Apoptosis in the Early Developing Periodontium of Rat Molars

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ABSTRACT

Development of the periodontium involves a series of complex steps that result in the formation of root dentine, cementum, bone and fibres of the ligament. These precisely controlled and timed events require the participation of the enamel organ derived epithelial cells of Hertwig's (HRS) and ectomesenchymal cells of the dental follicle. These events involve rapid turnover of the tissues and cells, including disappearance of epithelial cells of HRS. Thus, it seemed likely to us that programmed cell death (apoptosis) may play a role in the development of the periodontium.

Fragments of first molars, obtained from 14- and 29-day-old rats, were fixed in glutaraldehyde-formaldehyde and processed for light and electron microscopy. For the TUNEL method for detection of apoptosis, specimens were fixed in 4% formaldehyde and embedded in paraffin.

Results confirmed that epithelial cells of HRS maintain a close relationship with the forming dentine root, and that they may become trapped in the dentino-cemental junction. Some of the epithelial cells exhibited ultrastructural features which are consistent with the interpretation that they were undergoing programmed cell death, i.e. apoptosis. Periodontal fibroblast-like cells showed typical images of apoptosis and engulfed apoptotic bodies. TUNEL positive structures were present in all corresponding regions. It seems therefore that apoptosis of epithelial cells of HRS and fibroblast-like cells of the periodontal ligament constitutes an integral part of the developmental process of the tissues of the periodontium. Anat Rec 258:136–144, 2000.

Key words: apoptosis; Hertwig's root sheath; periodontal ligament: periodontium; cementum

Development of the periodontium involves a series of complex steps that result in the formation of root dentine, cementum, bone and fibres of the ligament. These precisely controlled and timed events require the participation of the enamel organ derived epithelial cells of Hertwig's root sheath (HRS) and ectomesenchymal cells of the dental follicle. Cells of the HRS play a fundamental inductive role in the formation of root dentine whereas the ectomesenchymal cells are mainly concerned with the production of cementum, bone and periodontal ligament (Cho and Garant, 1989; Schroeder, 1992; Ten Cate, 1996).

It has been generally accepted that after the end of the inductive phase and consequent fragmentation of HRS, the epithelial cells move away from the root surface and remain as clusters in the periodontal ligament, forming what is known as epithelial rests of Malassez (Hamamoto et al., 1989; Ten Cate, 1996; Bosshardt and Selvig, 1997). However, more recently, evidence available suggests that some epithelial cells of HRS remain in contact with the root dentine surface, for some time, and there secrete

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enamel-related proteins and other macromolecules, forming thereupon the innermost layer of the cementum (Mckee et al., 1996; Bosshardt and Nanci, 1998). The fate of these presumably cementum forming epithelial cells is unknown but they eventually disappear from the region. However, in rat molars, unlike primates, some of the epithelial cells become trapped inside the dentino-cemental junction (Lester, 1969; Hamamoto et al., 1989; Bosshardt et al., 1998). Another possibility, recently suggested, for the disappearance of the epithelial cells is that they undergo transformation to mesenchymal cells (Bosshardt and Schroeder, 1996; Bosshardt et al., 1998; Bosshardt and Nanci, 1998).

It is well known that during embryonic development of most tissues of the body, a great number of cells die by the process of programmed cell death, i.e. apoptosis, and are subsequently removed, disappearing therefore from the tissue (Kerr et al., 1974; Raff, 1998). In the oral cavity, several structures of the tooth germ—in particular of the enamel organ—undergo apoptosis during development (Nishikawa and Sasaki, 1995; Shibata et al., 1995; Bronckers et al., 1996; Vermelin et al., 1996; Baratella et al., 1999). Also, apoptosis has been associated with cell and tissue turnover and remodelling (Wyllie, 1987; Raff, 1998; Toescu, 1998). Thus, it seemed reasonable to us to consider that there might be a role for apoptosis in the intricate processes of formation of the structures of the periodontium. We therefore examined early stages of periodontal development using light and electron microscopy, and the TUNEL (Terminal deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling) method for the detection of the DNA fragmentation characteristic of apoptosis (Gavrieli et al., 1992). For this purpose we used rat molars in early stages of acellular cementum and cellular cementum formation.

MATERIALS AND METHODS

Wistar rats aged 14 and 29 days, fed ad libitum, were anaesthetised with chloral hydrate (400 mg/Kg). Fragments containing upper maxillary first molars were removed and immediately immersed in the fixative solution. Principles of laboratory animal care (NIH publication 85–23, 1985) and national laws on animal use were observed.

Light Microscopy

The specimens were fixed in a mixture of 4% glutaraldehyde and 4% formaldehyde (freshly derived from paraformaldehyde) buffered at pH 7.2 with 0.1 M sodium cacodylate at room temperature for 16 hr (Katchburian and Holt, 1972). After decalcification for 5 days in a 7% solution of EDTA containing 0.5% formaldehyde, in sodium phosphate buffer 0.1 M, at pH 7.2, the specimens were dehydrated in graded ethanols and embedded in glycol methacrylate (Historesin-JUNG, Germany). Sections 2 µm thick were stained with Gill's hematoxylin and eosin for light microscopy.

