Apoptosis in Chronic Adult Periodontitis Analyzed by *In Situ* DNA Breaks, Electron Microscopy, and Immunohistochemistry

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Background: Apoptosis is an evolutionary form of physiological cell death. Previous studies suggest that apoptosis is involved in the pathogenesis of periodontal diseases. Therefore, we studied the apoptotic events in the gingival tissue of chronic adult periodontitis patients.

Methods: Gingival tissue biopsies from 22 patients with chronic adult periodontitis and from 11 healthy controls were obtained. Criteria for patient inclusion in the periodontitis group were a minimum of 14 natural teeth, excluding third molars, with at least 10 posterior teeth; 5 to 6 sites with probing depth ≥5 mm; attachment loss ≥3 mm; and extensive radiographic bone loss. The control group included healthy subjects with no prior history of periodontal disease. Apoptosis was determined using the terminal TdT-mediated dUTP-biotin nick end labeling (TUNEL) technique; electron microscopic analysis; and expression of Caspase-3, Fas, FasL, Bcl-2, and p53 by immunohistochemistry.

Results: TUNEL-positive cells and cells exhibiting chromatin condensation by electron microscopy were observed in the inflammatory infiltrate of biopsies obtained from periodontitis patients. Most of the TUNEL-positive cells belonged to neutrophil cell populations as they were stained with anti-myeloperoxidase. Positive staining for active-caspase 3, Fas, FasL, and p53 was only observed in the inflammatory infiltrate from periodontitis biopsies, whereas Bcl-2 cells were present in both periodontitis patients and healthy controls.

Conclusions: Our findings establish that apoptosis is induced in the periodontal tissue by host and microbial factors and support the hypothesis that apoptotic mechanisms could be implicated in the inflammatory process associated with gingival tissue destruction observed in adult periodontitis patients. *J Periodontol 2001;72:517-525.*

KEY WORDS

Apoptosis; periodontitis/pathogenesis; inflammation.

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Adult periodontitis is a chronic disease characterized by the interaction between Gram-negative bacteria and the host inflammatory response, which results in tissue destruction and tooth loss.[1-3] In addition to the direct pathological effects of bacteria on periodontal tissues, it seems clear that periodontal damage is inflicted by indirect mechanisms. Bacterial products act on the cellular constituents of the gingival tissues, activating cellular processes that induce the destruction of the connective tissue and bone.[4-7] Immunohistochemical studies have established that the majority of mononuclear cells migrate towards the perivascular connective tissue to form an inflammatory infiltrate, which is mainly composed of neutrophils, T and B lymphocytes, and macrophages.[8-10]

Several periodontal pathogens have been reported to induce cytotoxicity in a variety of cellular components of the periodontium. Short-chain carboxylic acids (SCCA) present in *Porphyromonas gingivalis, Prevotella loescheii,* and *Fusobacterium nucleatum* induce apoptosis in T cells[11,12] and gingival keratinocytes.[13] Moreover, lipopolysaccharide (LPS), a common component of the cell wall of Gram-negative bacteria, stimulates butyric acid-induced apoptosis in human peripheral blood mononuclear cells,[11,14] and a toxin from *Actinobacillus actinomycetemcomitans* induces apoptosis in B lymphocytes present in the periodontal tissue.[15] All these data indicate that tissue destruction by apop-

tosis seems to play a relevant role in the pathogenesis of chronic adult periodontitis.

However, apoptosis is an highly controlled process affecting various components in the initiation, amplification, and execution of the destructive process.[16] Hence, during apoptosis, a cascade of events is triggered, such as the activation of the cytosolic proteases "caspases,"[17,18] chromatin condensation, and activation of specific endonucleases that cleave DNA at internucleosomal regions.[19] Furthermore, apoptotic stimuli can trigger apoptosis via different mechanisms, including specific cell death receptors and ligands, such as Fas/Fas-ligand complex,[20] or stress signals that induce molecules directly or indirectly implicated in apoptosis, such as p53.[21,22] Finally, members of the Bcl-2 family proteins, among others, have been shown to play a critical regulatory role in the apoptotic process. The Bcl-2 protein is a member of the anti-apoptotic proteins which can prevent or reduce cell death induced by a variety of stimuli.[23-26]

The results of one study performed in clinically healthy gingival tissues exposed to chronic low-grade bacterial challenge and inflammation indicated that apoptosis-associated DNA damage and expression of p53 and Bcl-2 apoptosis-regulating genes were prevalent phenomena.[27] The detection of apoptosis in the inflammatory gingival tissue of periodontitis patients in sites with probing depth ≥5 mm and attachment loss ≥3 mm could partially explain the mechanisms associated with the destruction of the supporting tissues of the tooth.

