). In older specimens, the network appears sparse with isolated strands observed (Simpson 1965).

As non-functioning cells usually disappear, the continued existence of these cells suggests that they are not inactive and may play a functional role. The potential for activity is supported by the presence of glycogen and glycolytic enzymes (Ten Cate 1965), indicating an active metabolism (Valderhaug and Nylen 1966). Ultrastructurally, the presence of abundant mitochondria (Valderhaug and Nylen 1966) and primary cilium in association with the Golgi complex (Hamamoto *et al.* 1989) also indicates the potential for activity.

Epithelial cell rests demonstrate rapid proliferation when cultured *in vitro* (Boyko *et al.* 1981) and have been implicated in the aetiology of periodontal cysts (Ten Cate 1996, Valderhaug 1972) and periodontal pocket formation (Grant and Bernick 1969). It has also been suggested that they exist to maintain the periodontal

ligament space by preventing ankylosis and root resorption (Löe and Waerhaug 1961, Spouge 1980, Lindskog *et al.* 1988).

In 1961, Löe and Waerhaug performed replantation of newly erupted permanent teeth in dogs and monkeys and observed that ankylosis and subsequent root resorption never occurred when periodontal ligament containing epithelial cells was retained. They suggested that the epithelial cell rests were the components limiting resorption.

Hamner *et al.* (1970) replanted 22 teeth in a baboon 20 minutes after extraction and endodontic therapy. They disced off the periodontal ligament and cementum on the distal surface of maxillary incisors and left the mesial surface periodontal ligament intact. After 12 months, the mesial surface maintained a good periodontal union between alveolar bone and cementum, with only focal microscopic resorption. The hypothesis is again offered that the epithelial cells of Malassez play a role in periodontal space maintenance.

Lindskog *et al.* (1983), suggested that endothelial cells and epithelial cells produced a collagenase inhibitor which was similar to the "anti-invasion factors" that are present in dental roots (Lindskog *et al.* 1980). These inhibitors may therefore protect the root surface from osteoclastic resorption, and contribute to the maintaining the integrity of the periodontal ligament.

Epithelial cells have demonstrated the capacity for proliferation and can be readily cultured. The capacity to prevent ankylosis has been related to a bone resorption factor (Birek *et al.* 1983). This was not prostaglandin, shown by Brunette *et al.* (1979) to be produced by epithelial cells.

However, administration of hydroxyethylidene bisphosphonate (HEBP), a drug that interferes with the homeostasis of the periodontal ligament, by depositing osteoids and causing ankylosis, appears to challenge the protective role of epithelial cell rests (Wesselink and Beertsen 1993). Furthermore, Reitan (1961) observed a lack of regeneration of these cells on the compression side in teeth subjected to orthodontic loading when regeneration of the connective tissue elements of the periodontal ligament occurred.

On an ultrastructural level, Brice *et al.* (1991) showed the presence of epithelial cells in resorption bays after orthodontic expansion. Reitan (1961) studied orthodontic movement and root resorption in the human and found the epithelial rests of Malassez absent on the pressure side of the periodontal ligament adjacent to the hyalinised tissue-resorbed areas and present on the tension side. Absence of epithelial cells is characteristic of a reorganised, formerly hyalinised fibrous tissue on the pressure side and also of a resorptive process in the cementum and the dentine of the tooth. Reitan (1961) demonstrated that in orthodontic manipulation direct bone resorption had no visible effect on the epithelial cells and that pressure had no effect other than to move the cells closer to the cementum.

Although most of the research have been performed in animals, the results are still applicable to humans as there is a similarity in the morphology of the epithelial root sheath in many species including rodents, pigs, sub-human primates and humans (Grant and Bernick 1971). This was confirmed in comparing cells from rats, dogs and humans (Owens 1978).

MAINTENANCE OF PERIODONTAL LIGAMENT LIMITS

A significant function of the periodontal ligament is the preservation of the width of the structure (McCulloch and Melcher 1983a). As the surrounding alveolar bone rarely colonises the periodontal ligament space the width of the periodontal ligament is preserved throughout life. Ankylosis, the condition in which the tooth root fuses to the bone, is thought to occur when mechanisms regulating periodontal ligament width are defective. Periodontal ligament cells as indicated by ankylosis resulting from destruction of periodontal ligament cells (Melcher 1976) express these regulatory mechanisms.

Studies *in vivo* have underlined the active role of periodontal ligament fibroblasts as regulators of periodontal ligament width; heat killing of periodontal ligament cells induces ankylosis (Line *et al.* 1974). Studies *in vitro* have shown that periodontal ligament cells can inhibit mineralised bone nodule formation by rat bone stromal cells (Ogiso *et al.* 1991), suggesting that periodontal ligament cells could control the extent of osteogenesis in physiological remodelling of periodontium.

However, if periodontal ligament cells are osteogenic (Roberts and Chase 1981), expression of the bone phenotype must in some way be blocked; otherwise the periodontal ligament would undergo bone formation, mineralise across the width, and spontaneously ankylose.

Some observations indicate that the block exerted by periodontal ligament cells on mineralisation may be due to prostaglandin production (Ogiso *et al.* 1991, 1992). Recent data have also shown that bradykinin as well as thrombin has the capacity to stimulate prostanoid biosynthesis in human periodontal ligament cells (Marklund *et al.* 1994), although the mechanisms involved in the activation of prostaglandin biosynthesis remain unclear.

Results of other studies have demonstrated that human periodontal ligament cells are capable of producing bone-like tissue *in vitro* (Arceo *et al.* 1991). Rat periodontal ligament cells can form mineralised nodules in a collagenous matrix and exhibit intense alkaline phosphatase activity (Mukai *et al.* 1993). The formation of mineralised nodules by periodontal ligament cells *in vitro* may reflect the existence of cementoblast precursors (Cho *et al.* 1992). Although the periodontal ligament likely contains osteogenic cells, the relative numbers of osteogenic progenitor cells with extensive capacity for division and self-renewal appear limited.

Current knowledge of the periodontal ligament does not rule out alternative models in which fibroblast and osteoblast precursors are mixed in the periodontal ligament, but the fibroblast population blocks expression of the osteoblastic phenotype in the body of the periodontal ligament (Lekic *et al.* 1996).

Other control systems are likely to be involved in the preservation of the junctional epithelium at the cemento-enamel junction (Magnusson *et al.* 1983) and in the maintenance of discrete cellular and acellular cementum zones of teeth (Schroeder, 1986). The presence of periodontal ligament cells affects the expression of gingival epithelial cell keratins (Carmichael *et al.* 1991) and influences epithelial differentiation and migration (MacKenzie 1988).

It should be noted that other cells also might contribute to regulation of periodontal ligament boundaries. For example, the epithelial cells may have a role in maintaining the width of the periodontal ligament space (Lindskog *et al.* 1988) and in neo-cementogenesis (Slavkin *et al.* 1989a). Collectively, the periodontal ligament

possesses detailed control mechanisms to preserve its hard and soft tissue limits; some of these control mechanisms are most likely mediated by periodontal ligament fibroblasts.

PERIODONTAL WOUND HEALING, RE-ORGANISATION AND REGENERATION

Initially, healing of wounds in the periodontal ligament is similar to processes occurring in other types of connective tissue with organisation of granulation tissue to form a new neurovascular supply and to re-form the other periodontal ligament components. However, periodontal regeneration represents a healing process, which results in the re-establishment of the previous architecture and function of the periodontal ligament, reproducing or reconstructing the lost or injured part (American Academy of Periodontology 1992). This implies that new cementum is formed with inserting collagen fibres and bone.

According to Amar (1994), the current belief in regeneration is that cells are derived either from a small population of multi-potential stem cells or from several populations of committed progenitor cells which may have a local or systemic origin. The precise origin of periodontal ligament fibroblasts, cementoblasts, and osteoblasts in mature tissues or the location of their progenitors is still unknown, and it is unclear whether they originate from a single, multi-potential stem cell or from multiple different ancestral cells for the separate lineages. There is little evidence available on the cellular hierarchies and lineage characteristics in fibroblast populations of adult mammalian periodontal tissues.

This ambiguity is partly due to a lack of clear phenotypic markers. Fibroblast populations are believed to contain a system of lineages resembling other cell renewal systems, which may include fibroblast stem cells (Francz *et al.* 1989). It has been suggested that the periodontal ligament fibroblast lineage is a renewal cell system characterised by balance (McCulloch and Melcher 1983a; McCulloch *et al.* 1989); new cells generated by proliferation are equal to the number of cells lost through cell death and migration.

Periodontal ligament cells in mammals resemble other recognised renewal cell systems such as blood cells and the epithelial cells of the small intestine lining where the most primitive cells are considered to be stem cells. These cells typically

show extensive self-renewal, responsiveness to regulatory factors, and can generate multiple types of more differentiated, specialised cells (Evans and Potten 1991).

Stem cells are usually found in a specific area within a tissue (Potten and Loeffler, 1990). Progenitor cell populations of periodontal ligament are also abundant in locations adjacent to blood vessels. They demonstrate features representative of stem cells, such as a small size, responsiveness to stimulating factors, and slow cycle time (Gould *et al.* 1980, McCulloch and Melcher 1983a, McCulloch 1985). The periodontal ligament appears to be the principal source of periodontal ligament fibroblasts in unwounded periodontium and may also contain sites for other progenitor cells including the cementum-related part of the periodontal ligament for cementoblasts and the bone-related portion for osteoblasts (McCulloch and Melcher 1983b). Studies of enamel organs, papillary, pulpal and follicular mesenchyme (Palmer and Lumsden 1987) have revealed that the tooth-related periodontal ligament contains cementoblast precursors and the bone-related ligament contains osteoblast precursors. These findings suggest that the developmental patterns established for the locations of progenitor cells are preserved in adult periodontal ligament.

Embryological studies have also suggested that periodontal regeneration recapitulates the process of morphogenesis, histogenesis, and cytodifferentiation, as well as cell field migration that occurs during development of the periodontium. Osborn and Price (1992) described events occurring during tooth and periodontal development and the interactions between tissues of different embryonic origin. They presented autoradiographic evidence that periodontal cells (i.e. cementum, periodontal fibres, and alveolar bone) originate from dental papilla cells migrating into the dental follicle. These results support the hypothesis that some progenitor cells remain undifferentiated and form a pool of self-renewing cells in the periodontal ligament space throughout life. Such cells originate from the late periodontal mesenchyme and proceed to terminal differentiation as needed.

In normal periodontal tissues, the daily rate of cell generation is between 0.5-2%, a relatively rapid cell turnover compared to other soft connective tissues (McCulloch and Melcher 1983a). Progenitor cells are probably responsible for maintaining homeostasis of the periodontal attachment apparatus, serving as a source of renewal cells for cementoblasts, periodontal fibroblasts, and osteoblasts. As only

some of the newly formed cells differentiate into osteoblasts, or cementoblasts, it is of interest to note that apoptosis balances cell generation (McCulloch *et al.* 1989). It is possible to increase cell formation rate and differentiation in the periodontal ligament by application of orthodontic forces or by wounding (Roberts 1975, Gould *et al.* 1980).

Although it is unknown whether the same or similar progenitor cell populations are activated by these stimuli, a substantial number of stem cells can be triggered to proceed towards terminal differentiation. During controlled orthodontic tooth movement, these cells can respond on the tension side to form cementum, new periodontal fibres, and bone (Reitan 1967). In this manner, integrity of the cementum and periodontal ligament space can be maintained.

Thus, the small population of purported periodontal stem cells may be responsible for regenerating and maintaining homeostasis in, the periodontal ligament. This cell, which may have its origin in the late periodontal mesenchyme of the dental papilla, is advocated as a tripotential cell capable of differentiating into cementoblast, osteoblast, and periodontal fibroblast.

Periodontal wound-healing studies demonstrated that after wounding, cells of the periodontal ligament begin to divide and migrate into the site of injury. It was observed that the cells with increased mitotic activity originate from within 200 μm of the wound margin (Melcher 1970, Gould *et al.* 1977, Igihaut *et al.* 1988). During the repopulation response following periodontal ligament extirpation there is a fivefold increase in the proportion of ^3H -thymidine labelled cells, which represents largely the proliferation of perivascular cells as well as cells associated with the adjacent alveolar bone marrow spaces. Most of this population of progenitor perivascular cells was located within 5 μm of the blood vessels.

It took on average 3 days before any dividing or migrating cells could be detected at the site of the wound (Gould *et al.* 1980). These cells appeared similar to stem cells in other renewal cell systems, thus the products of the paravascular cells could represent the division of primitive fibroblast progenitor cells. Daughter cells of these progenitors can repopulate wounded periodontal ligament (Gould *et al.* 1980). The majority of these proliferating paravascular cells are located mainly in the central portion of the periodontal ligament (McCulloch and Melcher 1983b).

Again, it is not clear if the perivascular progenitors actually migrate and contribute to periodontal ligament cell populations in normal conditions (McCulloch and Melcher 1983b). However, ultrastructural radioautography studies have revealed that these progenitor cells within the periodontium have marked differences in cytodifferentiation and constitute the cellular reservoir of new cells for repair and regeneration. Hakkinen and Larjava (1992) showed that fibroblasts in wound healing of the periodontal granulation tissue displayed increased levels of collagen and hyaluronic acid synthesis and are morphologically larger than other fibroblasts.

In studies involving mouse periodontal ligament, McCulloch and Melcher (1983b) demonstrated that dividing cells subsequently migrate to the cemental and alveolar bone surfaces. Under physiological conditions, it appears that a reservoir of undifferentiated cells is responsible for the maintenance of the homeostasis of the periodontium and its interface with the root surface (cementum) and the alveolar bone. These undifferentiated periodontal cells can also be triggered to achieve terminal differentiation under specific stimuli such as orthodontic movement or periodontal regeneration.

BONE

As the periodontal ligament is surrounded on one side by alveolar bone, an understanding of bone biology is necessary.

Bone is a specialised connective tissue of both mineral and organic material. The mineral content contributes about two-thirds of its weight, and the organic matrix consisting mainly of type I collagen contributes to the remaining one-third. The osteoblast and osteoclasts are the two main cell types responsible for the turnover of bone matrix. The current concept of bone remodelling is based on the hypothesis that osteoclastic precursors become activated and differentiate into osteoclasts, beginning the bone resorption process. This is followed by a bone formation phase (Hill 1998). The rate of tissue turnover is determined by the number of sites entering the bone formation phase, called activation frequency together with the individual rates of the two processes of bone resorption and formation (Charles *et al.* 1987, Ericksen *et al.* 1986).

BONE RESORPTION

The bone resorption cascade involves a series of steps directed towards the removal of both mineral and organic constituents of bone matrix by osteoclasts aided by osteoblasts. The progenitor cells are recruited from the haemopoietic tissues such as bone marrow to bone via the circulating blood stream. They proliferate and differentiate into osteoclasts through a mechanism involving cell-to-cell interaction with osteoblast stromal cells (Suda *et al.* 1996). The bone surface is prepared for the removal of the unmineralised osteoid layer by the lining osteoblasts, which produce a variety of proteolytic enzymes, facilitating the access of the osteoclasts to the mineralised bone.

Osteoclast polarisation is induced by recognition of extracellular bone matrix proteins forming the characteristic ruffled borders and clear zones of the osteoclasts (Roodman 1996, Suda *et al.* 1996). Activation of the osteoclasts at the surface of the mineralised bone is achieved by the classical concept of osteoblasts releasing osteoclast activating factors and a new concept of osteoblast cell-to-cell contact (Fuller *et al.* 1991). Activated osteoclasts resorb bone by producing hydrogen ions,

which dissolve minerals, and proteolytic enzymes, which degrade organic materials under the ruffled border of the cell.