TUNEL Method

For the TUNEL method, we used the Apop Tag-Plus Kit (Oncor, Gaithersburg, MD). The specimens were fixed in 4% formaldehyde (freshly derived from paraformaldehyde) buffered at pH 7.2 with 0.1 M sodium phosphate at room temperature for 16 hr. After decalcification for 5 days in a 5% solution of EDTA, prepared as above, the specimens were embedded in paraffin. Sections 6 µm thick were mounted in silanized slides. Deparaffinated sections were pretreated in 20 µg/ml proteinase K (Oncor-Protein Digesting Enzyme) for 15 min at 37°C, and after several washings in distilled water they were immersed in 3% hydrogen peroxide in PBS (50 mM sodium phosphate, pH 7.4, 200 mM NaCl) for 15 min; they were then immersed in the equilibration buffer. After incubation in TdT enzyme (terminal deoxynucleotidyl transferase) at 37°C for 1 hr in a humidified chamber, the reaction was stopped by immersion in a stop/wash buffer at 37°C for 15 min; the sections were then washed in PBS for 10 min. The sections were subsequently incubated in anti-digoxigenin-peroxidase at 37°C for 30 min, in a humidified chamber. After several washings in PBS, the sections were treated with 0.06% 3.3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) in the presence of 0.1% hydrogen peroxide for 3–6 min, at room temperature. The sections were counterstained with 1% methyl green in sodium acetate buffer for 10 min and dehydrated in 100% butanol, rinsed in xylol and mounted in Entellan medium. Involuting mammary or prostate gland sections were used as positive controls for the TUNEL method. Negative controls were incubated in medium lacking TdT enzyme. The specimens were examined and photographed in a OLYMPUS BX-50.

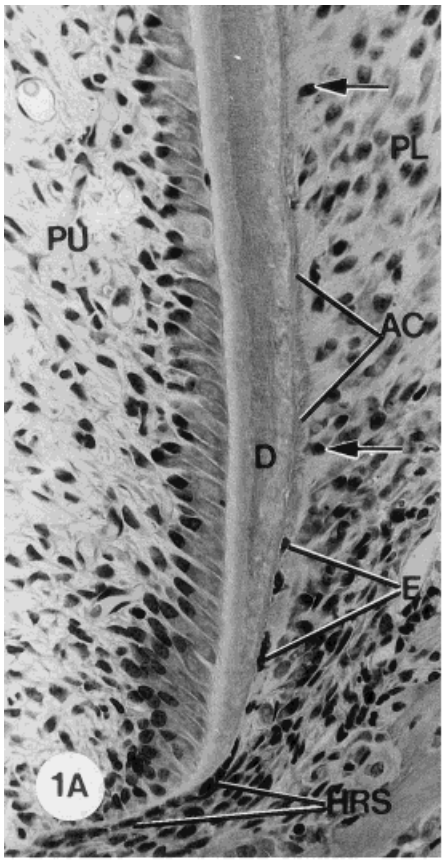
Transmission Electron Microscopy

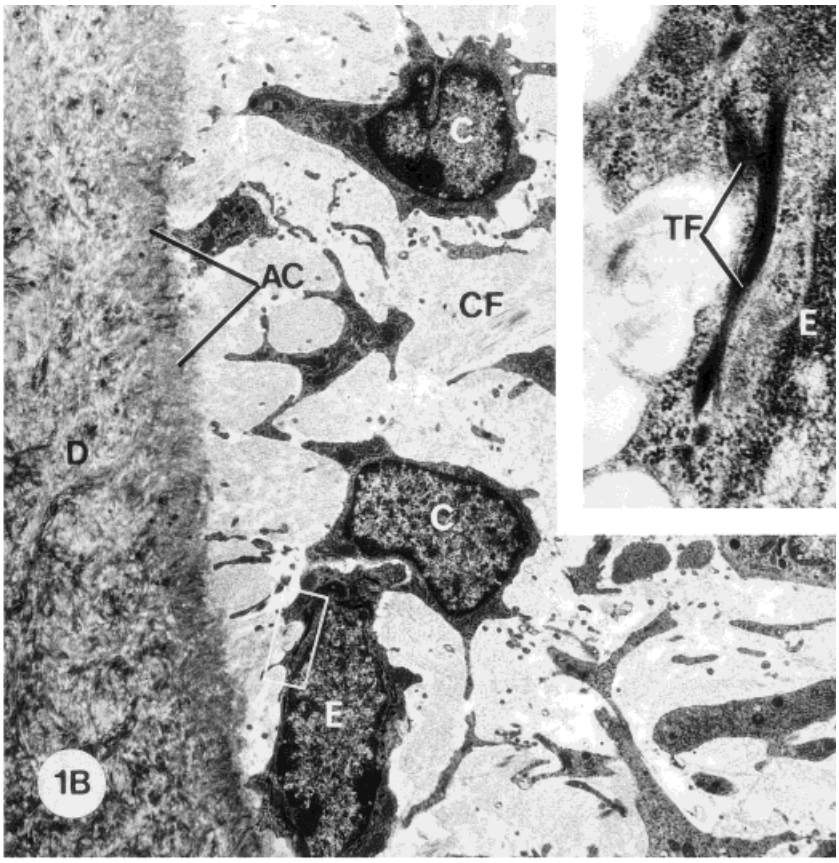
The specimens were fixed in a mixture of 4% glutaraldehyde and 4% formaldehyde (freshly derived from paraformaldehyde) buffered at pH 7.2 with 0.1 M sodium cacodylate at room temperature for 16–20 hr (Katchburian and Holt, 1972). After decalcification for 5 days in a 7% solution of EDTA containing 0.5% formaldehyde in 0.1 M cacodylate buffer, at pH 7.2, the specimens were washed in 0.1 M cacodylate at pH 7.2. They were then transferred to cacodylate buffered 1% osmium tetroxide for 1–1.5 hr at room temperature and subsequently treated with an aqueous solution of 0.5% uranyl acetate for 2 hr. All specimens were dehydrated in graded ethanols, treated with propilene oxide and subsequently embedded in Araldite.