The aim of this work was to establish, by morphological and immunohistochemical criteria, the presence of apoptotic cells in the gingival tissue of adult periodontitis patients as compared with healthy donors. Our data demonstrate the presence of apoptotic cells, mainly neutrophils, in the gingiva of periodontitis patients. These data are in agreement with the clinical pathology and suggest that apoptosis is one of the mechanisms involved in the pathogenesis of periodontitis.

MATERIALS AND METHODS

Collection of Gingival Tissues
Gingival tissue biopsies from 22 patients (7 males and 15 females, 36 to 55 years old; mean age 46.36 ± 5.80 years) diagnosed with chronic adult periodontitis and from 11 healthy subjects (3 males and 8 females, 33 to 49 years old; mean age 40.81 ± 5.47 years) were obtained under local anesthesia. Criteria for patient inclusion in the adult periodontitis group were a minimum of 14 natural teeth, excluding third molars, and including at least 10 posterior teeth. Patients had moderate to advanced periodontal disease (at least 5 to 6 teeth had sites with probing depth ≥5 mm, attachment loss ≥3 mm, and extensive radi-

ographic bone loss), and had received no treatment at the time of examination. Patients did not suffer from systemic illness and they had not received antibiotics or non-steroidal anti-inflammatory therapy in the 6-month period prior to the study. Subjects in the control group were healthy, had no prior history of periodontal disease, exhibited probing depth <3 mm, and had no attachment loss, clinical inflammation, sulcular bleeding, or radiographic bone loss. After informed consent was obtained, soft tissue gingival biopsy samples were taken from patients. Each subject contributed one biopsy. The incisions of the inflamed tissues were made 1 to 2 mm subgingivally. The specimens, therefore, consisted of the gingival margin, sulcular epithelium, and gingival connective tissue. Soft tissue biopsy specimens were immediately embedded in tissue freezing medium‖ and snap-frozen in liquid nitrogen slurry. Cryostat sections (4 to 6 μm thick) were obtained in cryostat microtome,¶ briefly prefixed in acetone,# and stored desiccated at −70°C until use. Three sections of each sample were used for immunohistochemistry, TUNEL, and electron microscopy.

Immunohistochemistry
Sections were fixed for 15 minutes in acetone at 4°C. A standard 3-stage immunoperoxidase ABC (avidin-biotin-peroxidase complex technique) technique was used to detect p53,** Bcl-2,** Fas,†† FasL,†† and caspase-3.†† In brief, non-specific tissue binding was blocked by incubation with 1.5% horse serum for 30 minutes. Specimens were then incubated and permanently mounted for 60 minutes with 40 μl of diluted primary antibody. All rinsing steps were performed with Tris-buffered saline (TBS pH 7.2-7.6). Biotinylated horse anti-mouse IgG secondary antibodies were used at a 1:200 dilution for 30 minutes.** Endogenous peroxidase activity was quenched by exposure for 30 minutes to 0.3% H_2O_2 in methanol. Sections were then incubated for 45 minutes with pre-formed avidin-biotin-horseradish peroxidase macromolecular complexes.** A black color was developed by exposure for 6 to 8 minutes to 0.5 mg/ml of the chromogen 3′, 3′-diaminobenzidine tetrahydrochloride (DAB).** Sections where counterstained with hematoxylin Carazzi, dehydrated, and permanently mounted. Positive and negative controls were processed with each series.

DNA Nick End Labeling of Tissue Sections
The presence of death-associated DNA fragmentation was assessed in situ by terminal TdT-mediated dUTP-biotin nick end labeling (TUNEL) using a commercial

kit,[‡‡] following a previously described method.[28] Briefly, slides previously fixed in acetone at 4°C were washed with TBS and treated for 30 minutes at 37°C with 20 μg/ml proteinase-K.[§§] Endogenous peroxidase was inactivated with methanol containing 0.3% H_2O_2[**] at room temperature (RT) for 15 to 30 minutes. Fifty μl of labeling reaction mixture (5 μl TdT enzyme in 45 μl of labeling buffer, prepared and cooled on ice prior to use) was applied to the slide and incubated for 60 to 90 minutes in a 37°C humidified incubator. The reaction was stopped by 3× washes for 5 minutes each with TBS. Then, 70 μl of anti-FITC conjugate was added, incubated at 37°C for 30 minutes and, subsequently, antibody excess was removed by 3× washes as previously described. Finally, the slides were stained with DAB[**] for 10 to 15 minutes at RT and the reaction stopped by washing with distilled water. Sections were counterstained with methyl green, dehydrated, and permanently mounted. Three experimental slides and positive and negative control slides were prepared for each biopsy. Positive control slides consisted of paraffin-embedded tissue sections of rat mammary gland.[‡‡] For negative controls, TdT was omitted during incubation. Double labeling for TUNEL/anti-myeloperoxidase,[**] TUNEL/anti-CD3,[**] or TUNEL/anti-CD20[**] was performed by sequential TUNEL with DAB as chromogen giving a brown reaction product, followed by alkaline phosphatase staining with a fast red reaction product.[**]

