In Situ Detection of Apoptosis at Sites of Chronic Bacterially Induced Inflammation in Human Gingiva

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Apoptosis is a key phenomenon in the regulation of the life span of terminally differentiated leukocytes. Human gingiva represents an established model to study immune responses to bacterial infection. In this investigation, we used the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) technique to evaluate presence and topographic location of apoptosis-associated DNA damage in human gingival biopsies along with the expression of the p53 and Bcl-2 apoptosis-regulating proteins. Qualitative data analysis showed high densities of cells expressing DNA damage and p53 both within the epithelial attachment to the tooth and in the perivascular infiltrate (infiltrated connective tissue [ICT]) immediately underlying the site of chronic bacterial aggression. Topographic analysis of DNA damage between ICT and p53-positive cells was consistently observed. Quantitative analysis of the ICT showed mean densities of DNA damage- and p53-positive cells of 345 ± 278 and 403 ± 182 cells/mm², respectively. Numerical consistency was confirmed by multivariate regression analysis; densities of DNA damage-positive cells were significantly predicted by densities of p53-positive cells (P = 0.001, r² = 0.84). In the ICT, cells displaying biotinylated DNA nicks were 3.8% ± 2.7% of total cellularity, while p53- and Bcl-2-positive cells represented 4.4% ± 1.7% and 15.4% ± 6.7% of total cells, respectively. It is suggested that p53 expression associated with DNA damage is a prevalent phenomenon in chronically inflamed human gingiva, and that apoptosis may be a relevant process for the maintenance of local immune homeostasis at sites of chronic bacterial challenge in vivo.

Supercritical periodontal tissues are constantly exposed to a mixed anaerobic gram-negative flora which can induce inflammatory responses leading to destruction of the tooth-supporting apparatus, i.e., periodontal diseases. Preservation of periodontal health is thus dependent on the establishment and the maintenance over time of a local host-bacterium equilibrium (9). In this respect, the existence of a high rate of epithelial cell turnover and a highly regulated local immune response are thought to concur in limiting the penetration of pathogenic microorganisms into the gingival tissues (8, 9, 27). Recent investigations have indicated that the recruitment of inflammatory cells at this site of bacterial challenge is dependent on the selective activation of subepithelial capillary loops to express specific leukocyte adhesion molecules (26, 41). Following diapedesis, polymorphonuclear leukocytes migrate into the gingival junctional epithelium along gradients of chemotactic and haptotactic molecules to reach the front of bacterial challenge (43, 44). The majority of mononuclear cells, on the other hand, enter the perivascular connective tissue to form an inflammatory infiltrate composed of a specific subset of T cells, B cells, and macrophages (37, 42, 45). Despite the chronic nature of the bacterial stimuli, in a healthy person, the size of the inflammatory infiltrate has been shown to remain fairly constant over the life of the individual (28, 35, 36). A specific mechanism(s) should therefore account for the observed stability in the size of the inflammatory infiltrate in spite of the continuous influx of leukocytes. One such mechanism may be programmed cell death of tissue-infiltrating leukocytes.

Apoptosis is a programmed form of cell death which results in the elimination of specific cells without disturbance of tissue structure or function (3, 17, 48). It is implicated in a wide variety of biological phenomena, including inflammatory responses (3, 12).

The apoptotic process can be modulated by various stimuli, including hormones, cytokines, growth factors, bacterial or viral infections, and immune responses. Among other factors, the products of two genes, those encoding the p53 and the Bcl-2 proteins, have been shown to play a fundamental regulatory role in this process (48). The Bcl-2 protein can prevent or markedly reduce cell death induced by a wide variety of stimuli (31). Under physiologic conditions, Bcl-2 expression seems to be associated with a pool of less differentiated cells and with cells undergoing terminal differentiation. In these cells, Bcl-2 will prevent apoptotic cell death and thus play a pivotal role in tissue development, cell maturation, and terminal differentiation (13, 20). Conversely, the p53 tumor suppressor gene, whose expression can induce apoptosis, has been implicated in almost all forms of inhibition of cell replication (19). Its expression has been found to be essential for the apoptotic response to the accumulation of DNA damage, p53 expression is also implicated in the regulation of tissue dynamics via its induction of apoptosis in terminally differentiated cells, including inflammatory cells (30).

Emerging evidence indicates that bacterium-modulated apoptosis appears to be an important phenomenon in the pathogenesis of infectious diseases (2). Specific pathogens or their exocellular products may directly induce apoptosis of host cells (23, 51). Conversely, phagocytosis of bacteria or exposure to bacterial components such as lipopolysaccharide may delay programmed cell death of terminally differentiated polymorphonuclear leukocytes (PMN) (1, 4).