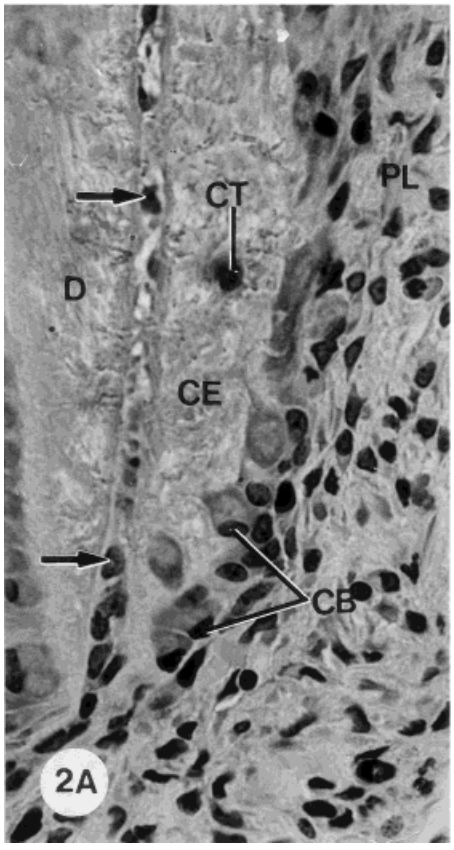
Semi-thin sections stained with toluidine blue were examined in a light microscope, and suitable regions were carefully selected for final trimming of the blocks. Ultrathin sections from selected regions were collected on grids and stained in alcoholic 1% uranyl acetate and in lead citrate prior to examination in a Jeol 1200 EXII electron microscope.

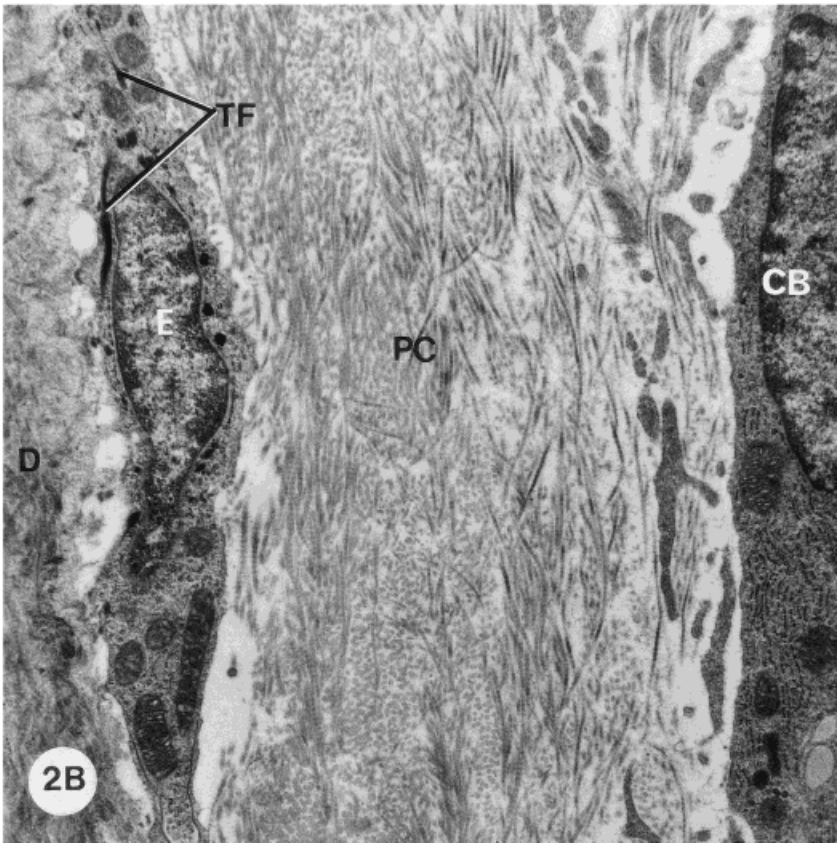
RESULTS

Early stages of root formation in upper first molars of 14-day-old rats showed a thin layer of acellular cementum on the surface of the root dentine. Fibroblast-like cells were abundant in the region, and some were in contact with the cementum surface. Epithelial cells from Hertwig's root sheath formed a continuous layer in the apicalmost region—the diaphragm—but were separated from each other at other levels, being in contact with the early cementum surface or mantle dentine (Fig. 1). The cytoplasm of the epithelial cells always showed conspicuous tonofilaments (Fig. 1B, inset). In 29-day-old rats, developing roots showed the forming layer of cellular cementum covered by typical and apparently active cementoblasts









Figures 1–2.

(Fig. 2). Several cells were enclosed within and along a slit-like dentino-cemental junction (Fig. 2A). These cells invariably exhibited bundles of tonofilaments in their cytoplasm (Fig. 2B).

After extensive examination of several ultrathin sections, we observed that some of the tonofilaments-rich epithelial cells, located in the dentino-cemental junction and on the cementum surface, contained varied and irregularly shaped masses of condensed chromatin in the nuclei (Fig. 3). In the cells located within the dentino-cemental junction, the condensed chromatin exhibited extremely irregular profiles and often appeared as fragmented and dispersed blocks. The convoluted nuclear envelope of these cells showed several large and small blebs, and their scarce cytoplasm contained some vacuoles and bundles of tonofilaments. Their plasma membrane was absent or not discernible in some regions. Frequently, these epithelial cells were partially surrounded by what appeared to be processes of other cells (Figs. 3B and C).

In the region of periodontal ligament, some fibroblast-like cells showed conspicuous peripheral condensed chromatin within the nuclei. Several finger-like projections extended from the nuclei towards a shrunken cytoplasm that contained apparently intact organelles. Long cytoplasmic extensions towards the matrix were also present (Fig. 4). Dense, round or fragmented structures, exhibiting varied shapes and sizes, were observed free in the tissue or inside vacuoles of periodontal fibroblast-like cells (Fig. 5). Also, cytoplasmic processes of the periodontal fibroblast-like cells appeared to be engulfing large and small dense bodies, next to the cementum surface (Fig. 5B) or in the central region of the periodontal ligament (Fig. 5C).

Examination of sections stained by the TUNEL method showed brown-yellow stained structures in the developing periodontium (Fig. 6). Round, TUNEL positive structures, were observed adjacent to early acellular cementum (Fig. 6A). In stages of cellular cementum formation, TUNEL positive structures were found within the dentino-cemental junction, closer to the apical root (Fig. 6B); these TUNEL-positive structures were irregularly shaped and sometimes the staining appeared to diffuse beyond the nucleus (Fig. 6C). Other round structures, of varied sizes, were found within lacunae of cellular cementum, near the outer surface (Fig. 6D).