Electron Microscopy Technique

Biopsies of gingival tissue were fixed with 2% glutaraldehyde in phosphate-buffered saline (PBS). After 3 PBS washes, the tissue was post-fixed in 2% osmium salt, dehydrated through a graded series of 30% to 100% ethanol dilutions, followed by 100% propylene oxide as clearing agent and finally infiltrated in 1:1 mixture of propylene oxide[‖‖] for 1 hour. After several changes of 100% resin over 24 hours, tissue blocks were enclosed in gelatin capsules and allowed to polymerize at 37°C overnight, followed by additional hardening at 65°C for 12 hours. Semi-thin sections, 0.1 to 1.0 μm in thickness, were cut with a glass knife in a microtome[¶¶] and stained with toluidine blue for light microscopy orientation. Ultra-thin sections, 60 to 80 nm thick, were cut with a diamond knife in a microtome, mounted on 200-mesh copper grids and stained with uranyl acetate and lead citrate. The sections were observed in a transmission electron microscope.[##]

Data Analysis

The presence and distribution of immunohistochemical signals were qualitatively assessed in adjacent sections. The presence of positive cells in the infiltrating connective tissue (ICT), oral sulcular epithelium (OSE), and oral gingival epithelium (OGE) was determined as previously described.[29,30] A range between 0 and

4 was used to establish the degree of antibody-positive cells present in the inflammatory infiltrate: 0 = no detectable cells; 1+ = less than 10%; 2+ = approximately 25%; 3+ = approximately 50%; and 4+ = >50%. The number of TUNEL-positive cells in a field was determined by counting 100 cells, evaluated for each single determination, and reported as the mean of analyses from the 3 sections cut and mounted on treated slides for each patient. All slides were coded by the same person (AS) and analyzed by another person (JG), making the measurements double blind. A second examiner (AA) verified the results.

RESULTS

Presence of Apoptotic Cells in Gingival Tissue of Periodontitis Patients

Tissue destruction is associated with the progression of chronic adult periodontitis. To investigate whether this tissue destruction is mediated by apoptosis, we analyzed the presence of different proteins implicated in the regulation of apoptosis. The presence of cells with death-associated DNA fragmentation was detected *in situ* by TUNEL labeling. Analysis of gingival biopsies from periodontitis patients demonstrated the presence of TUNEL-positive cells (Fig. 1A and B), whereas no positive cells were observed in the biopsies from healthy controls obtained after surgical removal of the third molar. The number of apoptotic cells detected in biopsies of periodontitis patients rarely exceeded 10% per field under a ×40 objective (Table 1).

In order to investigate the type of cells undergoing apoptosis in the inflammatory infiltrate, we used a double staining technique using TUNEL labeling in combination with markers for neutrophils (myeloperoxidase) and T and B lymphocytes (CD3 and CD20, respectively). Therefore, we observed that TUNEL-positive cells also stained with an anti-myeloperoxidase antibody, which indicates that these cells belong to neutrophil cell populations (Fig. 1A and C).