The aim of this investigation was to evaluate in situ the presence of cells with apoptosis-associated DNA breaks in the marginal portion of healthy human gingiva. Furthermore, we compared the distribution of cells with damaged DNA with the
TABLE 1. Phenotypic characterization of inflammatory infiltrate

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cells/mm² (mean ± SD)</th>
<th>Ratio</th>
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<tbody>
<tr>
<td>CD22 (B cells)</td>
<td>475 ± 403</td>
<td></td>
</tr>
<tr>
<td>CD3 (T cells)</td>
<td>4,434 ± 2,173</td>
<td></td>
</tr>
<tr>
<td>CD4 (helper cells)</td>
<td>3,539 ± 2,585</td>
<td></td>
</tr>
<tr>
<td>CD8 (suppressor cells)</td>
<td>1,281 ± 1,131</td>
<td></td>
</tr>
<tr>
<td>CD45RA (naive cells)</td>
<td>2,999 ± 1,771</td>
<td></td>
</tr>
<tr>
<td>CD45RO (memory cells)</td>
<td>3,153 ± 1,567</td>
<td></td>
</tr>
<tr>
<td>CD56 (NK cells)</td>
<td>152 ± 79</td>
<td></td>
</tr>
<tr>
<td>CD68 (macrophages)</td>
<td>1,556 ± 973</td>
<td></td>
</tr>
<tr>
<td>PMN elastase (neutrophils)</td>
<td>1,013 ± 754</td>
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and 0.01% H₂O₂ in the presence of nickel ions (15). Sections were lightly counterstained with methyl green, dehydrated, and permanently mounted. Primary antibodies against Ki67 and CD66 antigens were detected by an APAAP technique (5). Sections were incubated for 30 min with 1:25 dilution of rabbit anti-mouse IgG antibodies (Dako), washed, and exposed for 30 min at 1:50 dilution of APAAP complexes (Dako). Color was developed with the new fuchsin substrate kit (Dako) according to the recommended standard procedure in the presence of 4 mM levamisole to inhibit endogenous alkaline phosphatase. Slides were counterstained with hematoxylin and permanently mounted in Aquatex (Merck).

RESULTS

Degree of histologic inflammation and characterization of the infiltrate. The presence of an inflammatory infiltrate was consistently detected in all specimens. The infiltrate was located in a perivascular position subjacent to the junctional epithelium, near the expected site of bacterial plaque aggregation. All specimens demonstrated a Tagge inflammation score of 1, indicating the presence of a well-defined inflammatory lesion that did not extend deeply into the gingival connective tissue. The cell density within the inflammatory lesion was 5.764 ± 2.934 cells/mm². CD3+, CD4+, CD8-, CD22-, CD45RA-, CD45RO-, CD56- and CD68+ cells were all observed in the ICT. Table 1 presents the observed densities of cells positive for the different leukocyte phenotypes. T cells predominated over B cells (CD3/CD22 ratio = 11 ± 2.5), and cells displaying the T-helper phenotype predominated over cells with the suppressor/cytotoxic phenotype.
(CD4/CD8 ratio = 2.9 ± 0.9). No significant difference was observed between the densities of mononuclear cells displaying the memory/activated phenotype and the naive/quiescent phenotype (CD45RO/CD45RA ratio = 1.1 ± 0.4).

**Topographic distribution of cells with detectable DNA damage.** Incorporation of biotinylated-dUTP at the 3' end of DNA breaks was detectable both in the epithelium and in the ICT (Table 2). Particularly strong signals were present (i) in the suprabasal cells of the junctional epithelium, in close proximity with bacterial plaque aggregation (Fig. 1B), and (ii) in the perivascular inflammatory infiltrate. Sporadic positive cells were present in the upper layer cells of the sulcular and orogingival epithelium.

**Topographic distribution of p53-, Bel-2-, and Ki67-positive cells.** Immunoreactive p53 was detectable in all areas where DNA damage could be demonstrated. Strong p53 reactivity was shown within the junctional epithelium, and, in particular, in its more basal layers (Fig. 1C). High numbers of positive cells were also detectable within the ICT, while only rare positive cells could be found in the suprabasal layers of the sulcular and orogingival epithelium. Similar patterns of reactivity were observed with both PAB240 and PAB248 monoclonal antibodies; the signal appeared to be located mainly in the perinuclear (PAB240) or cytoplasmic (PAB248) position.

Bel-2-positive cells were located mainly within the perivascular inflammatory infiltrate (Fig. 1D); rare cells were observed in the basal layer of the gingival epithelium. No Bel-2-positive cells could be detected within the junctional epithelium or the suprabasal layers of the other gingival epithelium.

Detection of the proliferating cell-associated Ki67 nuclear antigen was confined to the epithelium. Strong reactivity was found in association with the epithelial basal layers.