Fig. 1. Light (A) and electron (B) micrographs of the mid-portions of developing roots showing early stages of acellular cementum formation (14-day-old rats). **A:** A thin layer of acellular cementum (AC) is observed on the root dentine (D); fibroblast-like cells apposed to the surface (arrows) or enclosed in the dentino-cemental junction at the apicalmost region (E) are observed. PU, developing pulp; HRS, Hertwig's root sheath; PL, developing periodontal ligament. ×139. **B:** An epithelial cell (E) in contact with the root surface shows tonofilaments (TF, **inset**). Developing fibroblast-like cells (C) are observed close to the early acellular cementum (AC). CF, collagen fibrils; D, dentine. ×4,200; inset, ×33,600.

Fig. 2. Light (A) and electron (B) micrographs of the apical portions of developing roots showing cellular cementum formation (29-day-old rats). **A:** There are several cells enclosed along the dentino-cemental junction (arrows); typical cementoblasts (CB) are apposed to the cellular cementum surface (CE). D, dentine; CT, cementocyte; PL, developing periodontal ligament. ×980. **B:** A region similar to that in A shows an elongated epithelial cell (E), containing bundles of tonofilaments (TF), enclosed in the dentino-cemental junction. A portion of a cementoblast (CB) is apposed to the precementum (PC). D, dentine. ×18,800.

TUNEL positive structures were not abundant in our specimens, and were not present in all sections examined. Controls for the TUNEL method using involuting mammary or prostate glands sections revealed positive structures. Sections incubated in medium lacking TdT enzyme were negative.

DISCUSSION

Our observations strongly suggest that programmed cell death, i.e. apoptosis, occurs in cells of Hertwig's epithelial root sheath (HRS) and also in fibroblast-like cells of the early developing periodontium of rat molars. The results, obtained at the onset of acellular and cellular cementum formation, also confirm that some epithelial cells—derived from HRS—maintain a close relationship with the forming dentine root. As expected for rat molars, in more advanced stages of development, many of the epithelial cells were found trapped inside the dentino-cemental junction (Lester, 1969; Bosshardt and Schroeder, 1996; Bosshardt et al., 1998). The epithelial cells, identified by the presence of tonofilaments in their cytoplasm, showed images which are compatible with the interpretation that they were undergoing a process of the cell death. These cells, mainly those in the dentino-cemental junction, showed condensed and irregular masses of chromatin within a convoluted nuclear profile which also exhibited protrusions and blebs. Their shrunken and irregular cytoplasm, often lacking portions of the plasma membrane, showed large and small vacuoles, and were full of tonofilaments. On the region of the cementum surface, the epithelial cells showed condensed and irregular masses of chromatin but no other signs of the death.

Although the images observed do not seem to fit exactly the classic description of apoptosis it is well recognized that morphological variations occur depending on the tissue (Milligan and Schwartz, 1996; Collins et al., 1997). Furthermore, apoptosis of cells that are inside cavities or lacunae, like chondrocytes, for example, is poorly understood. It has been reported that, when inside cavities or lacunae, it takes longer for apoptotic dying cells to be removed (Gibson et al., 1995; Blanco et al., 1998). This would, in turn, lead to advanced stages of degradation as observed by us in the dentino-cemental junction. How apoptotic cells or bodies are removed from the dentino-cemental junction is not known. However, some of our results showed that extensions and processes, presumably from neighbouring cells or macrophages, appear to surround and encircle the dying epithelial cells of the dentino-cemental junction, indicating perhaps that apoptotic epithelial cells or bodies may be somehow engulfed and removed. It is well known that the plasma membrane of apoptotic cells possesses signalling molecules to attract neighbouring cells or macrophages (Wyllie, 1987; Milligan and Schwartz, 1996; Ashkenazi and Dixit, 1998; Raff, 1998; Toescu, 1998). However, to be able to reach the dentino-cemental junction we must assume that there are communicating channels between the junction and the surface of the cementum, i.e. the region of the periodontal ligament, to allow cells to penetrate and engulf the dying cells. As mentioned earlier, the fate of apoptotic cells that are within lacunae or closed cavities, and thus not in contact with surrounding tissue, remains unclear. Furthermore, we did not observe macrophage-like cells in the stages of development examined in this study.