The presence of TUNEL-positive cells has been considered a reliable indicator of apoptosis; however, to further investigate the presence of active apoptosis phenomena, gingival biopsies were stained with an antibody which recognizes the active form of caspase-3 (Fig. 1A and D). The antibody used to detect the active form of caspase-3 recognizes a conformational epitope that is exposed by activation-induced cleavage of the inactive enzyme. Staining of the biopsies with this antibody demonstrated the absence of positive cells in healthy gingival tissues (data not shown). We observed a similar pattern of active caspase-3 positive cells to that observed with TUNEL, which con-
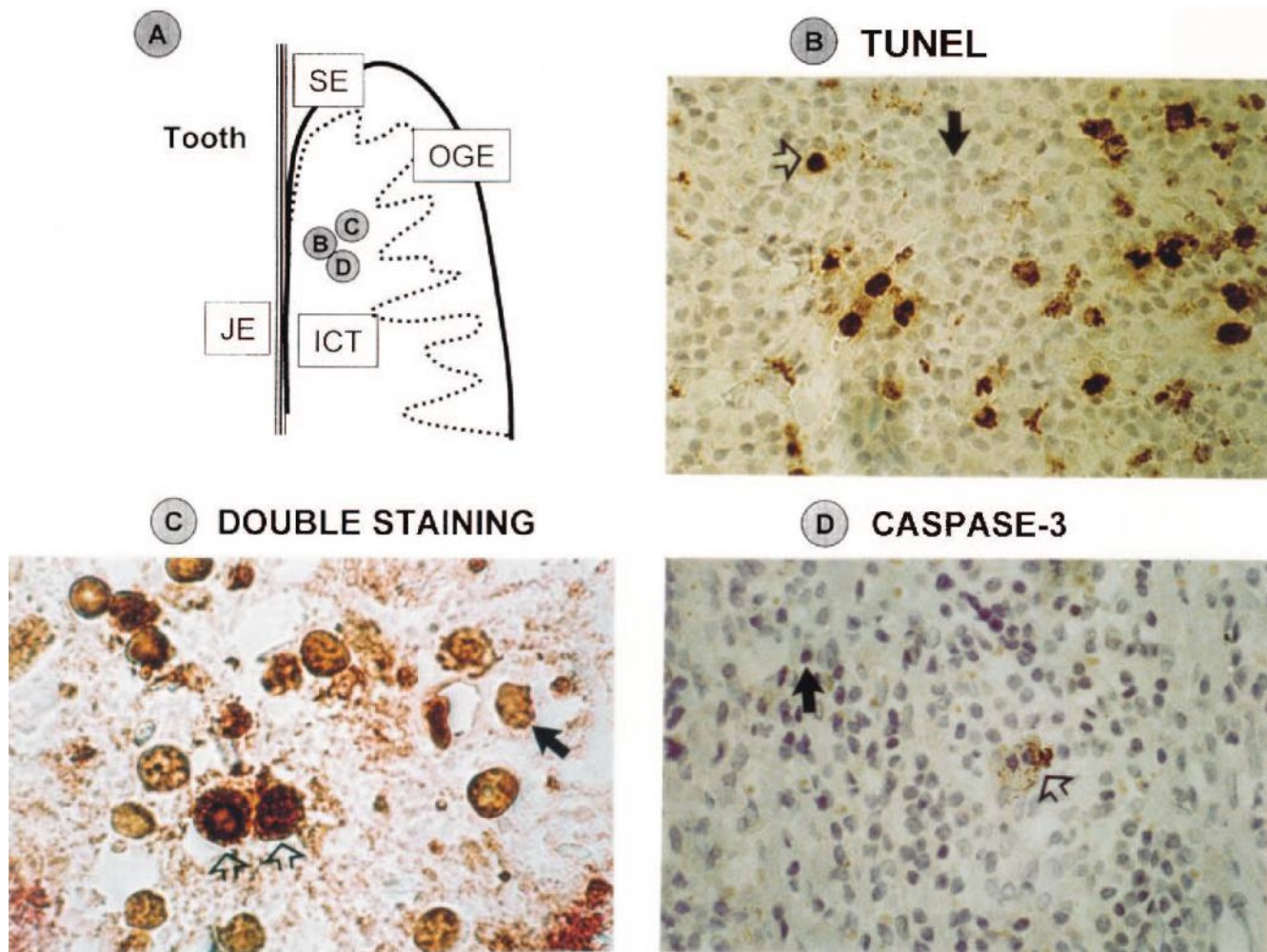
Figure 1.

*TUNEL and immunohistochemical detection in tissue-frozen sections of biopsies from periodontitis patients. **A.** Location of biopsies seen in B, C, and D. SE, sulcular epithelium; JE, junctional epithelium; OGE, orogingival epithelium; ICT, infiltrated connective tissue. **B.** TUNEL-positive cells (open arrow) and negative cells (filled arrow) are observed in chronic inflammatory cells in a gingival section from a periodontitis patient. Apoptotic cells are identified with the TUNEL method and DAB chromogen and methyl green counterstain (original magnification ×40). **C.** Double staining TUNEL/myeloperoxidase-positive cells (open arrows) of a gingival biopsy from periodontitis patients. TUNEL-negative cells are shown by the filled arrow (original magnification ×100). **D.** Active caspase-3 positive cells (open arrow) and negative cells (filled arrow) of frozen sections of gingival biopsy from a periodontitis patient stained with anti-human active caspase-3 using a DAB chromogen and hematoxylin counterstain (original magnification ×40).*

firmed the presence of a few apoptotic cells in the gingiva of periodontitis patients (Table 1).