Ultimately osteoclasts which have a life span of approximately two weeks, undergo apoptosis that is characterised by nuclear and cytoplasmic condensation and fragmentation of nuclear DNA into nucleosomal-sized units. TGF- β which blocks bone resorption can induce apoptosis in osteoclasts while parathyroid hormone and 1,23-dihydroxyvitamin D inhibit osteoclast apoptosis *in vitro* thus stimulating osteoclasts. This suggests that regulating the life span of osteoclasts plays a role in bone remodelling.

BONE HEALING

Bone is a highly vascularised tissue and when injured, considerable haemorrhage occurs. A clot fills the region between the two ends with formation of granulation tissue. Bone repair differs from other forms of repair in that it involves the activity of osteoblasts and osteoclasts derived from the periosteum or endosteum or alternatively by metaplastic transformation of primitive mesenchymal cells or fibroblasts in the adjacent connective tissues.

Bone repair follows a process of induction, inflammation, soft callus formation, hard callus formation and remodelling. After initial haemorrhage, molecular messengers enter the extracellular compartment, which stimulate cellular migration, proliferation and differentiation. This initially controls the healing response. These factors include platelet-derived growth factor (PDGF), transforming growth factor (TGF)- β , insulin-like growth factor (IGF) and prostaglandins. Mild acute inflammation is essential to the healing process. Neutrophils and macrophages are recruited to the site of injury by chemotaxis to remove bacteria and damaged tissue. These cells also express biochemical mediators affecting fibroblastic and angiogenic responses.

Fibroblasts proceed to deposit extracellular matrix followed by proliferation of cartilage formation and revascularisation. Calcification of cartilage follows which is subsequently resorbed and new woven bone forms by the differentiation of osteoblasts. Eventually this remodels into lamellar bone. Prostaglandins have

been shown to increase the number of osteoclasts available for this process (Yamasaki *et al.* 1980) and inhibition of prostaglandin synthesis by indomethacin blocks the appearance of osteoclasts (Sudmann *et al.* 1979).

BONE FORMATION

Bone formation also results from a cascade of events that involves proliferation of primitive mesenchymal cells, differentiation into osteoblast precursor cells, maturation, formation of matrix and mineralisation. Proliferation of osteoblast precursors is mediated by osteoblast-derived growth factors. These include members of the TGF- β superfamily and other growth factors sequestered in bone matrix including IGF-I and II, fibroblast growth factors (FGF's) and PDGF. These growth factors may have a role in preventing osteoblast apoptosis *in vitro* (Hill 1998). Differentiation of osteoblast precursor into mature cells is caused by IGF-I and bone morphogenic protein (BMP)-2, which is also a member of the TGF- β superfamily. Cessation of the osteoblast activity is thought to be due to negative feedback inhibition or the induction of osteoblast apoptosis by tumour necrosis factor released from adjacent marrow cells (Hill *et al.* 1997).

BONE REGULATION

The regulation by autocrine and paracrine factors e.g. FGF, PDGF, has its effect on differentiation, growth and regeneration of bone, however these factors do not induce cartilage or bone growth. Grafts (osteogenic) and osteoinductive materials can repair bone defects. Osteogenic materials contain osteoprogenitor cells or stimulate these cells and mature osteoblasts to proliferate. Examples include bone marrow, bone graft, TGF- β and demineralised bone matrix (DBM) containing growth factors that stimulate bone. Alternatively, osteoinductive cells stimulate cells to convert to chondroprogenitor and osteoprogenitor cells i.e. cellular differentiation. BMP, bone marrow, bone grafts and DBM have osteoinductive properties.

In 1965, Urist demonstrated bone induction with the use of DBM. This process follows a multistep cascade of chemotaxis and attachment of mesenchymal stem cells to the matrix. These cells proliferate and differentiate to chondroblasts,

chondrocytes and eventually osteoblasts. Formation of cartilage, bone and marrow results. New woven bone formation can be seen by day 11, which is then remodelled, and by day 21 bone marrow formation is complete. This induction process is regulated by BMPs that belong to a super family of differentiating molecules (tissue growth factors) and recapitulates embryonic development and morphogenesis.

Bone morphogenic proteins

BMP is a signalling molecular extract from bone matrix with a unique ability to induce the formation of cartilage and bone tissues when implanted into a soft tissue site. The osteoinductive ability resides in the solubilised component requiring appropriate carriers to exert activity (Sampath *et al.* 1981).

13 BMP sequences have been cloned, all of which except BMP 1 induce *de novo* bone formation in conjunction with a collagenous matrix, when implanted into extraskeletal sites in animal models (Reddi 1992, Wozney 1992) and demineralised matrix. BMPs are divided into three sub-families by their sequences: 2 and 4 (86% amino acid identity), 3, and 5-8 (71-80% identity), all of which are growth factors (Celeste *et al.* 1990). There is suggestion that the propeptide region play some role in controlling heterodimer formation between various BMPs, which interact differently with TGF- β receptors (Brunner *et al.* 1989).

A threshold dose exists for BMPs. Chemotaxis response has the lowest threshold followed by mitogenic response of mesenchymal cells to transform *in vitro* and at a higher yet threshold, bone differentiation is observed *in vivo*. Occupancy of 2-3 receptors per cell is sufficient to elicit chemotactic responses (Reddi 1994). BMP receptors are classified according to their molecular mass. Binding of the protein occurs via the protein core of the Type I receptors, this is then presented to the type II receptors. Signalling occurs by a receptor-ligand complex which is a heteromeric complex of type I and type II receptors (Franzen *et al.* 1993, Estevez 1993).

It is unlikely that the TGF- β superfamily members act alone. Growth factors such as IGFs, FGFs and PDGF are likely to have effects on osteoblast proliferation and differentiation as they are all bone growth stimulants and may have synergistic as well as inhibitory interactions on osteoblasts (Massagueu 1985, Roberts *et al.* 1985).

It has been suggested that BMPs will find clinical applications in a wide range of areas including periodontal regeneration. However, the need for prevention of ankylosis and the requirement for new cementum formation complicates this situation.

Cementoblasts express a similar phenotype to osteoblasts (MacNeil *et al.* 1994, Tenorio *et al.* 1993a, 1995) and pre-cementoblasts have been demonstrated to express BMP-2 during root formation (Tenorio *et al.* 1993b) suggesting that BMPs may induce cementum as well as bone formation.

The pleiotropism of BMPs has been demonstrated to also induce dentinogenesis (Nakashima 1990, 1994) as well as cementogenesis (Ripamonti *et al.* 1994). Expression and localisation of BMP during early development suggests a signalling role in epithelial-mesenchymal interactions during organogenesis (Helder *et al.* 1995).

Ripamonti *et al.* (1994) used a preparation composed mainly of BMP-2 and BMP-3 to treat periodontal defects in baboons and found induction of cementum with periodontal ligament-like structures in addition to alveolar bone. The periodontal ligament was oriented and inserted correctly in the cementoid.

Using aliquots of insoluble collagenous matrix as a delivery system, pellets containing BMP-2 and BMP-3 (osteogenin) were implanted into surgically created large molar furcation defects in baboons. Pellets without the BMPs were used in control sites. Histological analysis demonstrated that in conjunction with the collagenous matrix, the BMPs induced regeneration of cementum, periodontal ligament and alveolar bone after two months. The preparation allowed distinction between the newly formed and mineralised bone from the pre-existing bone.

Similar experiments carried out using BMP-2 on beagle dogs (Sigurdsson *et al.* 1995) and Wistar rats (King *et al.* 1997) also found increased rate of normal bone and cementum formation without an increase in ankylosis or resorption. However, BMP-2 induced bone formation at some distance from the defect suggesting an effective delivery system is required to harness the use in periodontal regenerative procedures (Sigurdsson *et al.* 1996).

Bowers *et al.* (1991) however, failed to find an effect for purified BMP-3 alone on the stimulation of periodontal tissue regeneration in humans. BMP-3 with DBM and DBM alone resulted in insignificantly more new tissue formation than BMP-3 and collagen matrix or the collagen matrix alone.

Ripamonti *et al.* (1994) showed increased bone healing around surgically created Class II furcation defects in baboons followed treatment with BMP-4 and in 1996 Ripamonti *et al.* also evaluated BMP-7 for its effect on cementogenesis. Following a two-month healing period, new cementum formation was seen with areas of a functionally oriented periodontal ligament without bone formation. It was suggested that the presence of exposed dentine serves as a substrate for direct cementum formation.

The expression and functions of BMPs have not been fully elucidated. It remains to be seen whether BMPs have a therapeutic advantage in combination or in single application. More research is also being done on finding a suitable delivery system for periodontal regeneration allowing optimal osteoinductivity with low doses of BMPs, which have appropriate surface characteristics. The solutions to periodontal regeneration may be found once these obstacles have been overcome (Kenley *et al.* 1993).

BONE VS. ROOT SURFACE REMODELLING

Remodelling processes similar to bone occur on the root surface. Although it is unclear whether osteoclasts and tooth-resorbing cells (odontoclasts) are the same, a number of similarities exist. Odontoclasts have a ruffled border, however, they are smaller with fewer nuclei and have a smaller or no clear zone. Both the enzymatic properties and acid phosphatase activities are also similar (Seltzer 1988, Pierce *et al.* 1991).

Root resorption

External root resorption can be classified based on clinical and histologic manifestations; external surface resorption, external inflammatory root resorption, and replacement resorption (Andreasen 1994). Ankylosis can occur as a result of

replacement resorption and can also be included as a type of external resorption (Ne *et al.* 1999).

External surface resorption is a transient phenomenon whereby the root surface undergoes spontaneous destruction and repair. It is considered as a normal physiological response as it is found in all teeth to varying degrees (Malueg *et al.* 1996).

External inflammatory root resorption represents the most common type of external root resorption and is at times described as a bowl-shaped resorptive defect that penetrates dentine (Barrett and Kenny 1997). Aetiological factors include injury or irritation of the periodontium from trauma, periodontal infection or orthodontic treatment; these initiate an inflammatory response within the periodontal ligament (Benenati 1997). The most common cause is trauma; injury leads to pulpal necrosis and damage to the root surface, exposing dentinal tubules. Bacteria and bacterial-products as well as tissue breakdown products within the root canal, stimulate inflammation in adjacent periodontal tissue and lead to inflammatory resorption of the root (Andreasen 1994).

It has also been postulated that pressure is another aetiological factor due to the resorption that occurs in orthodontic tooth movement, tooth eruption, bone lesions, tumours, cysts, impacted teeth and occlusal trauma. Resorption tends to cease once pressure is removed (Benenati 1997).

External replacement resorption is caused primarily by luxation injuries. It is a continuous process whereby the tooth is gradually resorbed and replaced by bone (Tronstad 1988). Large areas of the periodontal ligament are lost or damaged during avulsion, resulting in union between the tooth and alveolar bone, which proliferates during the healing process to meet this demand (Barrett and Kenny 1997). Prior to replacement by bone, inflamed connective tissue separates bone from tooth surface (Hammarström *et al.* 1989, Hammarström *et al.* 1992).

Ankylosis

After extensive necrosis of the periodontal ligament, often as a sequelae to luxation injuries and especially avulsion, bone forms on the denuded root surface. With no

intervening connective tissue, ankylosis is defined as the union of cementum or dentine and bone (Pindborg 1970). Mechanical removal of all or part of the periodontal ligament prior to replantation has consistently demonstrated ankylosis (Lindskog *et al.* 1985, Barrett and Kenny 1997).

When 20% or more of the root surface is damaged, the risk of progressive ankylosis is increased (Andersson *et al.* 1984, Tronstad 1988). Transient ankylosis occurs at sites where once-established ankylosis later disappears. This is possibly related to minor areas of damage upon the root surface (Andreasen and Kristerson 1981).

Physical and biochemical alterations to the periodontal ligament may influence the expression of progenitor cells resulting in ankylosis and replacement resorption (Barrett and Kenny 1997). This includes extraoral drying, mechanical removal, thermal damage by freezing, pH or osmolarity change, infection and any factors that leads to the loss of viable periodontal ligament on the root surface (Tsukiboshi 1993).

Ankylosis occurs when cells remodelling bone are unable to distinguish between cementum, dentine and bone. The root is therefore incorporated into the remodelling process of the alveolus and is replaced by bone (Tronstad 1988). This can be demonstrated histologically 2 weeks after replantation (Andreasen 1980a). It is assumed that the damaged periodontal ligament is repopulated from adjacent bone marrow cells with osteogenic potential and will consequently form ankylosis (Line *et al.* 1974)

Clinically, the ankylosed tooth is immobile with a high percussion sound (Andreasen 1975). Radiographically, ankylosis is represented by the disappearance of the normal periodontal space. Continuous replacement of root substance with bone can be detected two months after replantation in radiographs and histologically there is bony union between root surface and surrounding alveolar bone (Andreasen and Hjorting-Hansen 1966) which is apparent two weeks after replantation (Schatz and Joho 1994).

TOOTH REPLANTATION AND TRANSPLANTATION

Replantation is defined as the insertion of a tooth in its own alveolar socket, with or without endodontic therapy, after intentional extraction or avulsion by trauma whereas transplantation is defined as removing a tooth from its natural alveolar socket and placing it in a different alveolar socket (Anderson 1961).

HISTORICAL ASPECT

When a tooth is extracted, some portion of the periodontal membrane usually remains attached to it and the tissue containing epithelial cells is removed with the tooth in most cases (Simpson 1964). As early as 1755, Hunter reached the conclusion that a vital periodontal ligament was a prerequisite for a successful re-union between root and bone; Younger verified this conclusion in 1886. On the basis of animal experiments, Scheff in 1890 suggested that root resorption does not occur when teeth are replanted with their periodontal ligament.

Morris and Thompson (1963) recorded successful reattachment of human periodontally sound teeth re-uniting connective tissues to dentine and cementum with periodontal fibres aligned in a parallel orientation. In 1976, Burkland *et al.* completely disrupted periodontal ligament in rat incisors and showed some collagen fibres uniting with bone and cementum after 5 days. This repair process was however not maintained due to necrotic matter producing root resorption.

Thus the result of transplantation is intimately dependent on the preservation of the periodontal ligament remnants, as their vitality is a prerequisite for prevention of root resorption and replacement by bone (Löe and Waerhaug 1961, Cafresse *et al.* 1977, Andreasen 1980a,b, Karring *et al.* 1980, Andreasen and Kristerson 1981, Blomlöf *et al.* 1983).

EVENTS FOLLOWING REPLANTATION

The sequelae of tooth replantation has been described extensively (Butcher and Vidair 1955, Löe and Waerhaug 1961, Andreasen and Hjorting-Hansen 1966,

Sherman 1968, Anderson *et al.* 1968, Rothschild *et al.* 1969, Shulman and Kalis 1970, Hamner *et al.* 1970, Nordenram *et al.* 1970, Gamal 1970, Castelli *et al.* 1971, Hurst 1972, Nasjleti *et al.* 1975, Soder *et al.* 1977, Andreasen *et al.* 1978, Nasjleti *et al.* 1978).

One day after replantation, a zone of fibrin enmeshing erythrocytes and inflammatory cells was interposed between the root surface and any remaining periodontal fibres attached to the alveolar bone. Epithelium migrating rapidly along the root had reached the alveolar crest at 3 days, and was within the ligament space at 7 days (Proye and Polson 1982).

Many histological studies indicate three different modes of periodontal healing following localised injury to the periodontal ligament. The most frequent response is **root surface resorption** showing superficial resorption lacunae, spontaneously repaired with new cementum and without further sequelae. In cases with extensive damage to the periodontal ligament, cell repopulation from adjacent bone marrow cells with osteogenic potential will lead to **ankylosis**. Such ankylosis, also at times called replacement resorption can first be recognised histologically as soon as 2 weeks after replantation. Radiographically, it is apparent after 2 months. When resorptive processes are severe enough to connect dentinal tubuli and the periodontal tissues, an infected necrotic pulp can lead to an **inflammatory resorption**. The inflammatory resorption can already be observed after 2 weeks and can be controlled by endodontic treatment. The rate of repair depends on the degree of preservation and in dogs new Sharpey's fibres have been observed at 4-6 weeks after replantation (Hammer 1955).