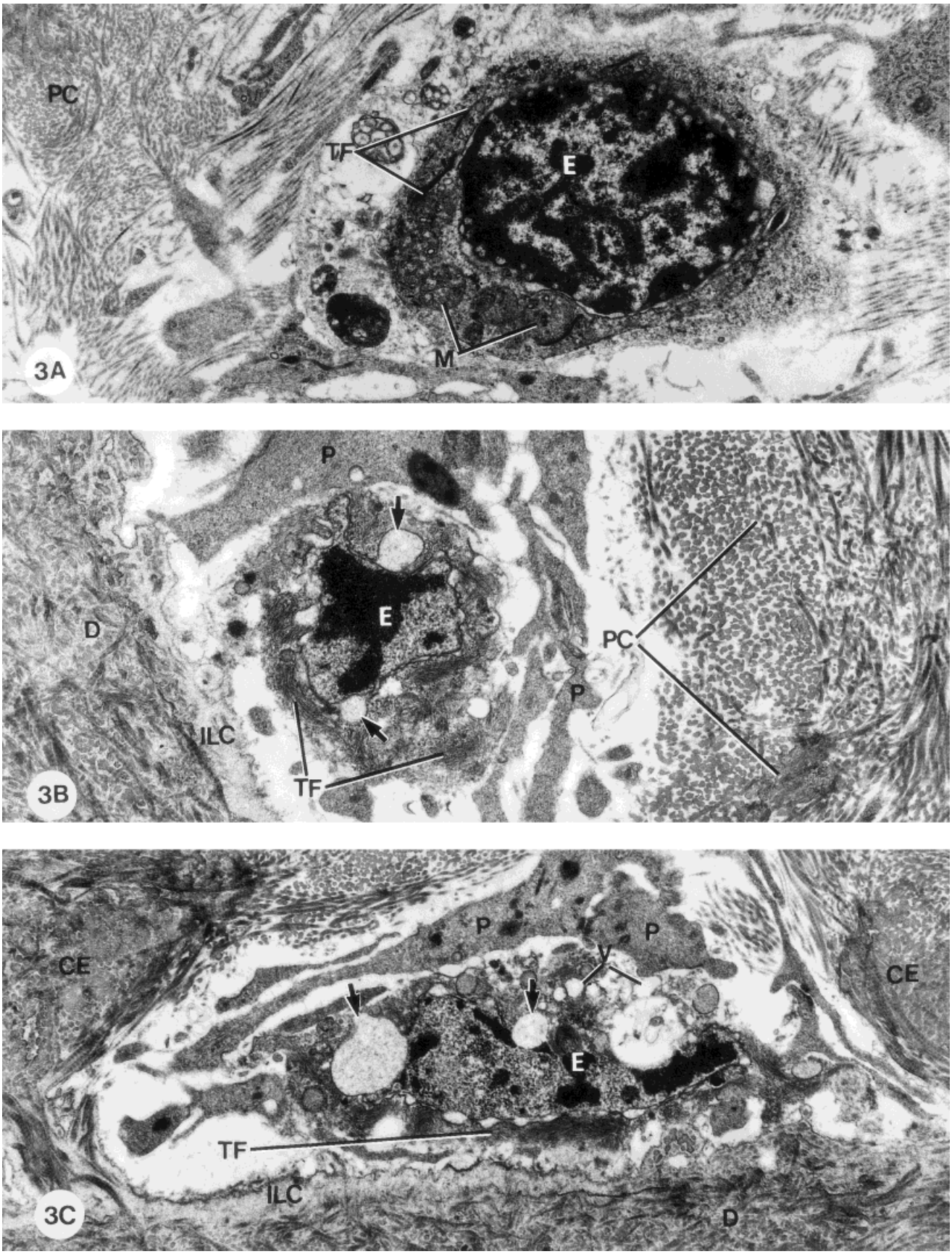


Figure 3.

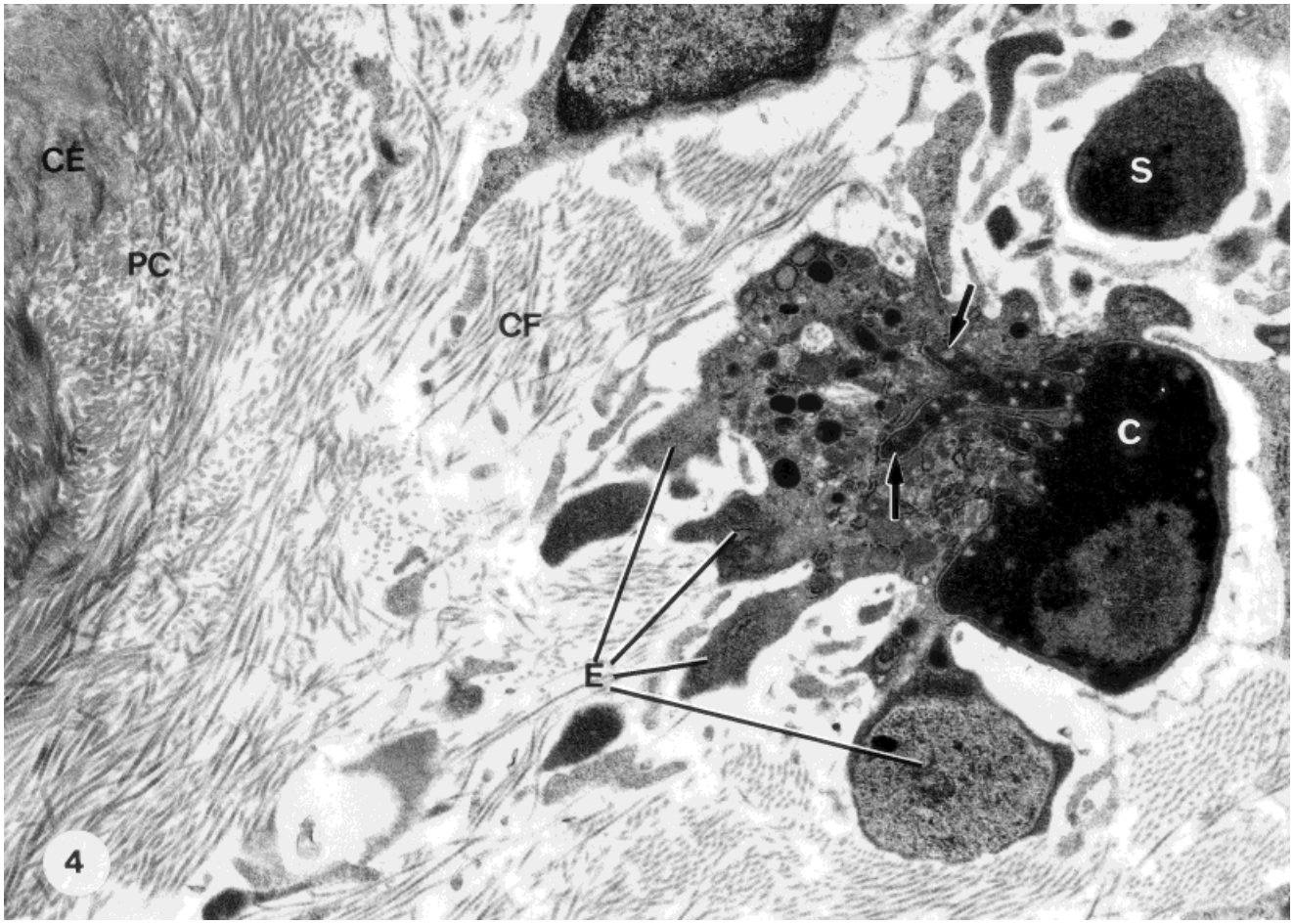
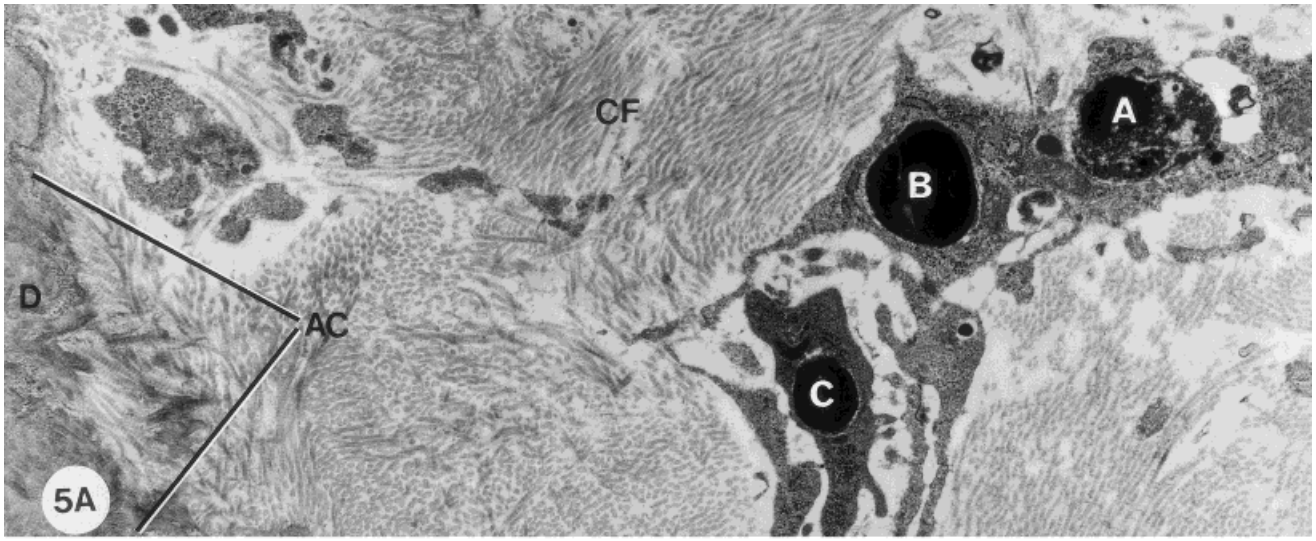