Direct evidence for the presence of periodontitis-associated apoptosis was provided by analyzing the apoptotic cells by electron microscopy. Cells showing chromatin condensation and nuclear bodies could be observed in the gingival biopsies of periodontal patients by electron microscopy (Fig. 2). Apoptotic cells were predominantly observed in ultra-thin cuts performed in deep sections of the biopsies and always in close proximity to the sites of bacterial plaque aggression.

Fas and Fas Ligand Could Be Involved in Periodontitis

One mechanism of apoptosis depends on the expression of cell death receptors and ligands.[20] To investi-

gate whether Fas/Fas ligand mechanisms could be involved in the tissue destruction associated with periodontitis, we stained gingival biopsies from periodontitis and healthy controls with an anti-Fas and anti-Fas-ligand antibody. As observed in Figure 3, some Fas- and Fas-ligand–positive cells were clearly present in the gingival biopsies from periodontitis patients.

Expression of Bcl-2 and p53-Positive Cells

Bcl-2 is an anti-apoptotic member of the Bcl-2 family. Its expression is associated with reduced apoptosis.[23,26] Considering the low levels of TUNEL- and active caspase-3 positive cells found in gingival biopsies of adult periodontitis patients presenting clinical criteria of tissue destruction, we investigated whether significant numbers of Bcl-2 expressing cells were

Table 1.

Characterization of Cells Undergoing Apoptosis in Periodontal and Healthy Patients

Tissue	N Samples	TUNEL-Positive (mean ± SD)	Inflammatory Infiltrate					
			TUNEL	Casp-3	Bcl-2	p53	Fas	Fas-L
Disease	22	5.63 ± 2.75	<10%	<10%	>25%	<10%	<10%	<10%
Healthy	11	0	0	0	0	0	0	0

detected. Bcl-2 positive cells were detected in gingival biopsies from both periodontal patients and healthy controls (Fig. 3). However, a higher number of Bcl-2 positive cells were located in the inflammatory infiltrate of periodontitis patients (Table 1), suggesting a role for Bcl-2 protein in the control of apoptosis of the inflammatory cells (Fig. 3).

Finally, to further investigate the involvement of apoptotic mechanisms, we studied p53 protein expression in the gingival tissue of periodontitis patients. p53 is a tumor suppressor protein, associated with the induction of apoptosis under situations of cell stress.[31] p53 protein may function as a transcription factor, inducing genes such as bax, which is directly implicated in apoptosis.[32] Staining with a polyclonal antibody anti-p53 demonstrated the presence of p53-positive cells in gingival biopsies from periodontitis patients (Fig. 3; Table 1). The number and distribution of p53-labeled cells correlate with the low expression of TUNEL-positive cells found in the gingival biopsies of periodontitis patients.

DISCUSSION

Apoptosis, or programmed cell death, plays a critical role in the regulation of inflammation and the host immune response.[16] The apoptotic process induces a series of coordinated morphological and biochemical events in the affected cell, resulting in its death and removal by scavenger phagocytes.[16] Characteristically, a cell undergoing apoptosis initially develops cytoplasmic shrinkage and membrane bleeding, which is subsequently followed by chromatin condensation and DNA fragmentation.[19,33] The present work demonstrates the presence of apoptotic cells in the gingival biopsies from periodontitis patients, in agreement with other studies showing that apoptotic cells constitute less than 10% of the total cells.[34,35] We examined sections of human gingival tissues from patients with chronic adult periodontitis by electron microscopy and performed immunohistochemical analysis to detect DNA fragmentation-positive cells and active caspase-3, Fas/FasL, Bcl-2, and p53-positive cells.

Cells specifically degrade their DNA, generating

DNA terminal ends which can be detected by means of a reaction catalyzed by exogenous TdT. Our findings showed that the number of TUNEL-positive cells was higher in periodontitis biopsies than in healthy tissue and was preferentially found in the inflammatory infiltrate. However, our observations are not consistent with other findings[27] reporting that apoptosis-associated DNA damage was a prevalent phenomenon in human clinically healthy gingival tissues. The different half-life of cell tissues, their different cell proliferative rate, and the fact that apoptotic cells are difficult to define[36] could be among the factors involved in these differences.

However, a more reliable indication of the activation of the apoptotic mechanism is the detection of cells expressing the active form of caspase-3.[37] One of the earliest and most consistently observed features of apoptosis is the induction of cytosolic proteases known as caspases, which cleave cellular substrates producing the characteristic apoptotic morphology. Caspases are found inside the cell as inactive proforms, which upon cleavage of an active caspase become the enzymatically active protein.[17,38] Among the caspase family, caspase-3 is considered an executor enzyme, because it can be activated by several other active caspases and due to its catalytic specificity for a relevant number of critical cellular substrates.[39] Therefore, the presence of positive cells for active caspase-3 is considered a hallmark of apoptosis activation. In our study we report the presence of active caspase-3 positive cells in the biopsies derived from adult periodontitis patients; their number and distribution are comparable to TUNEL-positive cells.