Under some circumstances the periodontal ligament may be regenerated either in whole or in part, and this apparently depends upon there being viable periodontal ligament attached to the replanted tooth (Löe and Waerhaug 1961, Sherman 1968, Hamner *et al.* 1970, Melcher 1970, Atrizadeh *et al.* 1971, Line *et al.* 1974). Under some circumstances the periodontal ligament may be regenerated either in whole or in part, and this apparently depends on there being viable periodontal ligament attached to the replanted tooth (Löe and Waerhaug 1961, Sherman 1968, Hamner *et al.* 1970, Melcher 1970, Atrizadeh *et al.* 1971, Line *et al.* 1974).

Much of the current knowledge regarding replantation has been advanced by Andreasen who cites the following 4 factors as having the strongest impact on

periodontal ligament healing, in descending order of significance (Andreasen *et al.* 1995):

- Stage of root development
- Length of the dry extra-alveolar storage period
- Immediate replantation
- Length of the wet period (storage in saliva or saline)

According to Løe and Waerhaug in 1961, other measures also include maintaining the cell rests of Malassez.

Andreasen showed that an extra-alveolar period of 18 minutes in saline does not increase root resorption when compared to immediate replantation (Andreasen 1981a). Another study showed that no vital cells remain on the root surface after drying periods exceeding 90 minutes at 20% relative humidity and 25 degrees (Soder *et al.* 1977). Replantation is the recommended therapeutic procedure following traumatic exarticulation of teeth although its long-term prognosis remains controversial. A higher rate of periodontal healing in teeth replantation (66.7%) within 1 hour is expected compared to replantation after 3 hours or more where a higher percentage (83.3%) of both inflammatory and replacement resorption is seen. Even if the whole sample was prevented from drying before replantation, the teeth were affected by different rates of root resorption (Schatz *et al.* 1995).

Among the various prognostic factors suggested in the literature, bacterial contamination during extra-alveolar storage seemed the most critical (Schatz *et al.* 1995). Less adverse healing after replantation has been found by removing non-vital soft tissue (Andreasen 1981b) and the use of antibiotics by Selvig *et al.* 1990.

Root surfaces play an important role in healing. No evidence of new connective tissue attachment is observed on the denuded root surface. The absence of fibres on the root surface results in apical migration of the epithelium, and precludes formation of new connective tissue attachment (Proye and Polson 1982).

EVENTS FOLLOWING TRANSPLANTATION

An autograph is a graft from one site to a different site in the same animal. An isograph is a graft from one animal to a genetically identical recipient of the same

species. The site may be orthotopic, a natural location or heterotopic, a different site.

Tooth autografts and isografts usually undergo degeneration after grafting (Agnew and Fong 1956, Nordenram 1963, Fong *et al.* 1967, Weinreb *et al.* 1967). This is usually accompanied by a polymorphonuclear leukocytic invasion by soft tissues of the tooth (Ivanyi and Vacek 1964, Irving and Bond 1968, Johansen 1970, Lemoine *et al.* 1970, Shulman and Kalis 1970, Klein *et al.* 1971, Bartlett and Reade 1973). Histological observations of tooth isografts have shown that degeneration commences in the part of the graft most remote from the blood supply (Atkinson 1972). Later changes in the dental papilla or pulp indicate that degenerative changes are often reversed and dentinogenesis is renewed (Agnew and Fong 1956, Nordenram 1963, Ivanyi and Vacek 1964, Grewe and Felts 1968, Irving and Bond 1968, Weinreb *et al.* 1968, Fitzgerald 1969, Klein *et al.* 1971, Atkinson 1972).

Initially, dentine of an abnormal variety, which contains cellular inclusions, is produced appearing first at the cervix or apex (Fong *et al.* 1967, Klein *et al.* 1971, Riviere *et al.* 1971, Atkinson 1972, Bartlett and Reade 1973). Cellular dentin deposition may then spread throughout the pulp (Nordenram 1963, Williamson 1964, Fong *et al.* 1967, Irving and Bond 1968) and may be superseded by regular or irregular secondary dentin (Weinreb *et al.* 1967, Fitzgerald 1969, Klein *et al.* 1971, Atkinson 1972, Bartlett and Reade 1973). Occasionally however, the pulp shows no sign of regeneration (Johansen 1970).

Degeneration appears to be universal at all stages of tooth development. Agnew and Fong (1956) used fully developed monkey teeth and Weinreb *et al.* (1968) transplanted 10-day-old rat teeth in which no root formation had taken place, both showed degeneration. Dentine repair initiated by the dental papilla seems to depend on the state of development of the transplant. Whereas the deposition of cellular dentine is more or less a universal observation, subsequent deposition of irregular or regular secondary dentine only occurs in teeth which have not begun root formation.

These results were obtained from 10-day old rat studies (Weinreb *et al.* 1967) and 10-day old or less mouse studies (Klein *et al.* 1971, Atkinson 1972 and Bartlett and Reade 1973). This suggests that more mature derivatives of the dental papilla may be too advanced to be able to carry out a retrograde reparative response. The age

of the transplanted tissue thus does have an effect upon the histopathological appearance of autografts and isografts. This effect is manifested in the kind of reparative tissue that is formed, and not in the reparative process itself, which occurs in teeth of any age.

In summary, tooth autografts and isografts degenerate after transplantation but then the dental pulp recovers and dentinogenesis is renewed. The enamel organ may form enamel or cysts but does not generally show the same powers of recovery as the dental papilla. There are, of course, exceptions to these general conclusions about the fate of teeth grafted into compatible animals.

EVENTS FOLLOWING HETEROTOPIC TRANSPLANTATION

Reorganisation of the periodontal ligament of mature tooth orthotopic isografts and replants has been reported in humans (Andreasen *et al.* 1978), monkeys (Shulman and Kalis 1970), and rats (Burkland *et al.* 1976). This event did not occur when teeth were transplanted into a heterotopic intrabony site (Bartrett and Reade in 1981) which may be related to the use of mature teeth in a heterotopic site rather than to a recently occupied tooth socket, as was the case in the other studies. In a recent extraction socket, there is close proximity between remnants of the periodontal ligament from both the tooth side and alveolar bone side of the wound, which can be considered as an incisional wound of the periodontium (Melcher 1969).

Andreasen (1972, 1976a,b) examined the pathogenesis of surgically induced ankylosis in rat molars and reported that, after 7 days, most animals exhibited ankylosis over various parts of the exposed root surface. However, 10 to 14 days after surgery, reversal of this process occurred, indicating the possibility of new attachment to the root surface where periodontal ligament was removed or injured. After 3 weeks, a normal periodontal membrane was restored in all specimens. In addition, Andreasen and Kristerson (1981) demonstrated the healing capacity of adjacent intact periodontal ligament to be 1-1.5mm. These combined effects could invalidate the interpretation of transplantation experiments.

Teeth transplanted to a heterotopic intrabony site have only the remnants of the periodontal ligament remaining on the tooth side of the wound. Reformation of the

periodontal ligament requires the healing of an excisional wound of the ligament with incorporation of fibres into the adjacent organising bone.

Little information is available regarding the capacity of the periodontal ligament to re-establish functional fibre connections when mature teeth are transplanted to intrabony sites, including the alveolar process, not previously acting as a tooth socket. A study in which mature teeth with attached remnants of the ligament were transplanted to the tibial shaft, suggests that mature teeth do not possess the ability to re-establish normal periodontal ligament fibre connections when transplanted to an intrabony site that was not a recent tooth socket (Bartlett and Reade 1981).

This was in contrast to teeth with partial root development isografted to the tibial shaft medulla (Bartlett and Reade 1973) and to tooth germs transplanted to other intrabony sites, including the femur (Atkinson and Layelle 1970) and parietal bone (Freeman *et al.* 1975). Although lack of tooth function at the sites used in the present study may have contributed to the lack of observable periodontal ligament reorganisation (Hoffman 1960), it does not explain its apparent complete absence.

In contrast, the finding of fibrous connective tissue resembling periodontal ligament attached to all of the roots, recovered after implantation with cultured periodontal ligament cells, but not in association with roots that had been implanted with gingival connective tissue cells or without cells is of interest (Boyko *et al.* 1981). This study replanted root discs into a newly created socket of the alveolus. The fact that orientated collagen fibres could be deposited in the absence of function may be surprising, particularly as the periodontal ligament of hypofunctional teeth is known to atrophy and the fibres to become disoriented (Cohn 1965).

Connective tissue was identified as a periodontal ligament when it fulfilled the following strict criteria:

1. presence of collagen fibres orientated at right angles to the surface of bone and tooth
2. presence of Sharpey's fibres attaching the connective tissue to bone and tooth
3. presence of cells occupying the expected location of osteoblasts and cementoblasts

Fibrous connective tissue was frequently found between bone and root but, unless it met the above criteria, it was designated a fibrous capsule and not a periodontal ligament.

There is strong circumstantial evidence to support the belief that the periodontal ligament was synthesised by progeny of the cultured periodontal ligament cells transplanted with the root. Criticisms include observations made at only one period of time after transplantation, use of only four transplants, and the lack of markers to indicate the transplanted cultured periodontal ligament cells gave rise to the new periodontal ligament. However, previous studies have not found periodontal ligament associated with teeth from which periodontal ligament has knowingly been removed or damaged (Löe and Waerhaug 1961, Sherman 1968).

Recently, Lang *et al.* (1995) showed formation of a connective tissue around periodontal ligament roots 2 weeks after implantation of root discs with cultured periodontal cells into the palate of mini-pigs. Fibre bundles within the connective tissue became oriented between the second and fourth weeks and after 12 weeks most of the total roots' surfaces were covered by an organised connective tissue resembling periodontal ligament *in vivo*. Oriented fibre bundles were attached to both alveolar bone and root, almost at right angles. Fibres penetrated the bone and root's surfaces in the same way as Sharpey's fibres. Only a few areas showed formation of a fibrous capsule (fibres parallel to the root), less than 1% of the total roots' surfaces showed calcified tissue formation and resorptions ($14.8 \pm 2.7\%$) were rarely found.

In summary, according to the current literature, there is conflicting evidence regarding the existence of epithelial cells after healing. Indeed, there seems to be marked acellularity subsequent to re-implantation, which might indicate an absence of any cells. Should this be the case, success in previous studies will have been due to the replantation in the extraction site where remnants of the periodontal ligament remain.

At present it is unclear whether re-implantation of root discs into a heterotopic site will successfully regenerate a structure which fulfils the criterion for functional periodontal ligament. Success has not been achieved in a previous study with mature rat molars. The potential for technical difficulties in maintaining a viable ligament is high. Previous studies have utilised cultured periodontal cells onto root discs to ensure viability.

Specific epithelial cell will ensure direct evidence for the involvement of epithelial cells in the reformation of the periodontal ligament after wounding. Use of inbred Wistar-Furth rats will avoid immunological rejection of grafts.

It has been well established that the biology of periodontal ligament shows significant similarity between rats, other animals and humans. An animal model is but the first step to examine a relationship before further work on human models.

AIMS AND OBJECTIVES

HYPOTHESIS

The hypothesis to be tested is that the presence of intact epithelial cells in the periodontal ligament prevents ankylosis, and contributes to the regeneration of a functional periodontal ligament, subsequent to traumatic disruption.

AIMS AND OBJECTIVES

1. With the use of a rat model, develop a model for heterotopic root transplantation studies.
2. Compare and contrast the bony healing response in the transplant area when the periodontal ligament containing epithelial cells is either left intact or denuded in this newly developed model.
3. View the various responses in a time-course study.

CHAPTER 3

METHODS AND MATERIALS

EXPERIMENTAL APPROACH

Mandibular incisor roots obtained from female Wistar-Furth rats were labelled with specific anti-keratin antibodies and detected with Alkaline-Phosphatase-Vector Red. The presence of an attenuated epithelial root sheath was determined histologically by exploiting the fluorescence of the dye Vector Red.

The region of root with periodontal ligament was identified and cylindrical sections 2mm thick cut using a diamond disc.

Transplantation of experimental and control root discs into syngeneic recipient rats was performed. At timed intervals, tissues were perfused fixed, decalcified, dehydrated and embedded in either glycol methacrylate or paraffin wax.

2 μ m sections (sagittal and transverse) were obtained from the glycol methacrylate embedded sections and stained with Toluidine Blue. Serial 7 μ m sections were obtained from the paraffin embedded sections and every tenth section was stained with Haematoxylin and Eosin.

Antigen retrieval with a microwave and pressure cooker was performed on serial sections obtained from paraffin wax.

Sections were then immunolabelled using the DAB - Peroxidase technique.

EXPERIMENTAL PROCEDURES

SURGICAL PROCEDURE

Donor rats

Female Wistar-Furth (WF) donor rats, 4-6 weeks old, were obtained from The University of New South Wales Biological Research Laboratories. After arrival, they were acclimatised in plastic boxes with a wire cage cover for at least 24 hours.

The rats were sacrificed by exposure to 2ml of Fluothane. The two mandibular incisors were removed by first dissecting the entire mandible with a No. 22 scalpel blade, then removing all soft tissues.

The mandible was split into two pieces through the midline symphysis. The incisors were gently lifted out of the mandible by elevating mandibular bone off the root surface using college tweezers commencing from the cemento-enamel junction through to the apical region in the ramus of the mandible.

The pulp was extirpated with the use of a barbed broach (Beutel Rock BE-333XF) placed through the apical foramen in pilot studies only. The pulp was maintained in subsequent experiments.

The region of root with periodontal ligament was previously identified and confirmed microscopically and histologically to be in the middle third of the root. Two sections of tooth root discs 2mm in length were cut in this area using a diamond disc (Horico 345 Diaflex #190) under copious irrigation with phosphate buffered saline solution.

The viability and sterility of ligament cells was maintained by immersion in phosphate buffered saline solution containing penicillin and streptomycin. Discs were stored by refrigeration at 4°C as necessary when recipient rats were being prepared. Denuded root discs were prepared as controls by planing the periodontal ligament with a sharp scalpel until no periodontal ligament remnants were observed. Control discs were prepared prior to experimental discs.

Recipient rats

Female WF recipient rats, 9-10 weeks old, were prepared one-day prior to surgical implantation. Fur was removed from the midline above the eyes extending from the frontal bone between the ears to the occipital bone.

On the day of the implantation the rats were anaesthetised with Ketamine (30mg/kg). The shaved area was rubbed with 70% alcohol and a midline incision was made with a No.22 scalpel blade 5mm above the eyes on the frontal bone extending for 20mm. The soft tissue and periosteum was reflected and two cavities were prepared on the frontal bone 5mm apart and 10mm from the eyes.

A No.8 round tungsten carbide surgical bur of 1.5mm at low speed was used to create a circular cavity under copious irrigation with phosphate buffered saline solution and in later experiments the cavities were extended longitudinally by 2mm in length. The objective was to create a cavity deep and long enough to contain the root cylindrical discs that were 2mm in length. This involved the entire thickness of the cranial bone. Care was taken not to puncture the dura.

Using aseptic technique, the experimental and control root cylinders were placed by blunt dissection in the prepared cavities and the wound closed using 4(0)-coated Vicryl absorbable suture for the periosteum and 3(0) chromic soft-catgut suture for the fascia.

At intervals of 3, 10, 21, 56 and 63 days post-transplantation the recipient rats were sacrificed for specimen collection (Table 2).

SAMPLE SELECTION

Table 2. Sample size

	3 days	10 days	21 days (3weeks)	56 days (8weeks)	63 days (9weeks)	TOTAL
Donors	4	4	10	8	4	30
Recipients	6	6	21	15	6	54

In the experimental period, a total of 30 donor and 54 recipient rats were used in the five post-transplantation observational periods (Table 2).