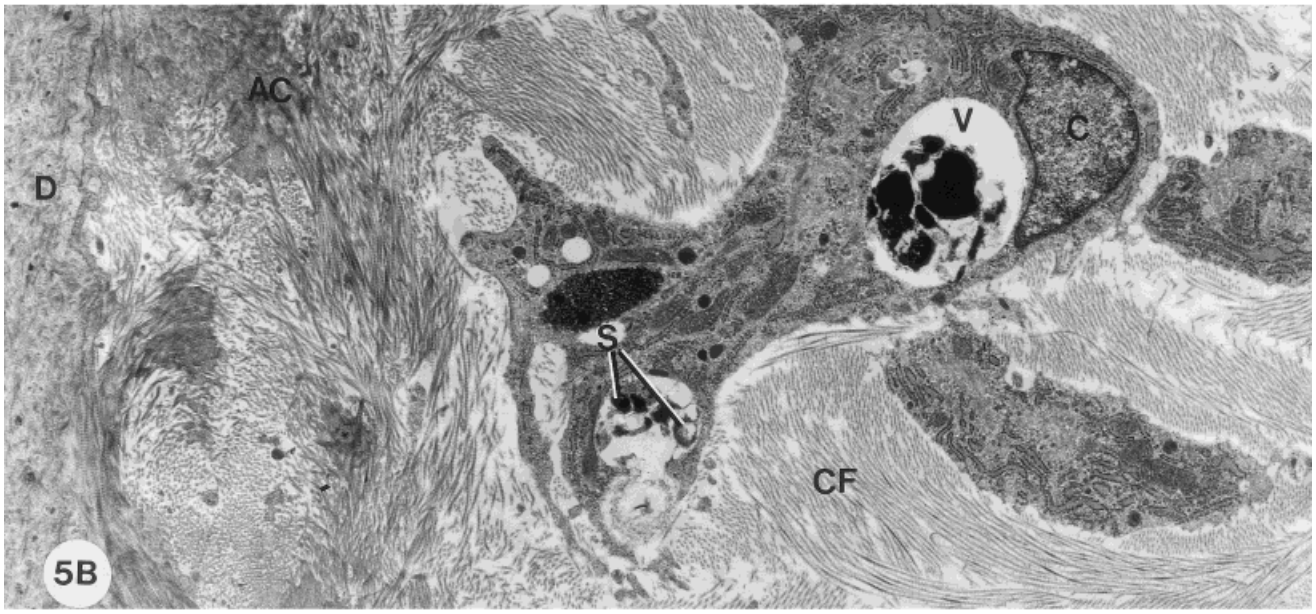
Fig. 4. Electron micrograph of the apical portion of a developing root (29-day-old rat). A fibroblast-like cell (C) adjacent to the surface of the cellular cementum (CE) shows condensed peripheral chromatin from which several finger-like projections (arrows) protude towards the cytoplasm. Several extensions (E) towards the matrix emerge from a cytoplasm which contains apparently intact organelles. A globular dense structure (S) adjacent to the cell ''C'' is also observed. PC, precementum; CF, collagen fibrils. ×12,800.