Electron microscopy provides the most precise data to study cell morphology, and hence the most accurate method to analyze apoptosis in tissues. In this study, we demonstrate the presence of apoptotic cells by electron microscopy in the deep area of biopsies from sites with probing depth ≥5 mm and attachment loss ≥3 mm. These results are in agreement with another study showing that apoptotic cells were detected only in the gingiva adjacent to >6 mm sulci, but were not found in gingiva adjacent to 4 mm probing depths.[40]

The presence of active caspase-3 positive cells, TUNEL-positive cells, and morphological apoptotic cells analyzed by electron microscopy supports the hypothesis that apoptotic mechanisms could be implicated in the gingival tissue destruction observed in adult periodontitis patients. Double staining using a combination of TUNEL and different markers for cell
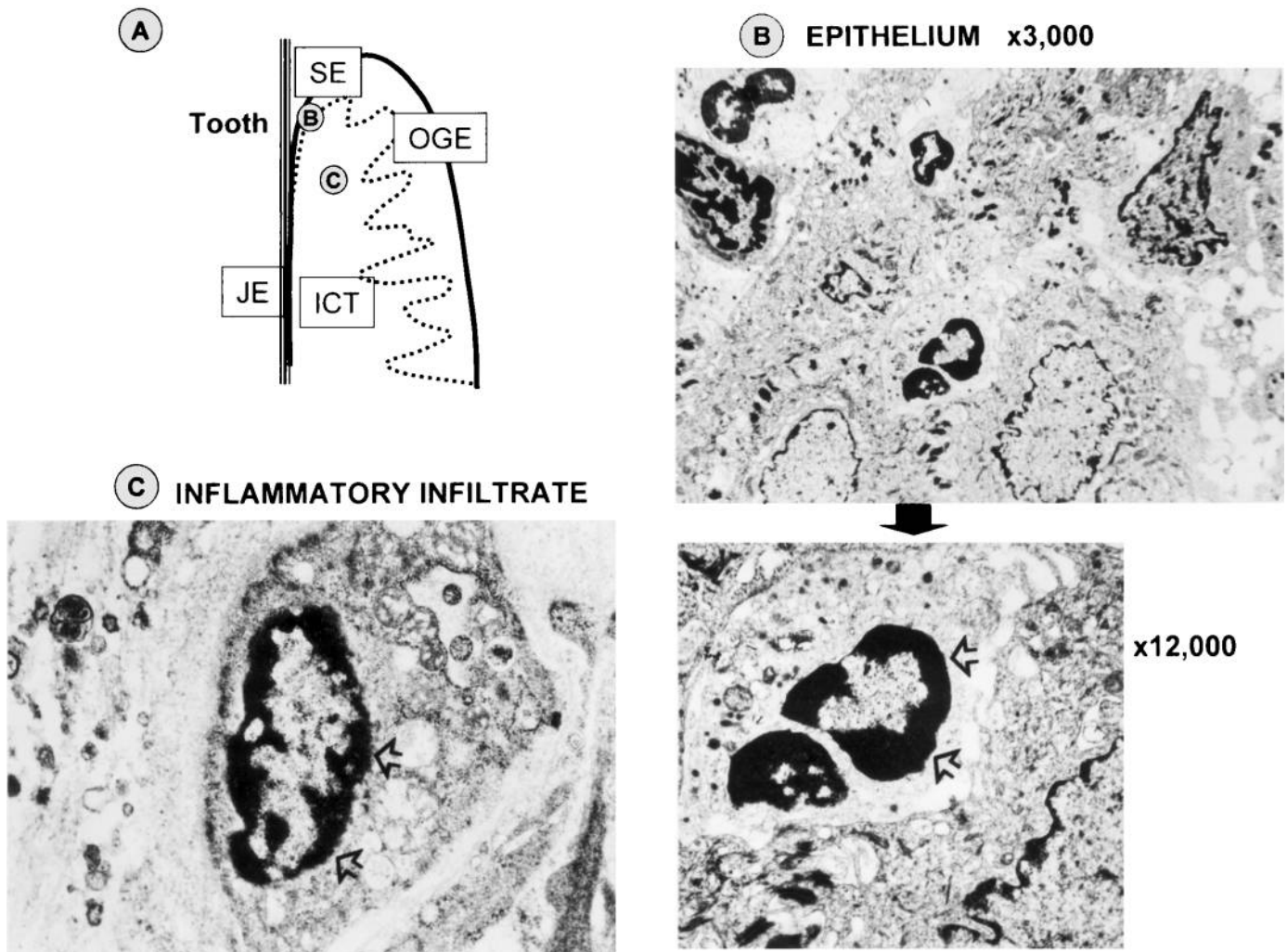
Figure 2.
*Transmission electron microphotographs of the gingival biopsy from a periodontitis patient **A.** Location of biopsies in B and C. SE, sulcular epithelium; JE, junctional epithelium; OGE, orogingival epithelium; ICT, infiltrated connective tissue. **B.** Electron microphotograph of the sulcular epithelium amplified ×3,000 and a detailed amplification (×12,000) of a cell undergoing apoptosis showing chromatin condensation (arrow). **C.** Ultrastructural features of an inflammatory apoptotic cell, with nuclear chromatin condensation and margination (arrow) with cytoplasmic vacuolization (original magnification ×10,000).*