SPECIMEN COLLECTION

WF recipient rats were anaesthetised with 1ml of Fluothane in a gas jar. Prior to dissection, the right jugular vein was severed, a 25 gauge needle was inserted into the left ventricle and 25 ml of sterile phosphate buffered saline was flushed through the vasculature at a rate of 20 ml per minute to clear the blood system. The tissues were perfused and fixed with 50ml of 4% paraformaldehyde in phosphate buffered saline which was flushed through the vasculature (see Appendix).

The cranium was removed from the body of the rat with a No.22 scalpel blade. The mandible was removed and the maxilla detached with the use of surgical scissors. The remaining elements of the cranium were fixed for 24 hours in 4% paraformaldehyde in phosphate buffered saline after which all soft elements were removed.

SPECIMEN DECALCIFICATION

The specimens of cranial tissue were placed into 50 ml jars and decalcified in a solution of 10% EDTA, pH 7.2, at 4°C for 42 days, changed daily (see Appendix). Specimens were radiographed after four weeks to determine the amount of mineralised tissue remaining. The specimens were radiographed at 60KV, for 0.2 seconds, at a source-object-distance of 35 cm.

When decalcification was completed after six weeks, the specimens were trimmed to a block 5mm by 10mm with one corner cut at an angle for identification purposes. The blocks were then dehydrated in graded alcohols (50%, 70%, 90%, 95%, 100%).

SPECIMEN EMBEDDING IN GLYCOL METHACRYLATE

For rapid analysis of results in the pilot studies, specimens were embedded in glycol methacrylate and stained with Toluidine Blue. Initial information regarding bone healing and viability of periodontal cells after surgical manipulation were obtained using this technique.

Embedding the specimens required infiltration with JB-4 catalysed infiltration resin (Polysciences). Specimens were placed into wells containing 10 ml of the catalysed infiltration resin for 24 hours at 4°C (see Appendix).

The resin was poured around each specimen in plastic trays and sealed with paraffin wax. The specimens were left for 24 hours to allow complete polymerisation before removal from the plastic trays.

SPECIMEN EMBEDDING IN PARAFFIN WAX

In the experiment proper, specimens were embedded in paraffin wax and immunolabelling and Haematoxylin and Eosin stains were used in histological analysis.

The specimens were placed into a Tissue-Tek VIP (Miles Scientific) instrument for dehydration, "clearing" (removing the dehydrant with a substance which is miscible with paraffin wax) and infiltration with paraffin. The tissues were then embedded in paraffin wax.

SPECIMEN SECTIONING

Glycol methacrylate sectioning was performed using a Sorvall Porter-Blum microtome. 2µm sections were cut with a dry glass knife, collected and transferred with tweezers onto a room-temperature water bath. Sections were then collected on pre-cleaned glass slides and air-dried.

Paraffin sectioning was performed using a Microm HM 325 (D and A Lab Services, Sydney, Australia) microtome with a stainless steel knife. Serial sections of 7 μ m were cut and transferred onto a 35°C water bath to allow flattening of the section. Sections were collected onto pre-cleaned Superfrost Plus microscope slides (Biolab Scientific) and dried in a 35°C oven for a minimum of 48 hours, followed by a 60°C oven for at least 1 hour.

SPECIMEN STAINING

Glycol methacrylate sections were stained with 1% Toluidine Blue in Borax for 20 seconds. Following staining, the sections were rinsed with water, dried and coverslipped with mounting medium.

Every tenth paraffin section was removed and stained with Haematoxylin and Eosin according to traditional methods (see Appendix). The rest were left for immunolabelling.

IMMUNOCYTOCHEMISTRY

Immunolabelling was obtained with a three layer Biotin-Avidin-Alkaline phosphatase, which was visualised with Vector Red (Vector Laboratories), and a two-layer diaminobenzidine (DAB)-peroxidase method.

Biotin-Avidin Alkaline Phosphatase Labelling

The following method was used in conjunction with Vector Red staining to allow immunofluorescent analysis:

- I. The primary antibody Dako-AE1/AE3 was applied at a concentration of 1:50 for 18 hours.
- II. Sections were rinsed in phosphate buffered saline (PBS) for 10 minutes.

- III. Biotinylated antibody conjugate, goat anti-mouse immunoglobulin (Ig)-G-biotin (Biosource International, code no: AM 13709) was applied at a concentration of 1:100 for 18 hours. The antibody is in solution with saline, pH 6.2, with 0.1% sodium azide as a preservative.
- IV. Sections were rinsed in phosphate buffered saline for 10 minutes.
- V. Streptavidin-alkaline phosphatase conjugate (Biosource International, cat no:6567) was applied to the sections for 30 minutes at a concentration of 1:1000. The antibody was diluted with 50mM Tris buffer, pH 8.0 with 1mM Mg Cl₂, 0.1 % sodium azide and 40% glycerol.
- VI. Sections were rinsed in distilled water for 5 minutes.
- VII. Vector Red alkaline phosphatase substrate was applied following the manufacturer's instructions for 20-30 minutes. Vector Red forms a precipitate producing a coloured diazo compound.

Antigen Retrieval

Antigen retrieval was required prior to immunolabelling of tissues, which had been previously fixed. Cross-links between protein end groups occur when fixation has been performed altering the structure of the antigen. The method using microwave and pressure cooker seemed to give reasonable results although many sections were wasted due to folding.

- I. Slides were heated for 1 hour at 60°C.
- II. Slides were de-waxed and placed in PBS to equilibrate the pH of the tissues, facilitate antibody binding and prevent non-specific binding of antibodies, thus reducing background staining.
- III. Slides were placed into a plastic slide holder and plastic rectangular receptacle containing 250ml of Antigen Unmasking Solution (Vector; cat no: H-3300). The unmasking solution was mixed as per the manufacturers' instructions resulting in a sodium citrate buffer of pH 6.0, at a concentration of 5 ml of Vector unmasking solution per 500 ml of distilled water.

- IV. The plastic receptacle was placed into 300ml heat sink of distilled water in the pressure cooker (Nordic Ware - h 2104 Microwave Tender Cooker). The distilled water heat sink was previously heated for 7 minutes on high (900W), with the lid loosened.
- V. The lid was tightened on the pressure cooker and placed into the microwave oven. The samples were heated for 2.5 minutes on high (900W), then 3 minutes on medium (450W).
- VI. The pressure cooker was then removed from the microwave and placed into a sink with cold running water until the pressure equalised.
- VII. The lid was then removed and the samples were placed into phosphate buffered saline.

DAB-Peroxidase Immunolabelling

The primary antibody used was a monoclonal mouse anti-human cytokeratin, AE1/AE3 (Dako-AE1/AE3). Monoclonal antibody AE1 recognises 56.5, 50, 50', 48 and 40 kD keratins of the acidic sub-family and anti-keratin AE3 reacts with the basic keratins of molecular weights 65-67, 64, 59, 58, 56 and 52 kD (Woodcock-Mitchell *et al.* 1982). The Dako-AE1/AE3 reacts with simple epithelia (42/52 and 46/54), hypoproliferative keratinocytes (48/56), stratified epithelia (50/58), stratified squamous epithelia of internal organs (51/59), keratinised (56.5/65-67) and corneal (55/64) epidermis (Sun *et al.* 1984).

The primary antibody was added to the following:

- a. Bovine serum albumin 1%. (The bovine serum albumin acts as a protein carrier when diluting large volumes, and acts as a competitive inhibitor)
- b. Goat serum 5%. (The goat serum is a blocking agent to prevent non-specific staining)
- c. Azide 0.1%. (preservative).
- d. Phosphate buffered saline (pH 7.3).

The secondary antibody used was peroxidase-conjugated goat anti-mouse immunoglobulins (Dako, P 0447). This antibody reacts with mouse IgA, mouse IgM and all mouse IgG subclasses. The cross-reaction with rat immunoglobulins is about 30%.

1. The primary antibody is applied to the tissue sections at a concentration of 1:100 for 1 hour.
2. Sections were washed in phosphate buffered saline for 10 minutes.
3. The sections were then placed in a 3% aqueous solution of hydrogen peroxide for 5 minutes. (Non-specific reaction is prevented by hydrogen peroxide which destroys any endogenous peroxidase activity.)
4. The secondary antibody was applied to the sections at a concentration of 1:50 for 30 minutes.
5. Sections were washed in phosphate buffered saline for 10 minutes.
6. The chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) was applied at a concentration of 0.03%, for 10 minutes. (DAB produces a brown pigment, insoluble in organic solvents, at the site of antigen localisation.)
7. Hydrogen peroxide at a concentration of 50 μ l of hydrogen peroxide per 10 ml of DAB was added acting as a substrate for the enzyme on the peroxidase-conjugated secondary antibody.
8. The sections were then washed in water for 5 minutes and counterstained with Haematoxylin and Scott's Bluing solution prior to mounting.

EXPERIMENTATION EVALUATION

A Heine magnifying spectacles (2.5X) were used during all surgically procedures.

An Olympus microscope was used to view sample extracted mandibular roots after labelling with specific anti-keratin antibodies and detected with Biotin-Avidin Alkaline Phosphatase.

A Dialux Leica, Germany light microscope was used for microscopy of labelled sections.

Histological sections were evaluated for the presence of inflammatory response, ankylosis and regeneration of the periodontal ligament.

The percentage of ankylosis was measured with the use of a grid overlying the entire root surface at low power magnification to delineate the amount of bone fusion along the root surface.

Photography was performed using a Leica camera mounted on the light microscope.

CHAPTER 3

RESULTS

The characteristics of the Wistar-Furth mandibular incisor root were histologically examined previously in a pilot study. Epithelial cell rests were present in the mid-third of the root. Another pilot study to determine bone healing within the surgically prepared cavities in the cranium revealed that new bone formation filling the entire cavity was radiographically evident by 3 weeks.

The rats were tolerant to surgical manipulation of the cranial bone with rapid recovery and healing. Wound healing after transplantation was uneventful, sutures were not disturbed and the rats did not demonstrate untoward behaviour post-transplantation.

Light microscopy demonstrated a continual change in histological appearance within and around the root discs within the 3 to 63 days post-transplantation observational period. Initially after transplantation, degeneration within the pulp chamber of both experimental and control discs and of viable periodontal ligament, was detected in experimental discs.

This was followed by secondary dentine formation within the pulp chamber of both types of discs as a result of pulpal remnants. External root resorption and ankylosis were present around control discs and some areas of experimental discs. A fibrous capsule and the periodontal space and was maintained adjacent to most root surface areas of experimental discs.

Within the experimental group, regularly spaced epithelial cells lying in close relation to the root surface were maintained at 3 and 10 days post-transplantation but were consistently absent in 21 day and later samples. This indicated that, related to the persistence of a fibrous capsule epithelial cells have disappeared by 21 days post-transplantation.

In another experiment involving only 21 and 56 days post-transplantation samples, cranial cavities were enlarged to facilitate insertion of root discs and prevent further damage to the periodontal ligament. Although as a result of this, the presence of

ankylosis was not detected in either control or experimental groups during the observational periods, this did not have not have any effect on the disappearance of epithelial cells as mentioned above.

The following chart represents the result from retrievable histological sections from a sample of 24 out of a total of 54 recipient rats in the various observational periods demonstrating the increasing presence of ankylosis in control discs as the observation period progressed. Ankylosis, as determined by fusion of bone to root surface in the transplant area, was recorded as a percentage of the total root surface area. Although there were variations within each observation period, a trend for increasing ankylosis with the longer observational period is clearly demonstrated.

As evidenced from Figure 2, the periodontal ligament remained intact despite the manipulation in sectioning 2mm discs from the surgically extracted mandibular incisor.

Immunological labelling of sample discs clearly demonstrates the presence of epithelial cells in a regularly spaced pattern along the root surface (Figure 3).

LEGEND
Figure 2. Incisor root in cross section. H & E 25X.
Figure 3. Epithelial cells of the periodontal ligament in cross section of incisor root. DAB-Peroxidase 250X.