The above results are supported by the observation that TUNEL-positive cells were present in corresponding regions of the dentino-cemental junction and the periodontal ligament. The TUNEL method is widely used to identify apoptosis in histological sections since it reveals characteristic DNA breaks (Gavrieli et al., 1992). The diffuse cytoplasmic TUNEL staining observed in some our preparations most probably represents leakage of DNA fragments out of the nucleus of apoptotic cells (Wijsman et al., 1993; Wheeldon et al., 1995; Rojo and Gonzalez, 1998). As in most other tissues, images of apoptosis as observed by the TUNEL method or by electron microscopy were not abundant in our specimens. Apoptotic cells quickly disappear from the tissue and the likelihood of detecting apoptosis in tissue sections is therefore quite small (Wyllie, 1987; Majno and Joris, 1995; Raff, 1998).

It is generally accepted that epithelial cells from HRS that remain on the root dentine surface, in early cementogenesis, probably produce constituents of the innermost layer of the cementum, mainly enamel-related proteins (Slavkin et al., 1988; Fong et al., 1996) but possibly bone sialoprotein (BSP) and osteopontin also (Bosshardt et al., 1998). More recently, it has been proposed that the epithelial cells undergo mesenchymal transformation to cementoblasts, thereby becoming able to secret cementum organic constituents (MacNeil and Thomas, 1993; Bosshardt and

Fig 3. Electron micrographs of the apical portions of developing roots (29-day-old rats) showing epithelial cells (E) identified by the presence of bundles of tonofilaments (TF) in their cytoplasm. **A:** The epithelial cell (E), adjacent to the surface of the newly formed precementum (PC), shows irregular and tortuous masses of condensed chromatin. Bundles of tonofilaments (TF) and mitochondria (M) are observed in the cytoplasm. ×12,000. **B:** The epithelial cell (E) present at dentino-cemental junction, i.e., between dentine (D) and precementum (PC), shows irregularly shaped condensed chromatin within a deformed nucleus. Blebs of various sizes emerge from the nuclear periphery (arrows). The cytoplasm is full of tonofilaments (TF). Processes (P) from other cells surround the epithelial cell (E). ILC, Innermost layer of cementum. ×20,800. **C:** The elongated epithelial cell (E) present in the dentino-cemental junction shows blocks of fragmented condensed chromatin and numerous blebs emerging from the nuclear surface (arrows). Vacuoles (V) and bundles of tonofilaments (TF) are also present. Cytoplasmic processes (P) from other cells appear to surround the epithelial cell (E). D, dentine; ILC, innermost layer of cementum; CE, developing cellular cementum. ×16,000.





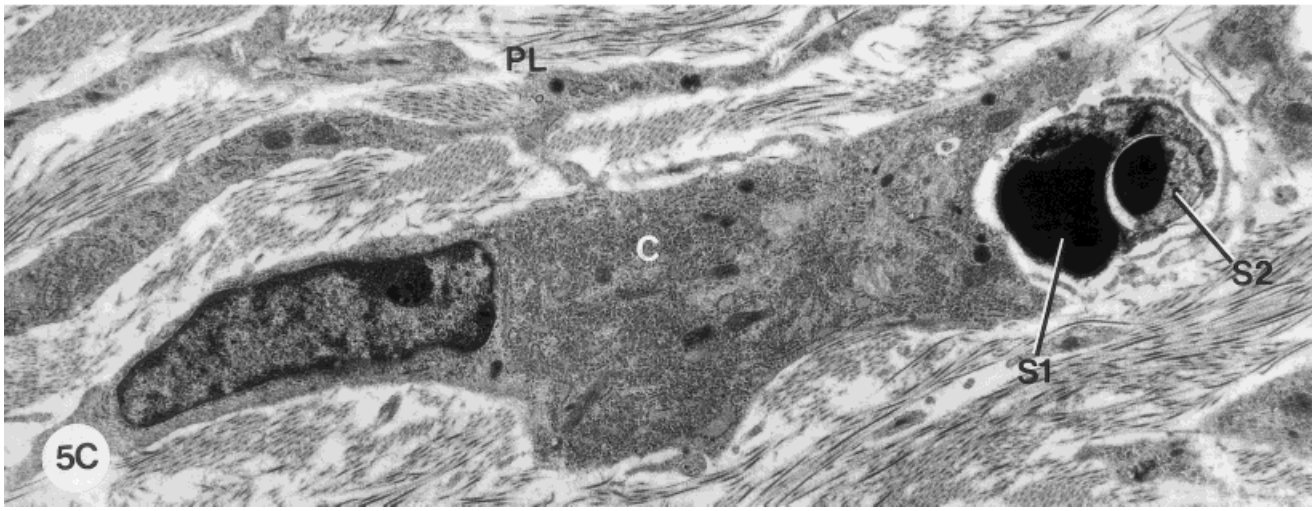


Fig. 5. Electron micrographs of the cervical portion (A, 14-day-old rat) and the mid-portions of the developing root (B, 29-day-old rat), and of the developing periodontal ligament (C, 29-day-old rat). **A:** Three fibroblast-like cells adjacent to the acellular cementum surface (AC) contain compact dense globular structures (A, B and C). D, dentine; CF, collagen fibrils. ×12,500. **B:** An irregularly shaped fibroblast-like cell (C), next to the root surface, contains dense structures inside a large vacuole (V). Cytoplasmic processes extending towards the matrix, appear to be engulfing small, dense and fragmented structures (S). The cytoplasm of the cell (C) shows intact organelles. CF, collagen fibrils; D, dentine; AC, acellular cementum. ×8,000. **C:** A fibroblast-like cell (C) of the periodontal ligament appears to be engulfing large and dense structures that consist of a major portion (S1) and a smaller round structure (S2). PL, developing periodontal ligament. ×8,000.