populations shows the presence of neutrophils undergoing apoptosis which implicate this cell population in this process (Fig. 1). Neutrophil apoptosis and subsequent ingestion by macrophages are the major mechanisms for clearing neutrophils that have been recruited to the inflamed sites and thus for promoting resolution of the inflammation.[41,42]

Polymorphonuclear neutrophils (PMN) are the most abundant immune cells in the inflammatory sites of periodontal patients, and their pathogenic role in this setting has been suggested.[43,44] They predominate in the junctional epithelium and gingival crevice, forming a leukocyte wall between plaque and tissue. The interactions between plaque bacteria and PMN are relevant for periodontal disease activity.[8] Circulating neutrophils have a short half-life, and the onset of apoptosis in these cells is associated with the loss of several important functions, such as adhesion and phagocytosis.[45] Inflammatory mediators, such as GM-CSF, IFN-γ,[41,46] or LPS,[47] delay the apoptosis of neutrophils by increasing mitochondrial stability and reducing caspase-3 activity, and by downregulating the gene expression of Bax, a pro-apoptotic member of the Bcl-2 family.[41] In contrast, anti-inflammatory cytokines such as IL-10 accelerate the apoptosis of LPS-activated neutrophils.[48] Recent studies show that the bacterial products isolated from different strains of *Porphyromonas gingivalis* delay neutrophil apoptosis in a dose-dependent fashion.[49]

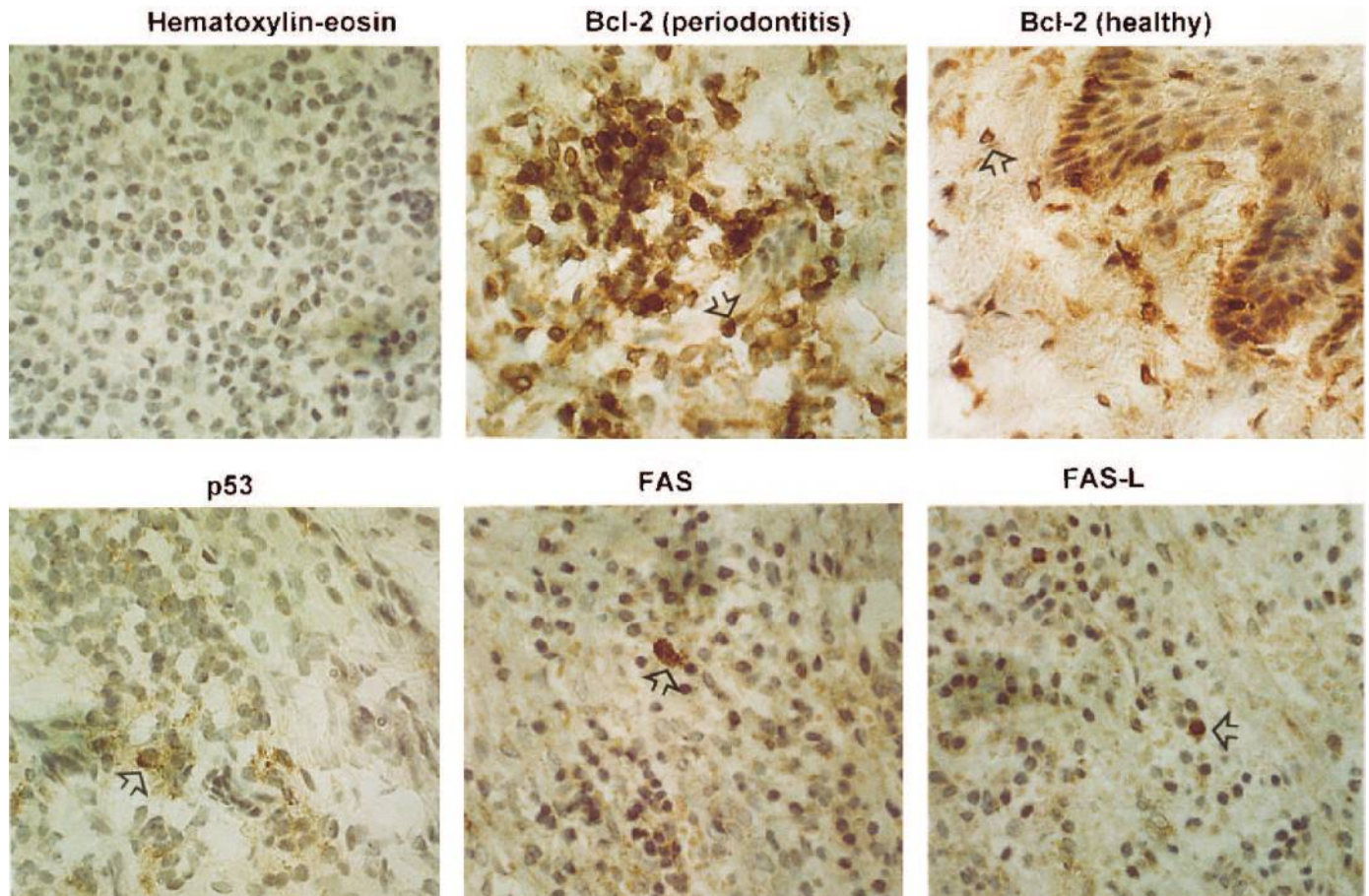Further study is required to determine whether a neu-