BF

Bone Formation

EC

Epithelial Cell

OR

Odontoblast Reaction

PDL

Periodontal Ligament

RD

Root Dentine

RR

Root Resorption

Figure 2

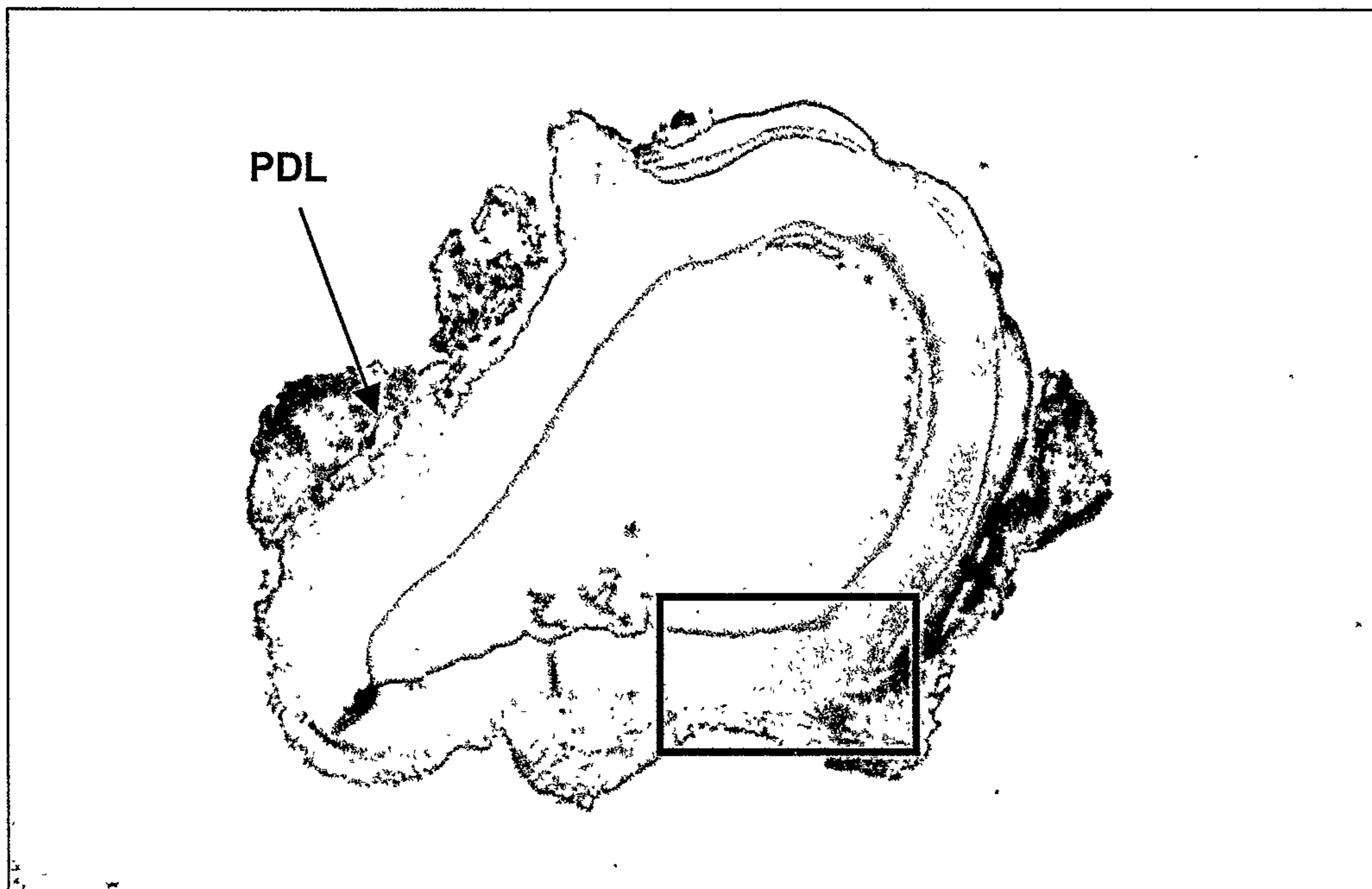
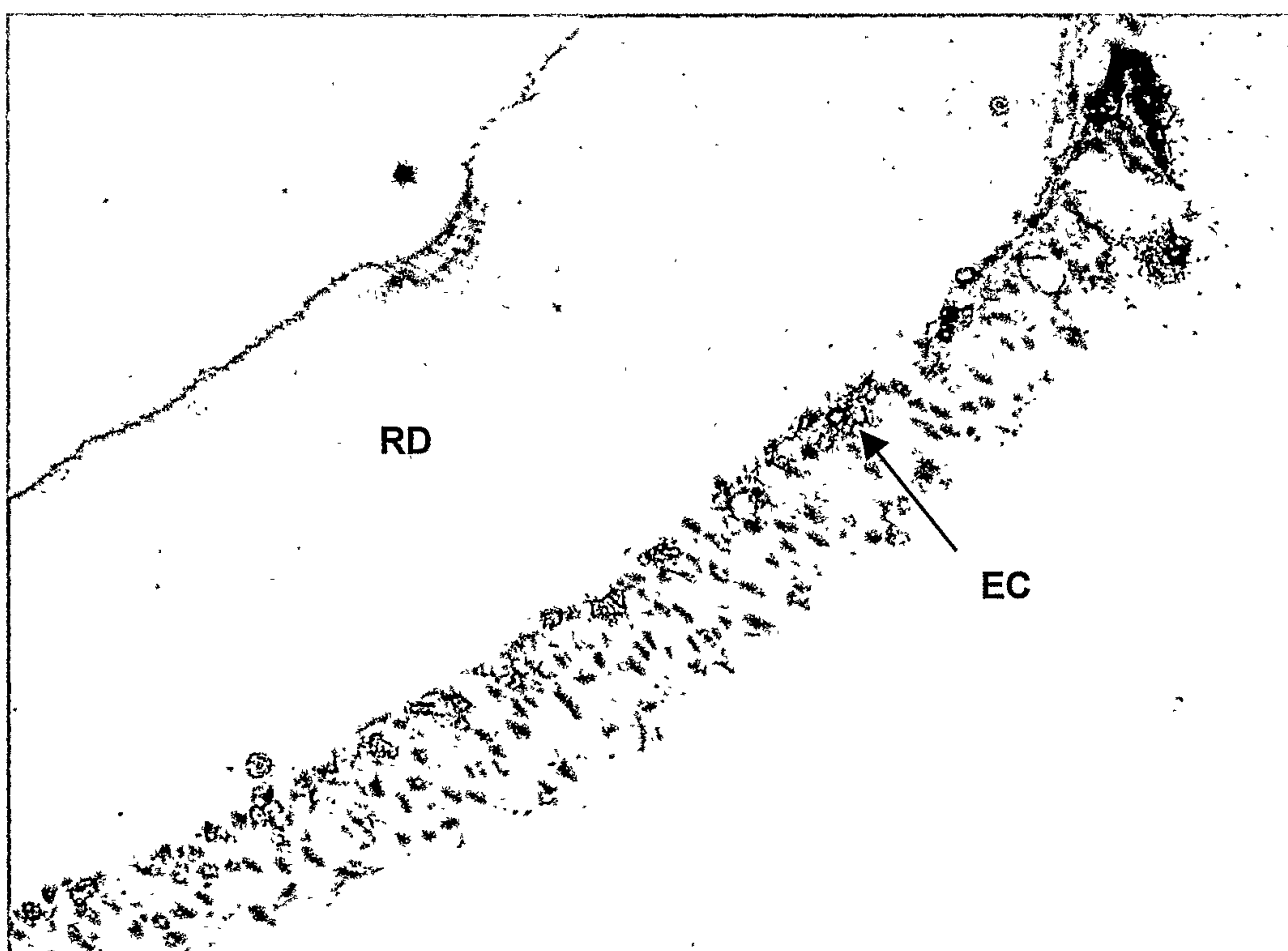


Figure 3



At 3 days post-transplantation, there is extensive degeneration both within the periodontal ligament and the pulp chamber. Although there were neither signs of periodontal regeneration nor ankylosis to adjacent bone, the regular pattern of epithelial cells remained (Figure 4).

Epithelial cells are also present within the periodontal ligament in 10 days post-transplantation samples (Figure 5).

LEGEND
Figure 4. Epithelial cells in cross section of incisor root 3 days post-transplantation. DAB-Peroxidase 250X:
Figure 5. Epithelial cells in cross section of incisor root 10 days post-transplantation. DAB-Peroxidase 250X.

BF Bone Formation
EC Epithelial Cell
OR Odontoblast Reaction
PDL Periodontal Ligament
RD Root Dentine
RR Root Resorption

Figure 4

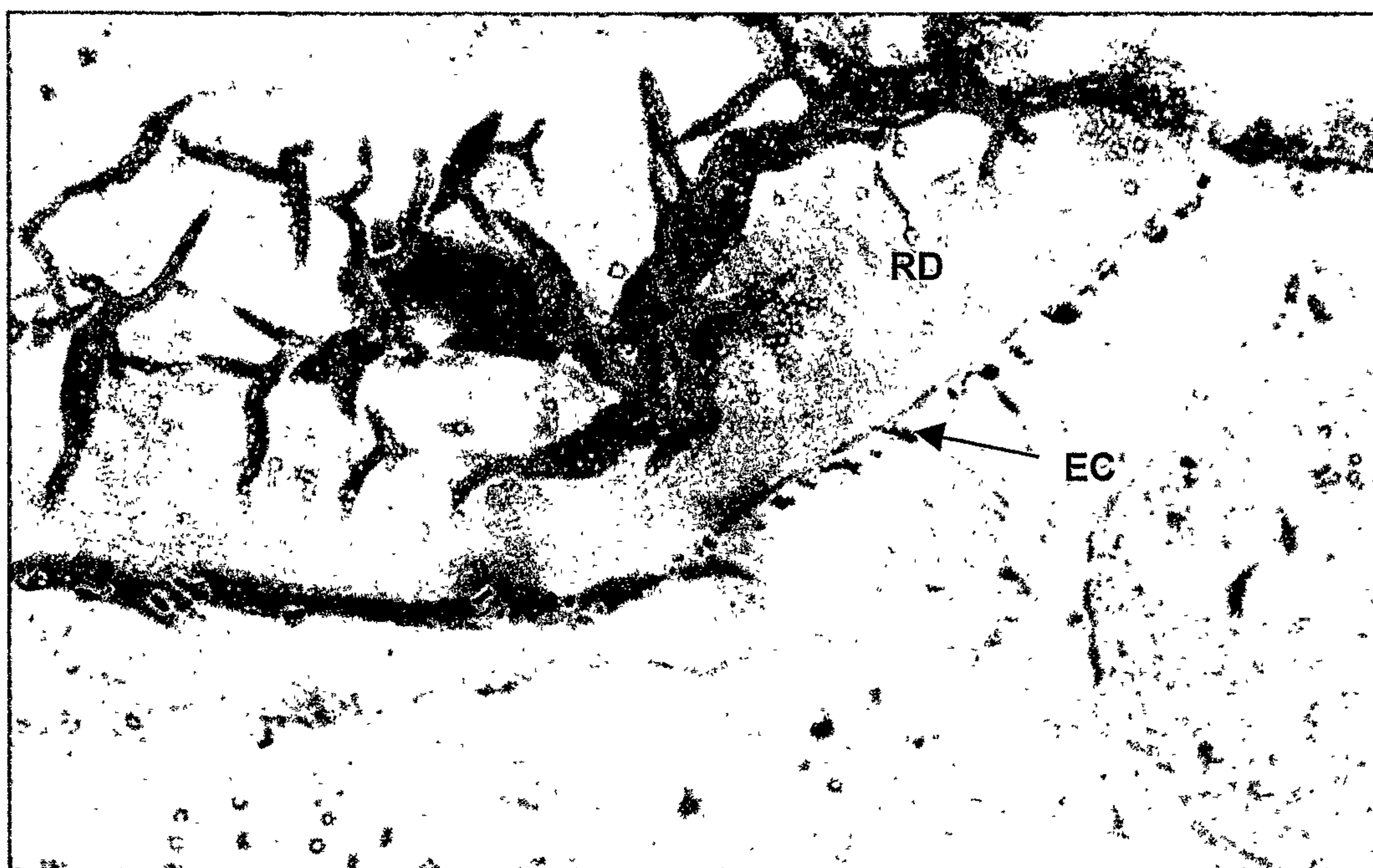
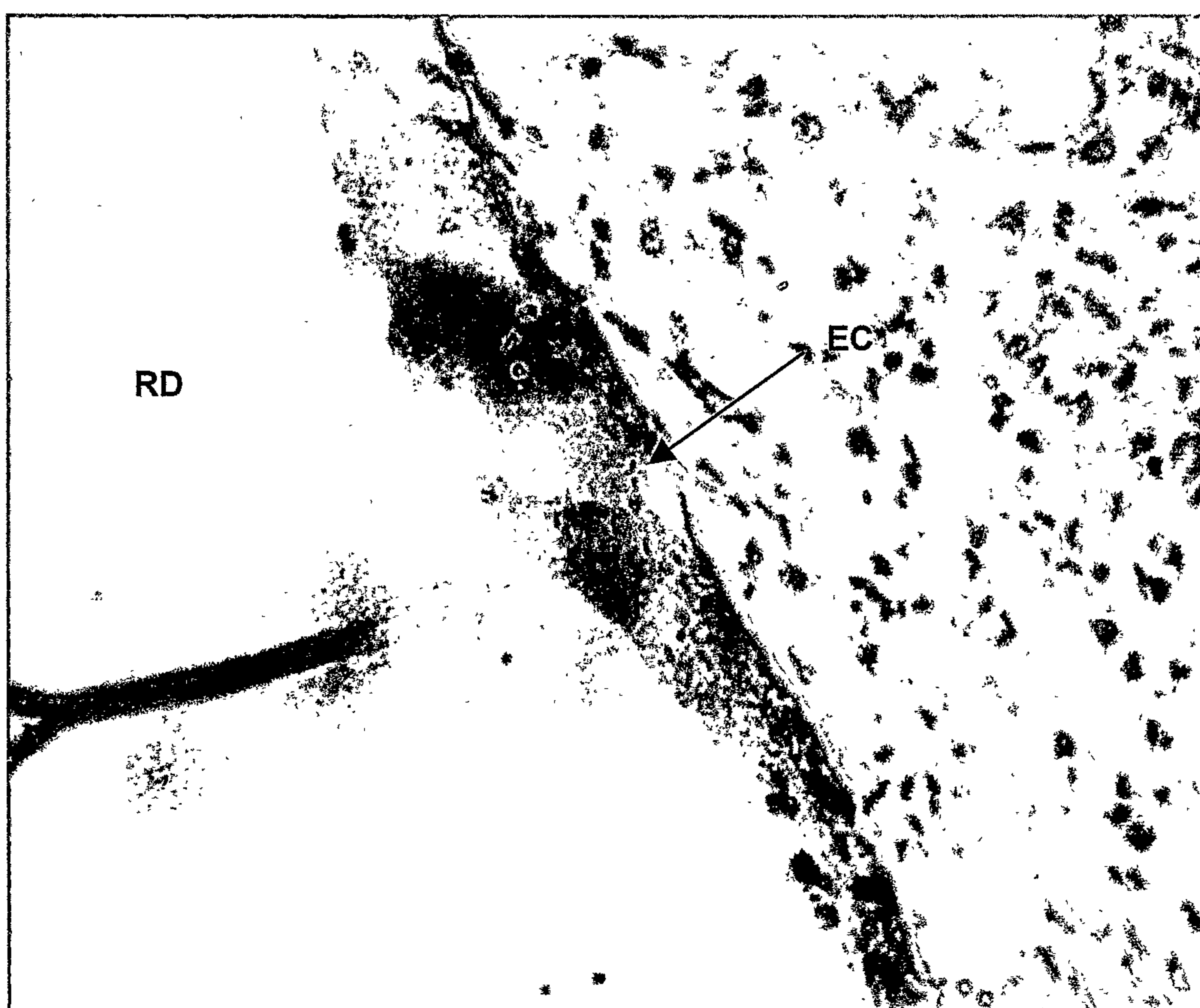


Figure 5



After 10 days, bony fusion has occurred around and within the control disc denuded of periodontal ligament (Figures 6 & 7). There is also evidence of root resorption on the external surface of the control disc.

LEGEND

Figure 6. Control disc 10 days post-transplantation. H & E 25X.

Figure 7. Control disc 10 days post-transplantation. H & E 250X.

- BF Bone Formation
- EC Epithelial Cell
- OR Odontoblast Reaction
- PDL Periodontal Ligament
- RD Root Dentine
- RR Root Resorption