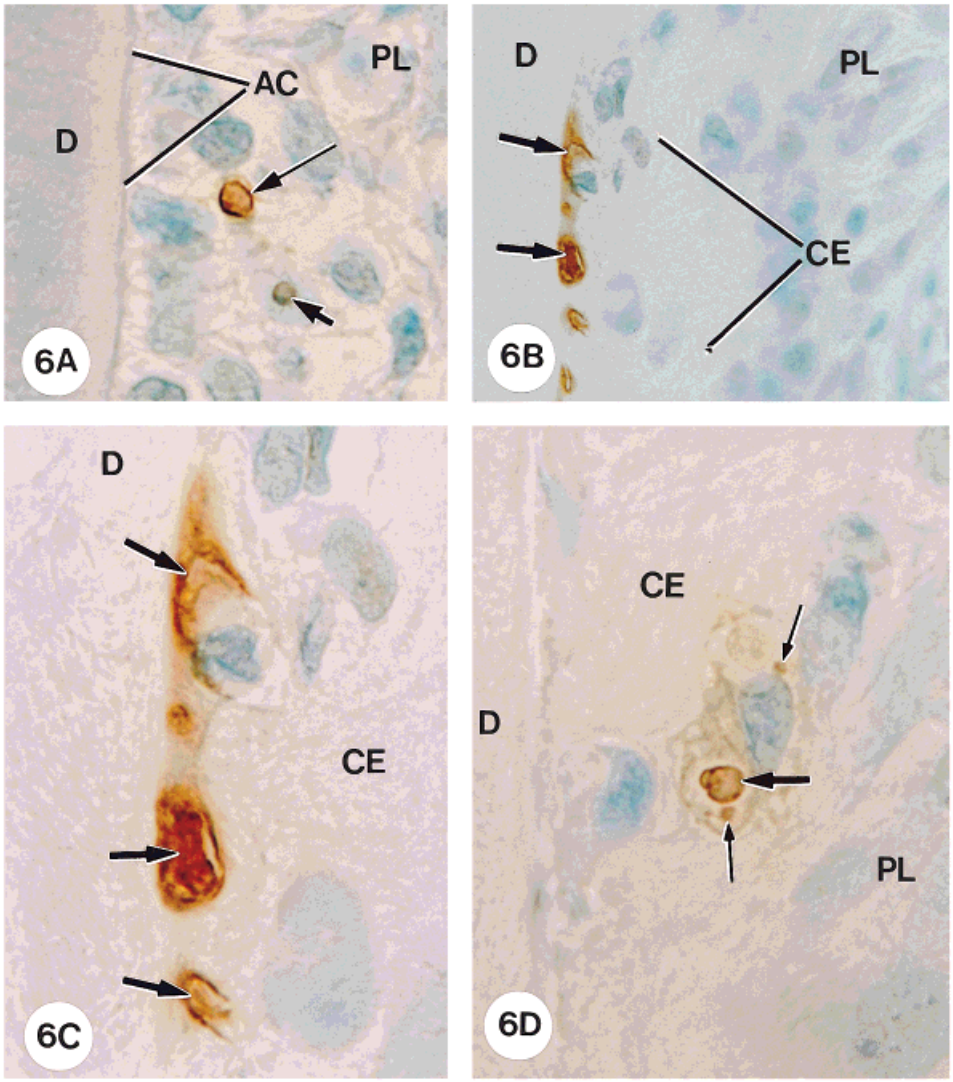


Fig. 6. Light micrographs of the cervical (A, 14-day-old rat) and apical (B, C and D, 29-day-old rats) portions of early developing roots stained by the TUNEL method. **A:** A TUNEL-positive round structure (arrow) is present adjacent to thin layer of early acellular cementum (AC). Another round but smaller structure shows a weak TUNEL-positivity (short arrow). D, dentine; PL, developing periodontal ligament. ×550. **B:** A region of early cellular cementum (CE) shows several TUNEL-stained structures in the dentino-cemental junction (arrows). D, dentine; PL, periodontal ligament. ×550. **C:** Higher magnification view of a region of B showing the TUNEL-positive structures (arrows), within the dentino-cemental junction in which the stain appears somewhat diffuse. D, dentine; CE, cellular cementum. ×1,390. **D:** Round TUNEL-positive structure (arrow), with small bleb, is present within an incomplete lacuna of the developing cellular cementum (CE). Other structures faintly stained by TUNEL method are also observed in the vicinity (short arrows). D, dentine; PL, developing periodontal ligament. ×1,390.

Schroeder, 1996; Bosshardt and Nanci, 1998). This transformations would, if true, explain why epithelial cells of HRS disappear from the region (Bosshardt and Nanci, 1998). Our results, however, open up a new possibility, i.e. that the cementum related epithelial cells undergo apoptosis and thereafter disappear from the region. Hence, it does seem reasonable to suggest a sequence of events in which these epithelial cells firstly secrete molecules for the innermost layer of the cementum and soon after die by apoptosis.

In the region of the periodontal ligament—near of far from the newly formed cementum surface—several ultrastructural images depicting different stages of apoptosis were observed. Periodontal fibroblast-like cells showed typical condensed peripheral chromatin and nuclear protrusions/blebs towards an intact cytoplasm. Apoptotic bodies of various appearances were observed in the process of being engulfed by fibroblast-like cells or found within them. These ultrastructural observations, together with the results of the TUNEL method, demonstrate that apoptosis occurs in fibroblast-like cells of the early developing periodontal ligament. We can not exclude, however the possibility that some apoptotic bodies observed derive from other—perhaps non-fibroblastic—cells of the developing periodontium. Altogether these results probably reflect the high turnover of cells and tissues in the region, perhaps necessary for remodelling and accommodation of the multiple, simultaneous and intricate interactions and movements taking place during root formation and tooth erup-

tion (Ten Cate and Deporter, 1975; Rippin, 1976; Beertsen and Everts, 1977; Berkovitz, 1990; Beertsen et al., 1997).

In conclusion, our findings show that programmed cell death, i.e. apoptosis, of epithelial cells of HRS and of fibroblast-like cells of the periodontal ligament constitutes an integral part of the developmental process of the tissues of the periodontium. However, examination of different regions, in different stages of development and in other species, using a quantitative approach, will be required to establish to full extent of programmed cell death during the formation of the periodontium.

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