Figure 3.
Characterization of inflammatory infiltrate by immunohistochemistry on sections of gingiva of periodontitis and control patients. Detection of positive cells (open arrows) stained with anti-human Bcl-2, p53, Fas, and FasL antibodies in frozen tissue sections of biopsies from periodontitis and control patients using a DAB chromogen and hematoxylin counterstain (original magnification ×40).

trophil apoptotic-inhibition mechanism is taking place; however, we initially investigated the expression of Bcl-2 in gingival tissue. Bcl-2 protein is an inhibitor of apoptosis and its upregulation is considered a main mechanism for apoptosis inhibition.[25] Immunohistochemical labeling of gingival tissues with an anti-Bcl-2 antibody demonstrated the presence of a significant number of Bcl-2 positive cells in the gingival tissue of periodontitis patients.[40] The presence of apoptotic cells raises the question about the mechanisms of apoptosis induction. It is established that bacterial plaque is implicated in the inflammatory response by inducing the secretion of soluble factors; however, there is no evidence on the mechanisms that induce cell apoptosis. A well-known inductor of apoptosis is the Fas-ligand, a member of the tumor necrosis factor-α cytokine superfamily, which, when bound to its receptor, provokes apoptosis. Therefore, we analyzed whether the Fas/Fas-ligand complex system could be involved in the apoptosis of the gingival tissue previously observed in peri-

odontitis patients.[34,50,51] In fact, Fas-positive and Fas-ligand–positive cells were observed in the biopsies of periodontal disease tissue and were not detected in biopsies from healthy samples, suggesting the involvement of this apoptotic mechanism. The use of specific apoptosis peptide inhibitors in ex vivo experiments with fresh tissue biopsies is currently being examined in our laboratory to further investigate the exact role of Fas/Fas-ligand in the induction of apoptosis in periodontitis gingival tissue.

Finally, we considered the possibility that p53 could be involved in apoptosis observed in periodontitis. Certain activators of apoptosis require the presence of a functional p53 protein. p53 is a tumor suppressor protein which, when active, induces genes related to cell cycle regulation, DNA repair mechanisms, and the induction of apoptosis.[32] Although p53 is present in normal tissues and cells, its short half-life make its expression almost undetectable in healthy normal tissues.[21] Upon activation, p53 is stabilized so that its

expression can be detected with anti-p53 antibodies using standard immunohistochemical techniques. Our data show the presence of few p53-positive cells in gingival biopsies of periodontitis patients that could support a p53-dependent apoptosis mechanism not yet clearly defined.

This study strongly supports the relevance of the apoptotic mechanisms in the selection of immuno-competent cell population in periodontal tissues. Further studies on the cellular immune regulation and apoptosis should add to a better understanding on the control of the inflammatory response involved in the pathogenesis of periodontal disease.

ACKNOWLEDGMENTS

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