Figure 6

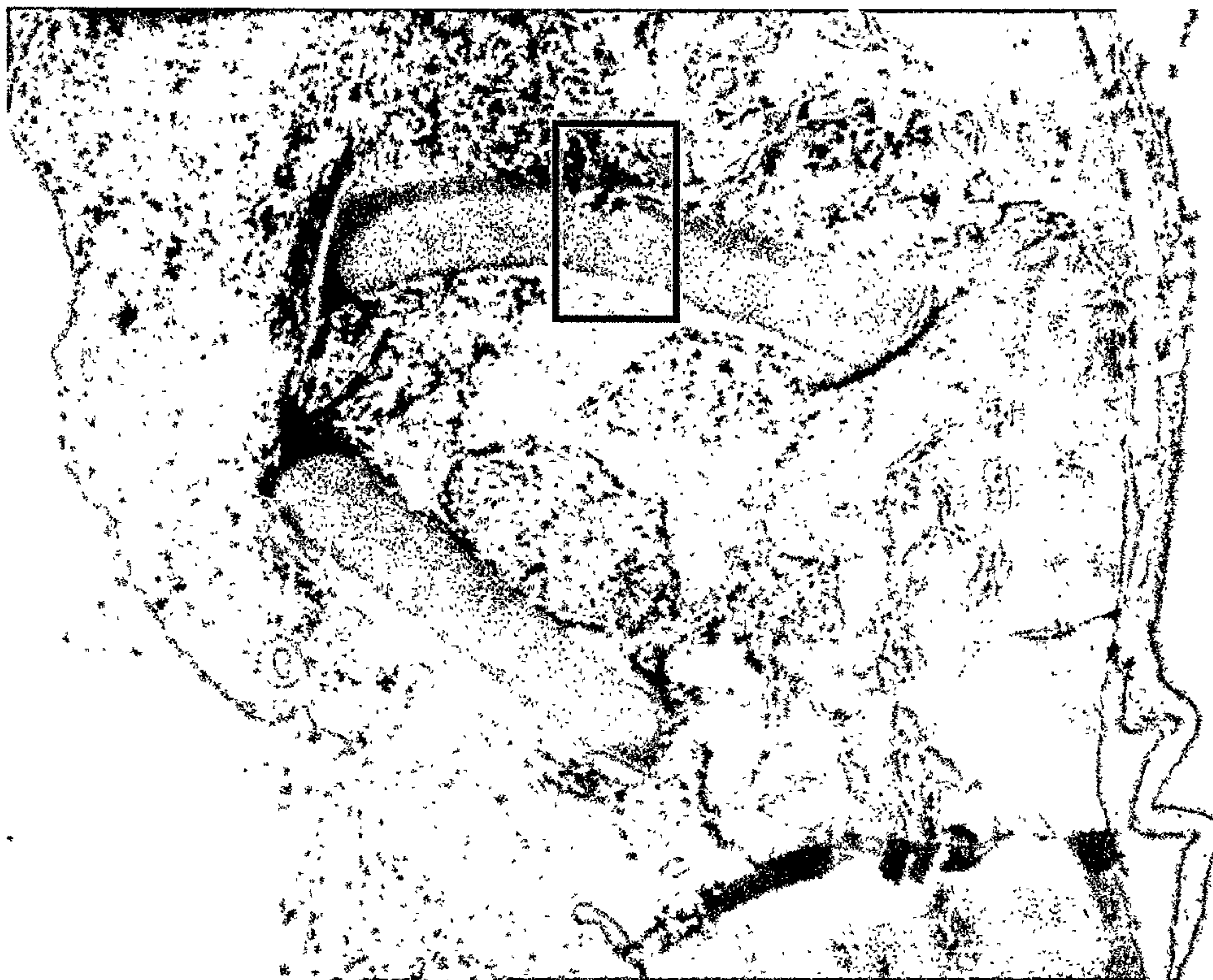
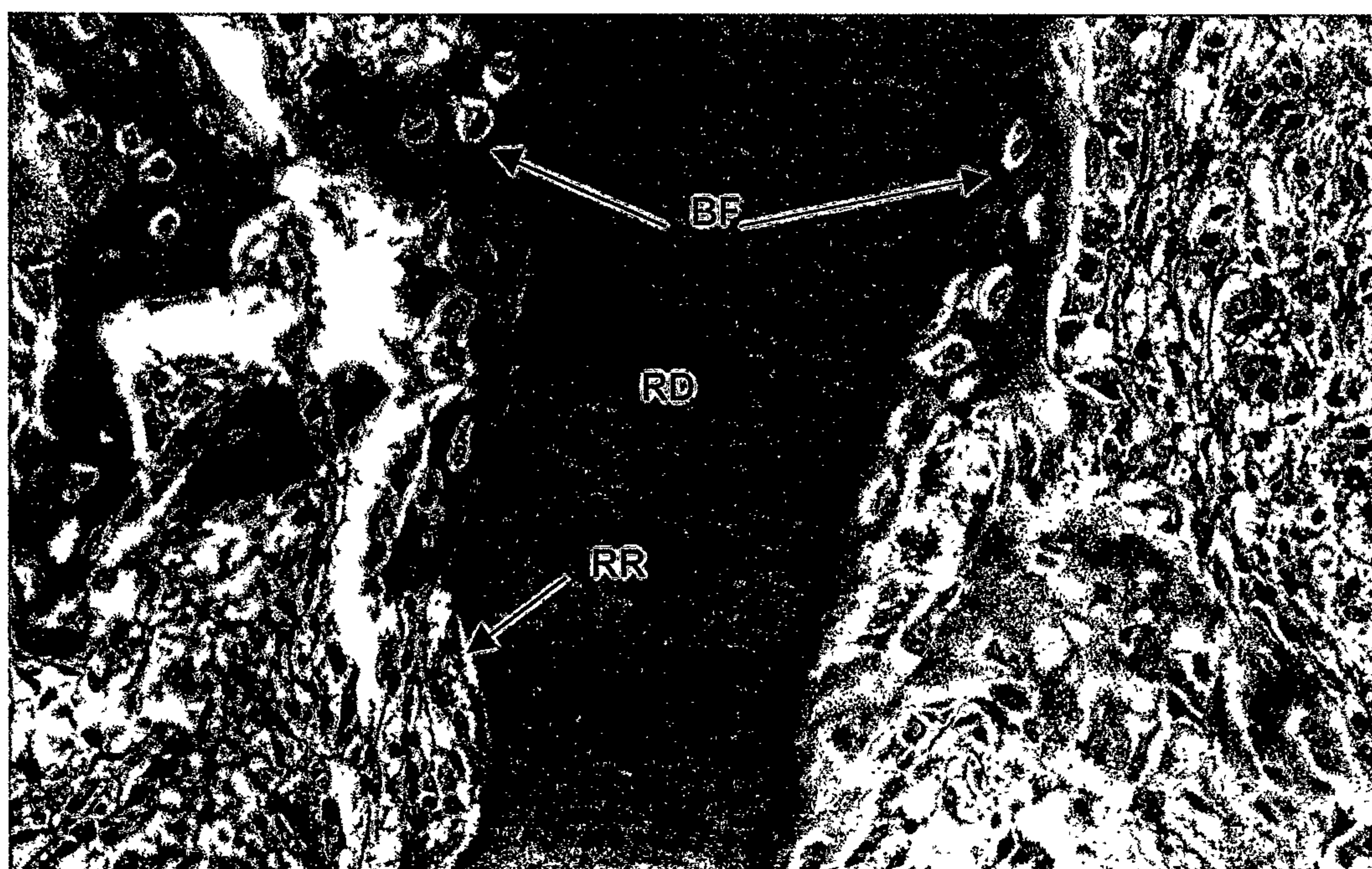


Figure 7



A fibrous structure is apparent in some areas around test discs (Figures 8 & 9).

An odontoblastic reaction has taken place within the pulp chamber not fully devoid of pulpal tissue (Figure 9).

This pulpal dentinogenesis within the pulp chamber is apparent in both control and test discs where there are pulpal remnants.

LEGEND

Figure 8. Test disc 10 days post-transplantation. H & E 25X.

Figure 9. Test disc 10 days post-transplantation. H & E 250X.

- BF Bone Formation
- EC Epithelial Cell
- OR Odontoblast Reaction
- PDL Periodontal Ligament
- RD Root Dentine
- RR Root Resorption

Figure 8

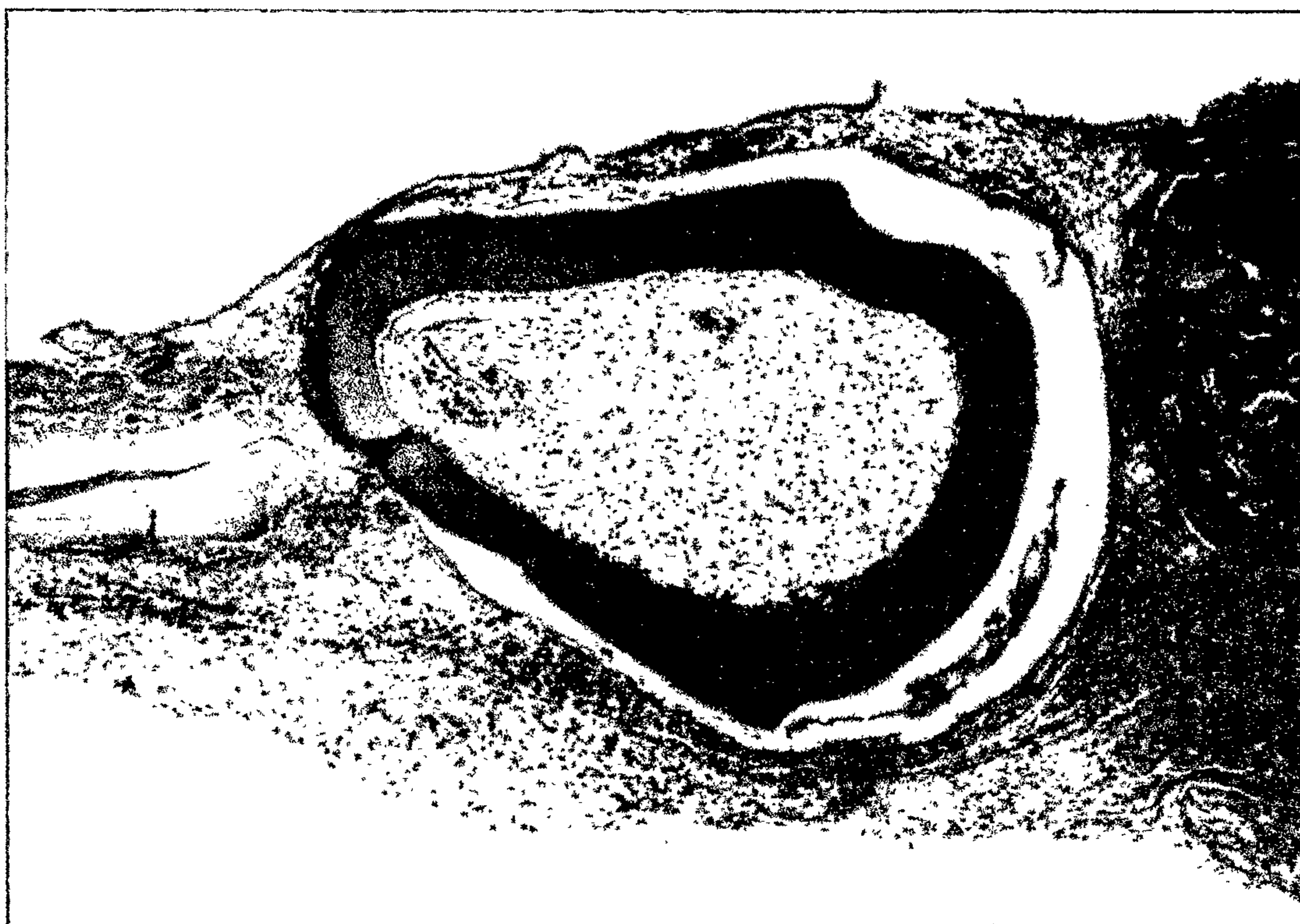


Figure 9



At eight weeks post-transplantation, there is significant bone formation and fusion between bone and the tooth surface around and within the control root disc (Figures 10 & 11).

Around the test disc, a fibrous structure resembling the periodontal ligament appears (Figures 12 & 13).

Figures 14-17 provides further examples of the extensive ankylosis that occurs in nine-week samples.

LEGEND

Figure 10. Control disc 8 weeks post-transplantation. H & E 25X.

Figure 11. Control disc 8 weeks post-transplantation. H & E 250X

Figure 12. Test disc 8 weeks post-transplantation. H & E 25X

Figure 13. Test disc 8 weeks post-transplantation. H & E 250X

Figure 14. Control disc 9 weeks post-transplantation. H & E 25X

Figure 15. Control disc 9 weeks post-transplantation. H & E 250X

Figure 16. Control disc 9 weeks post-transplantation. H & E 25X

Figure 17. Control disc 9 weeks post-transplantation. H & E 250X

- BF Bone Formation
- EC Epithelial Cell
- OR Odontoblast Reaction
- PDL Periodontal Ligament
- RD Root Dentine
- RR Root Resorption

Figure 10

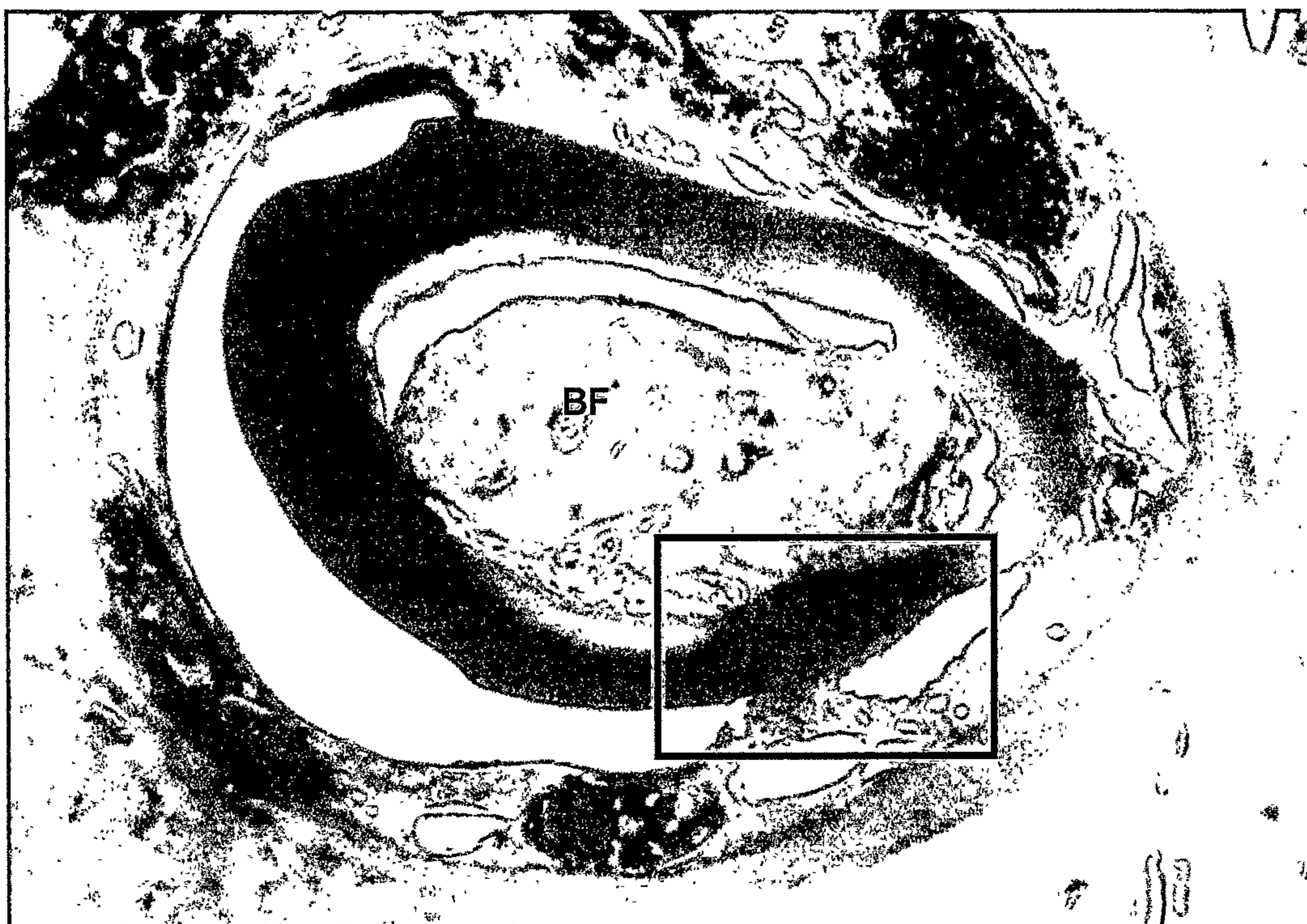


Figure 11

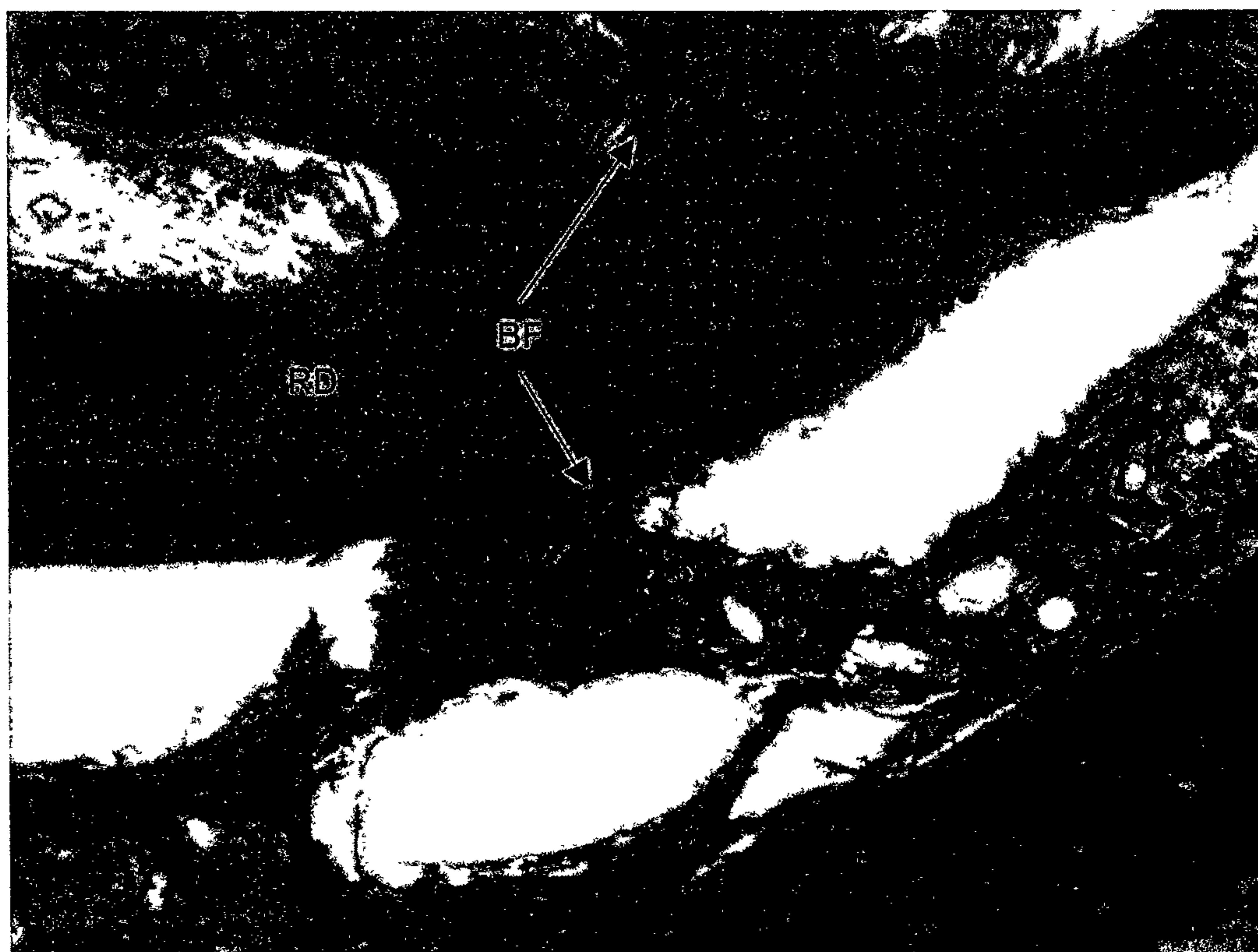


Figure 12



Figure 13

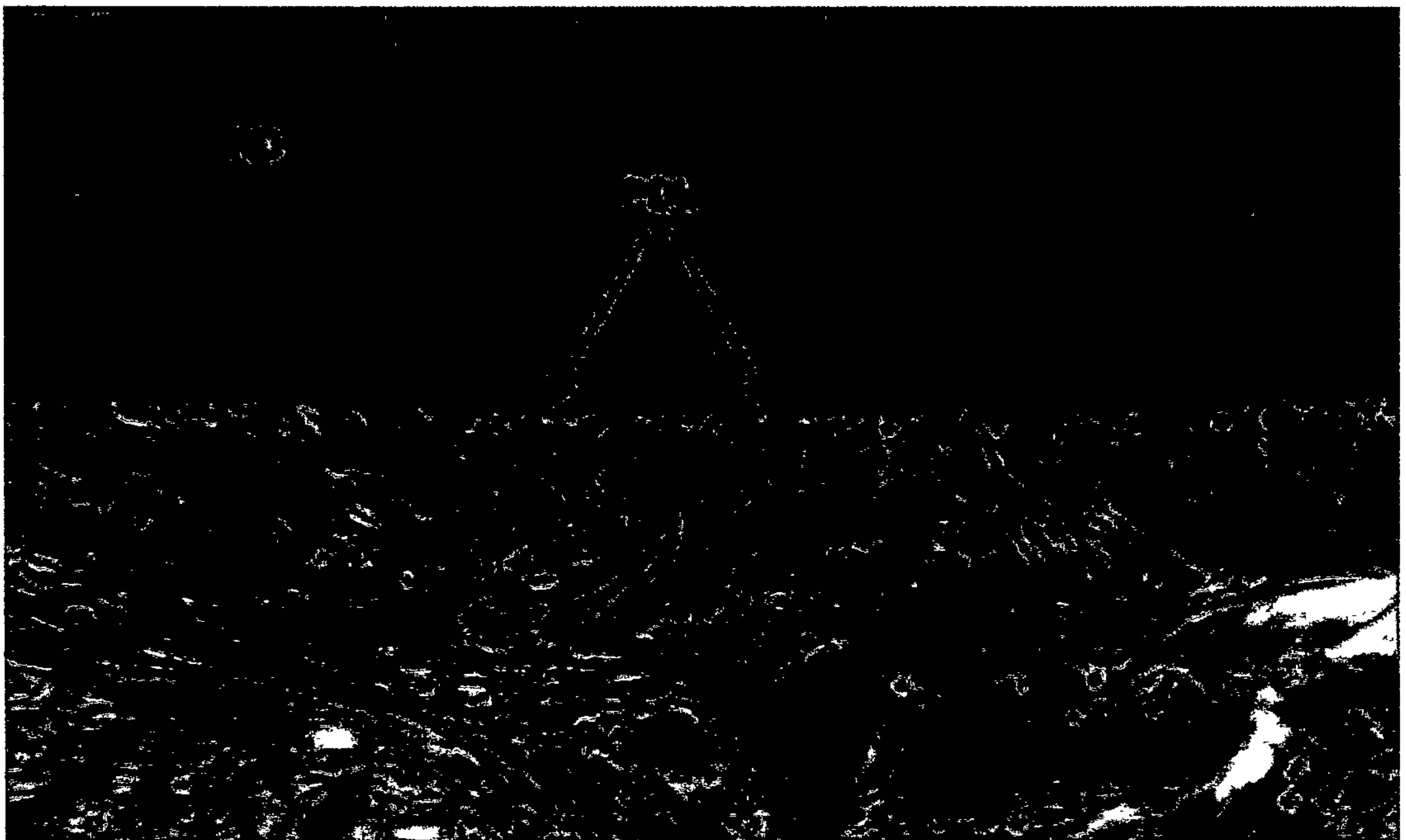


Figure 14



Figure 15

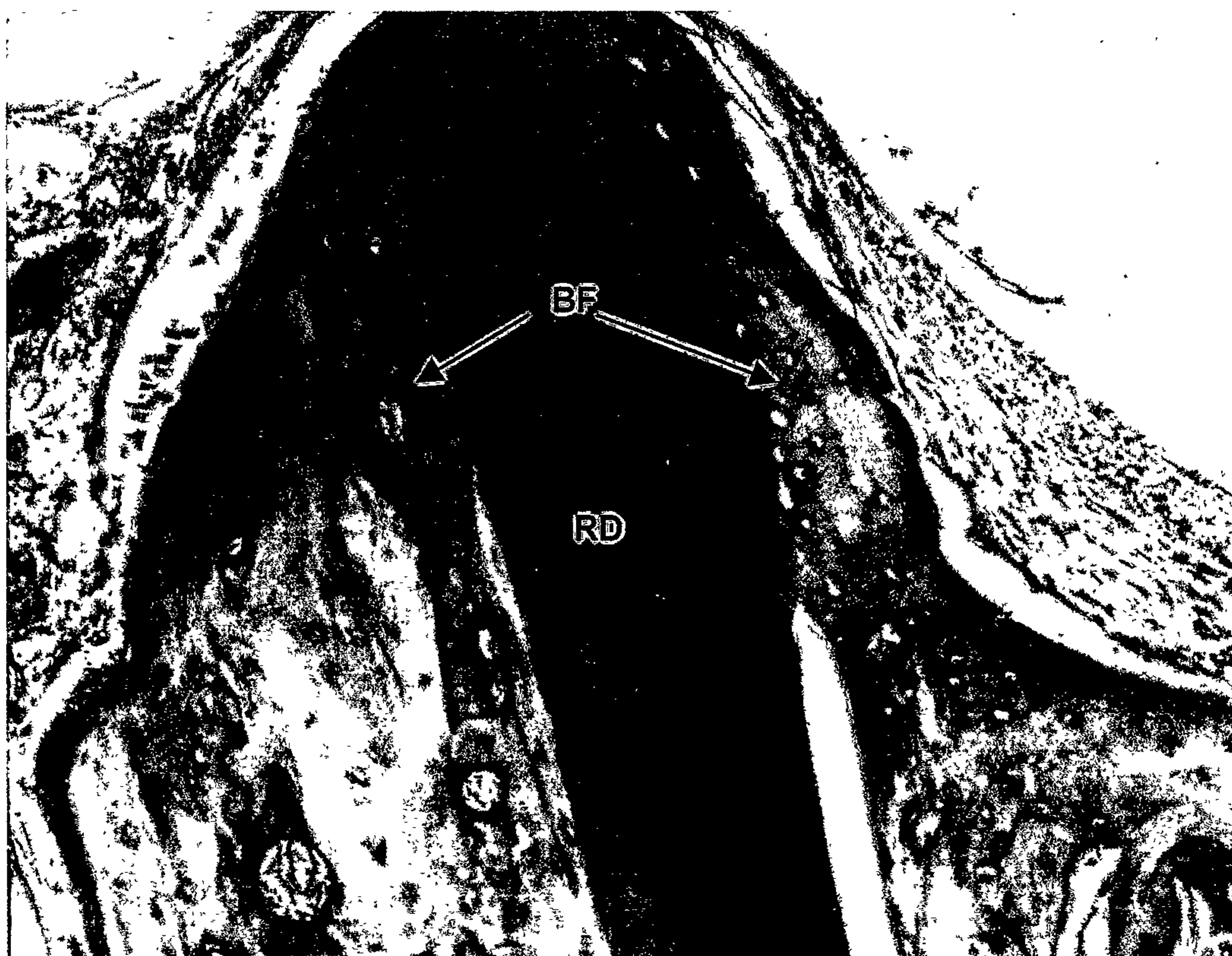


Figure 16

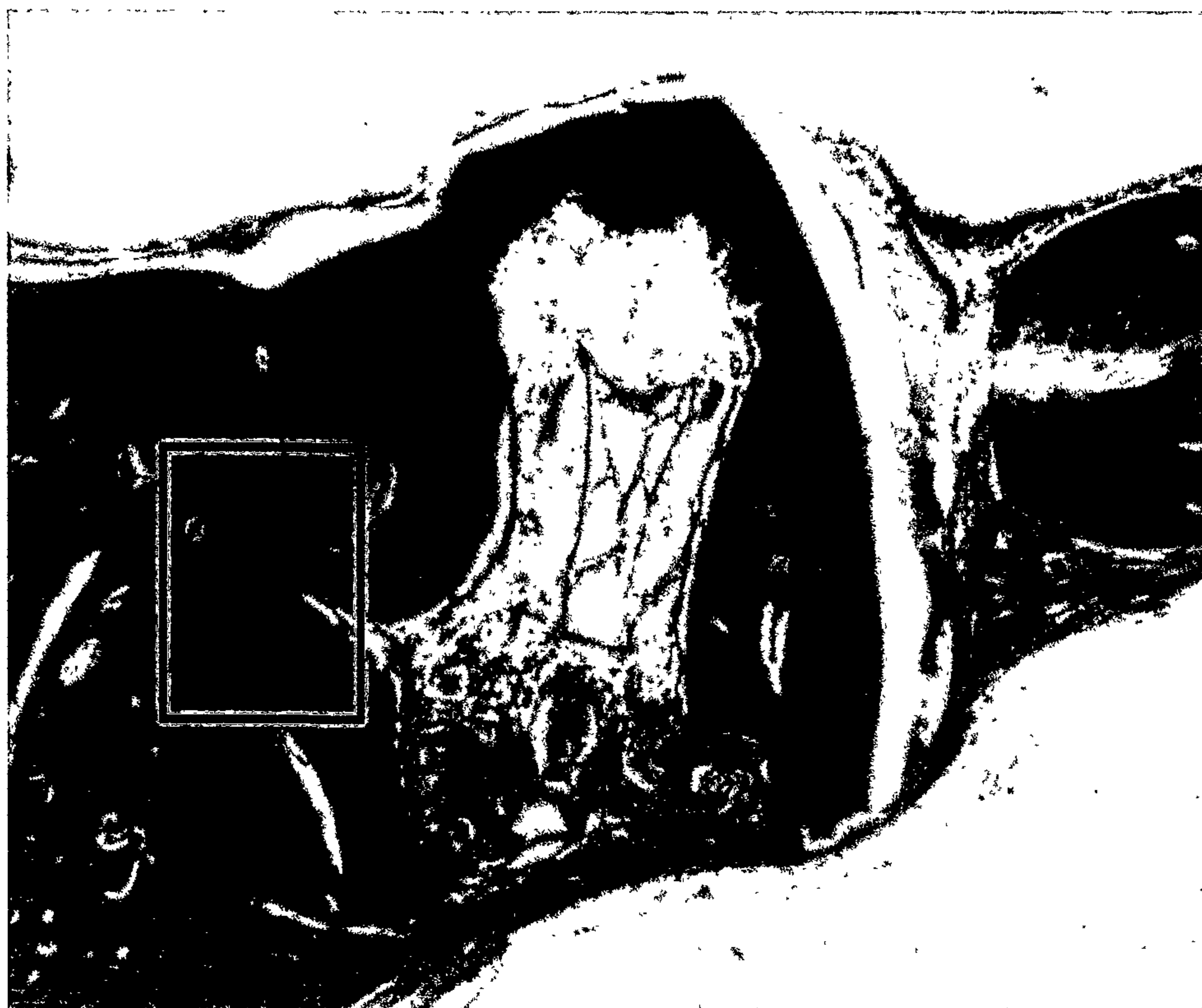
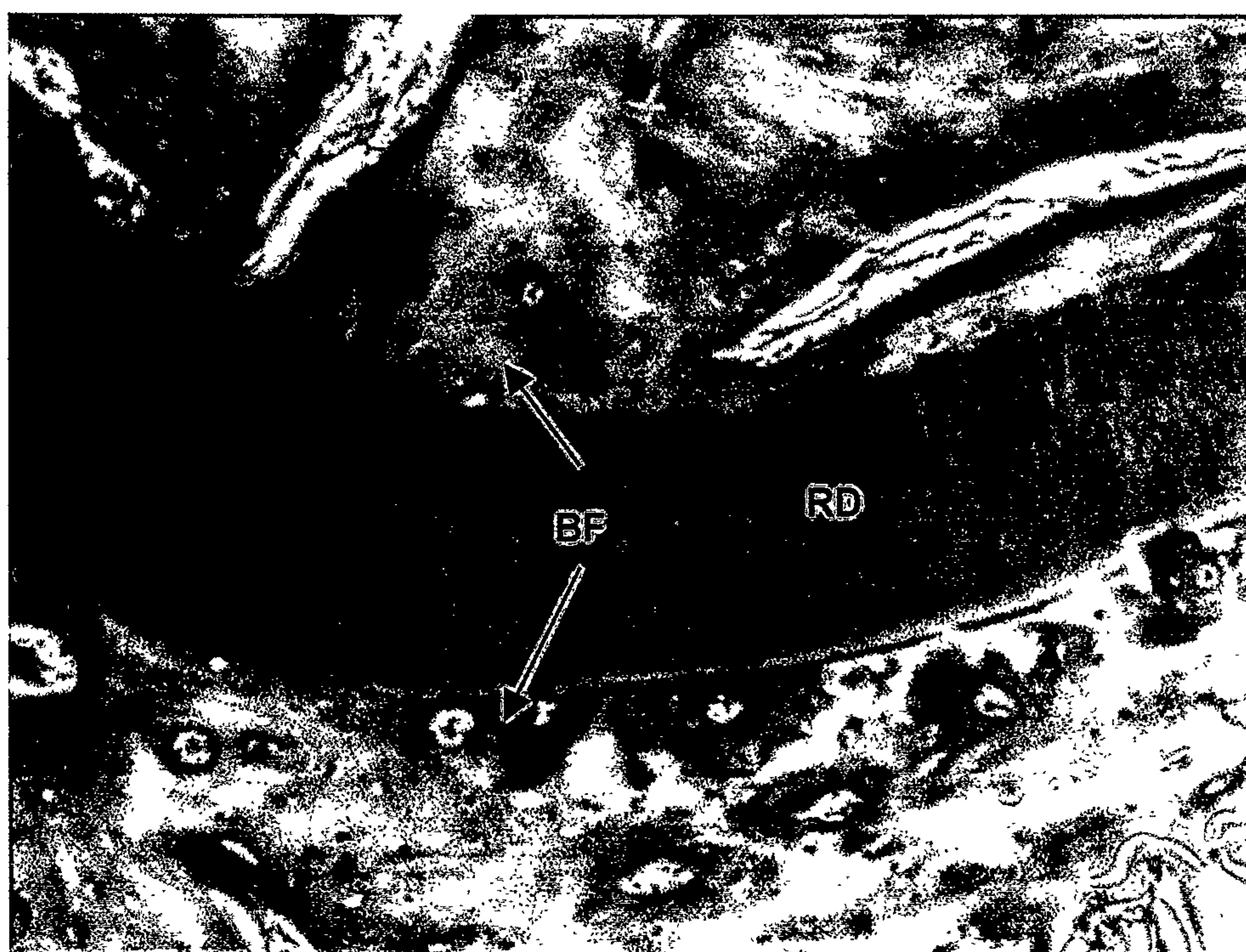


Figure 17



CHAPTER 4

DISCUSSION

The purpose of this project was to develop a model to study the fate of the periodontal ligament subjected to disruption, in relation to the behaviour of the epithelial cells. The objective was to develop a heterotopic model that eliminated the contamination problems hampering previous studies (Butcher and Vidair 1955, Loe and Waerhaug 1961, Andreassen and Hjorting-Hansen 1966, Sherman 1968, Anderson *et al.* 1968, Rothschild *et al.* 1969, Shulman and Kalis 1970, Hamner *et al.* 1970, Nordenram *et al.* 1970, Gamal 1970, Castelli *et al.* 1971, Hurst 1972, Nasjleti *et al.* 1975, Soder *et al.* 1977, Andreassen *et al.* 1978, Nasjleti *et al.* 1978). This includes variables such as effects of infiltrating oral bacteria and gingival epithelial ingrowth into the wound space.

The present study has shown that the existing model in this working form provided the expected results in terms of ankylosis and formation of a fibrous capsule. It has shown ankylosis in areas where the periodontal ligament is absent with occasional areas of ankylosis where the periodontal ligament has not been deliberately removed. The use of immunolabelling techniques has illuminated the presence and subsequent disappearance of epithelial cells in the different observation periods.

Periodontal ligament in all animals appears to have similar characteristics (Grant and Bernick 1971, Owens 1978) thus the results may be duplicated and extrapolated to different species and humans. Rats were chosen as the appropriate subjects, as they are readily obtainable at all times and for their ease in handling. Use of syngeneic Wistar-Furth rats minimised the risk of transplant rejection. Younger donor rats with pliant alveolar bone were chosen for ease of incisor removal; older recipient rats were chosen for their thicker cranial bone.

Of the cranial bones, the frontal bone has the greatest thickness. This is also an area difficult for the rats to scratch and loosen any sutures. Calvarial bone regeneration not only occurs from the rim and periosteum but also from the underlying dura (Mabbutt *et al.* 1979). Thus, maintenance of an intact dura was important for complete bone regeneration (Reid *et al.* 1981).

The cross section of the mandibular root is ovoid in shape with the largest diameter approximately 1.5mm. The bony cavity in the frontal bone was prepared to accommodate the discs. Difficulties were encountered in matching the shape of the cavity to the discs. Additional trauma may have been induced when placing a tight fitting ovoid disc into the circular bur hole, thus enlargement of the cavities was necessary.

In these samples, a large cavity with fibrous tissue around both experimental and control discs resulted within the present observational period. This may have been due to insufficient time for bone formation or periodontal regeneration to occur across the span between the root disc. Alternatively, fibrous repair tissue may have set in preventing desirable outcomes. Although there were different types of bony tissue adjacent to the disc along the root surface, a similar bone healing response was expected as a 2mm cavity is below the critical size of 8mm in which bony union will not occur in rat calvarial bone (Mulliken and Glowacki 1980).

An appropriate modification of the design would include forming a trench on the surface of the bone and laying the root longitudinally along it with an overlay of surgical gel foam to dissipate excessive pressure from wound closure. The root surface would thus be within the medullary bone and situated close to the periosteum. However, interpretation of the results may require separation and comparison of the two distinct sources of bone formation.

As it has been established that periodontal regeneration would only occur in the presence of viable periodontal ligament cells, it was thus essential to maximise viability. Studies have recommended endodontic treatment to prevent inflammatory resorption (Cvek *et al.* 1974, Andreasen and Kristersson 1981, Hammarström *et al.* 1986), thus initially, in the present study, the pulp tissue was removed. However, deleterious effects in samples where some remnants remained were not found. In fact, in the presence of pulpal remnants dentinogenesis occurred and provided a positive indicator that specialised dental tissues remained functional and were indeed stimulated within the experimental period. This concurs with Breivik's (1981) observations that odontoblasts can survive reimplantation with noticeable reparative dentine after two weeks.

Control discs denuded of periodontal ligament were prepared in advance to allow immediate transplantation once extracted viable root discs were sectioned. These

discs provided a remarkable indicator of the extensive ankylosis and root replacement that can occur. Experimental discs were stored refrigerated at 4°C whilst the recipient rat was being prepared for transplantation.

The observation periods were determined based on certain expected outcomes and the time given to complete this Masters program. The 3-day observation period was selected, as the time required for detection of an inflammatory reaction and to assess the viability of periodontal ligament after transplantation. It was expected that at 10-days, some healing processes would have occurred and from previous pilot studies, bone formation was found after 21-days.

Nine weeks was the longest observation period realistically possible for this project as the amount of time required for processing the data before analysis was significant. After overnight fixation, a minimum of six weeks is required for decalcification of the specimen blocks. Overnight processing is necessary before embedding and sectioning on the blocks requires constant cooling of both specimen blocks and the microtome knife.

For identification of epithelial cells, immunolabelling was necessary along with the challenges this technique entails. Antigen retrieval techniques were required due to the fixation techniques used. The microwave and pressure cooker antigen retrieval method was successful, however section folding occurred frequently. To ensure quality control, slides were processed in batches of 40 of which 10-20% yielded acceptable morphology.

Results are in agreement with past research and concepts of periodontal healing. The periodontal ligament of the lower incisor remained intact despite surgical manipulation during extraction as suggested by Simpson (1964). Epithelial cell rests were found located in the mid-third of the root, the region used by Brunette in 1976 for successful culture of epithelial cells.

Within 3 days, the acute inflammatory reaction was moving into its late phase as the neutrophil infiltrate decreased while the influx of macrophages increased, in agreement with findings by Wikesjö *et al.* (1991).

There was an odontoblastic reaction commencing from day-10 with formation of new dentine in the pulp chamber of both control and test discs in which pulpal

remnants had not been removed. It is known that terminal differentiation of odontoblasts does not occur when the dental papilla exists in isolation. Specific epigenetic signals are required to trigger differentiation (Kollar and Baird 1970, Ruch and Karcher-Djuricic 1971, Kollar 1972, Ruch *et al.* 1973).

It has been suggested that a contiguous basement membrane connection is involved in epithelial-mesenchymal interactions controlling odontoblast differentiation. However, the components of the basement membrane change during odontoblast differentiation, implying possible epithelial-mesenchymal transformation (Ruch *et al.* 1995). Thus, evidence of an odontoblastic reaction indicates that the environment of the transplantation site is amenable to epithelio-mesenchymal interactions.

Bone formation present at 21-days was indicated radiographically as well as histologically in accordance with Craft *et al.* (1974) and others (Simmons 1985, Kenley *et al.* 1993). Similar to the results obtained by Andreassen (1981) and Hammarström *et al.* (1989), there was ankylosis and bone replacement in control discs with only occasional sites of ankylosis in experimental discs.

Evidence of a functioning periodontal ligament appeared at 9 weeks post-transplantation in some samples. *In vivo* and *in vitro* studies on transplantation and replantation have implied that periodontal ligament cells can survive and proliferate in cultivating medium or tooth socket. The periodontal ligament regains normal and functional alignment between the root and bone and final reattachment is usually accomplished approximately two weeks after replantation (Inoue *et al.* 1987). However, this involves replantation into an artificial medium or an existing socket where a pool of periodontal ligament cells remains.

In conjunction with bone and ligament formation the of epithelial cells disappear by 21-days post-transplantation. This disappearance could be related to apoptosis or epithelial-mesenchymal transformation (EMT) as suggested by Boyer and Thiery (1993). Evidence exists that the epithelial cells remaining on the root surface for prolonged periods resemble mesenchymal cells, which may be the result of EMT (Thomas 1995).

Fitchett and Hay (1989) have provided convincing evidence to show EMT in the medial edge epithelium (MEE) after fusion of the two secondary palatal shelves in

the midline. The outer edge of the two-layered MEE disappears permitting basal cells to contact and give rise to mesenchyme. Immunocytochemistry of the area demonstrates the change of the intermediate filament profile from keratin to vimentin usually found in mesenchymal cells. A similar process may exist in epithelial cells of the periodontal ligament.

The periodontal ligament plays a vital role in maintaining the function of tooth within the alveolar bone, however there is incomplete understanding regarding the contributions and functions of the components of the ligament. Exploratory steps have been taken to provide an answer regarding the role and fate of epithelial cells during regeneration through development of the present transplantation model. This has provided information regarding response of the transplant area and the eventual disappearance of epithelial cells. Although the precise timing and mechanisms by which this occurs is at present uncertain, it is expected that with further research, the enigma that surrounds these cells may be unmasked.

Future Studies

Modification of the current model is necessary to overcome current difficulties in matching bony cavity to root disc. A template could be made of the average cross section of the incisor root at the middle third to provide guidance for cavity design. External tracing of this template could be rigidly linked to the bur and handpiece to provide accurate cranial cavity preparation. Alternatively, root cylinders could be laid along a bony trench as discussed above.

The use of a cytosine analogue to selectively eliminate epithelial cells prior to transplantation could be used to compare the response when selective cellular components of the periodontal ligament are removed. This would establish the importance of their presence during regeneration.

The sequence of events occurring between 10 and 21 days post-transplantation could be examined in detail and morphological changes within the epithelial cell network could be assessed by confocal microscopy. A 3D re-construction of this area will then be possible. An understanding of structure would also provide a basis for understanding of function.

The recent advances in gene knockout technology would allow the use of transgenic mice lacking in functional expression of a gene. Discs from a donor with a full complement of genes would be used as transplants into a recipient lacking a keratin gene for example, and the specific keratin tracked with a monoclonal antibody probe. So far, three groups of keratin genes have been knocked out; epidermal keratin genes 14 and 10, the corneal-specific gene K12 and the simple epithelial keratin genes 8, 18 and 19 (Magin 1998). The phenotype is affected depending on the genetic background of the mice due to the penetrance of the targeted genes. For example, in the C57/B16 strain of K8 knockout mice, 94% of animals died at embryonic day 12 whereas 55% of the FVB/N strain survived to adulthood. The C57/B16 strain of K18-knockout mice did not show any phenotypic abnormalities in embryonic or young adult states (Galou *et al.* 1997) and would therefore constitute an acceptable strain for experimentation.

This model would allow objective assessment of epithelial cells as donor-derived but an additional, non-epithelial marker would be required to facilitate investigation of a possible epithelial-mesenchymal transition.

The potential of gene therapy in periodontal regeneration has been considered and evaluated for design and construction of targeting vectors (Dunbar 1996). The appropriate DNA can be transferred directly by methods which insure uptake of DNA into cells or via virus-mediated vectors to transcribe the gene of interest. For example, the gene of interest would target expression of epithelium and not connective tissue in a specific area.

Another possibility is through the use of cell-base therapy whereby stem cells are seeded directly onto the tissue surface. Interest has recently been focused on marrow stromal cells as stem cells for tissues of mesenchymal origin. A pilot study has reported the use of seeding periodontal ligament fibroblasts in beagle dogs (vanDijk *et al.* 1991). More research is necessary to overcome the difficulties of obtaining and maintaining the viability of stem cells prior to placement.

Biologic modifiers, which are materials or proteins such as BMPs, and factors that have the potential to alter host tissue, to stimulate or regulate the wound healing process are currently used. The effect of BMPs on ankylosis and periodontal regeneration could be examined in an ectopic site and a model developed for therapeutic use.

In particular, a suitable delivery system is required. The biocompatibility, toxicity, ease of handling, pharmacokinetics, resorbability and retrievability must be considered. Current vehicles, which include insoluble collagen matrix, demineralised bone matrix, collagen sponge, granules, microparticles, fibrous sheets, and other synthetic materials, provide transient exposure of a few hours and have not been without problems.

Ultimately, the ideal clinical objective is the enhancement of bone and periodontal regeneration. This will be achieved through further investigations of the biology of the periodontal ligament including the contribution from epithelial cells.

CHAPTER 5

CONCLUSION

1. In pursuit of an understanding of the biology of cells within the periodontal ligament and in particular epithelial cells, a model has been developed to address the conflicting evidence regarding the fate of the epithelial cells subsequent to trauma. The heterotopic model utilises commonly available resources in a manner that can be readily accomplished and duplicated.
2. With the use of antibodies specific to cytokeratins within epithelial cells, this study has also revealed the regularly spaced pattern of epithelial cells adjacent to the root surface and their absence by 21 days post-transplantation.
3. Although epithelial-mesenchymal transition may be the mechanism by which this disappearance occurs, continual research will be necessary to elucidate the signalling processes present. Possible methods to achieve this have been suggested together with refinement of the present model to facilitate further use.

APPENDIX

Fixative

In 1 litre of water dissolve the following:

- 1 tablet of phosphate buffered sodium (PBS)
- 40 grams of paraformaldehyde (PFA)

Boil solution and cool in ice bath

Decalcifying Solution

In 1 litre of water dissolve the following:

- 3.63grams of Tris (to make 30 mmol solution)
- 100 grams of EDTA.

Mix until dissolved with a magnetic stirrer

Adjust pH to 7.2

Refrigerate at 4°C

Catalysed Infiltration Resin

Dissolve 100ml JB-4 Solution A with 0.90 grams of dry catalyst C

Mix until dissolved with a magnetic stirrer

Refrigerate at 4°C

Embedding Technique

- Add 1ml of Solution B to 25ml of fresh catalysed solution A (described above)
- Stir and place into ice bath (prevent premature polymerisation)
- Place specimens in the moulds
- Seal blocks with paraffin wax (anaerobic conditions are needed for polymerisation)
- Leave overnight

Haematoxylin and Eosin stain

Sections are de-waxed and rehydrated as follows:

- Histolene for 5 minutes.
- Histolene for 5 minutes.
- 100% alcohol for 3 minutes.
- 100% alcohol for 3 minutes.
- 95% alcohol for 3 minutes.
- 70% alcohol for 3 minutes.
- 50% alcohol for 3 minutes.
- 30% alcohol for 3 minutes.
- Distilled water for 1 minute.

The staining procedure is as follows:

- Formalin for 4 minutes.
- Washed in water until the slides appeared clear.
- Haematoxylin for 40 seconds.
- Washed in water until the slides appeared clear.
- Scott's bluing solution for 1 minute.
- Washed in water for 1 minute.
- Eosin for 40 seconds.
- 95% alcohol - 2 dips.
- 100% alcohol - 2 dips.
- 100% alcohol - 2 dips.
- Histolene - 2 dips.
- Histolene - 2 dips.
- Coverslipped and dried.

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