**Quantitative evaluation.** Box plots of the densities of ICT cells displaying biotinylated DNA nicks and detectable p53 and Bel-2 expression are shown in Fig. 2. Scoring of adjacent sections revealed similar densities of DNA damage- and p53-positive cells (345 ± 278 and 403 ± 182 cells/mm², respectively). Significantly more Bel-2-positive cells, however, were observed (1,457 ± 898 cells/mm²). The association between DNA damage-positive cells and the expression of the Bel-2 and p53 apoptosis-regulating proteins was further evaluated by least-squares regression analysis. The association was highly significant and explained 84% of the observed variability in the number of DNA damage-positive cells per micrometer squared (P = 21.142; P = 0.001 [Table 3]). Results indicated that the density of DNA damage-positive cells in the inflammatory infiltrate could be significantly predicted by a model including the density of cells expressing the p53 apoptosis-inducing protein (P = 0.017 [Table 3]). Conversely, the density of Bel-2-positive cells did not significantly contribute to the regression equation (P = 0.281). Furthermore, the calculated regression coefficient was close to 1 (β = 0.92), indicating a strong numerical consistency between DNA damage-positive cells and cells expressing the p53 protein.

**Comparison of labeled DNA damage-, p53-, and Bel-2-positive cells with the inflammatory cell density in the ICT indicated that positive cells represented a small yet significant fraction of the infiltrate.** Cells displaying biotinylated DNA nicks were 3.8% ± 2.7% of total cells; similarly, p53 and Bel-2 positive cells represented 4.8% ± 1.7% and 15.4% ± 6.7%, respectively.

**DISCUSSION**

The results of this investigation indicated that apoptosis-associated DNA damage and expression of the p53 and Bel-2 apoptosis-regulating genes were prevalent phenomena in human clinically healthy gingival tissues exposed to chronic, low-grade bacterial challenge and inflammation. This represents, to our knowledge, the first in situ study indicating the relevance of the apoptotic process in chronic, low-grade, bacterially induced inflammation. Cells positive for DNA damage, p53, or Bel-2 were selectively found in precise topographical locations: much of the expression was observed in the subepithelial inflammatory infiltrate and within the junctional epithelium and thus close to the area exposed to the oral microflora.

In situ detection of DNA damage at these sites of inflammation is an important observation since it may relate to a variety of biological phenomena, including programmed cell death. Use of the TUNEL technique allows the in situ detection of cells with DNA damage in a variety of tissues (7). Some investigations, however, have suggested that DNA damage evidenced with the TUNEL technique is not specific for the detection of apoptotic cell death but may also give positive results in areas of tissue necrosis (11). In this respect, it is important to underline that (i) in our material no necrosis was observed in the characteristic histopathological signs of necrosis; (ii) the selective and consistent tissue distribution of DNA damage-positive cells, as well as the appearance of positive and negative controls, strongly indicated the non-artifactual nature of the signal; and (iii) the topographic consistency of p53 expression with the areas displaying DNA damage, as well as the strong statistical association between the density of p53-positive cells and the density of TUNEL-positive cells, supports the conclusion that at least some of the cells with detectable DNA damage may be apoptotic.

The presence of DNA damage-positive cells associated with the expression of the wild-type p53 apoptosis-inducing protein in the subepithelial inflammatory infiltrate suggests that apoptotic cell death may be an important phenomenon in the regulation of the inflammatory response to a chronic bacterial challenge. About 4% of the cells present in the subepithelial mononuclear inflammatory infiltrate displayed apoptosis-associated changes. Such a high prevalence is striking since in vitro the apoptotic process has been shown to be quite rapid and leading to cell fragmentation in a few hours (16). The high percentages of apoptotic cells in the inflammatory infiltrate detected in this study may speak for a significant role of apoptosis in preventing increases in cellularity and topographic extension of the infiltrate. In this respect, it should be observed that in this investigation the infiltrate consisted of macrophages as well as B and T cells and some PMN (Table 1). The lesion was a T-cell-dominated one with T-helper cell-to-suppressor ratios of 3 and substantially equal densities of activated/memory and naive/quiescent T cells. These observations are consistent with the results of previous studies and suggest that the analyzed material could be considered representative of the chronic inflammatory reaction consistently found in clinically healthy gingiva.

The current understanding of the limited permeability of junctional epithelium to bacterial plaque and its products un-
FIG. 1. In situ detection of DNA damage, wild-type p53, and Bcl-2 in human gingiva. (A) Topographical orientation of the sections displayed in panels B to D. In all sections the tooth surface is on the left. Abbreviations: JE, junctional epithelium; SE, sulcular epithelium; OGE, orogingival epithelium; ICT, infiltrated connective tissue. (B) TUNEL-positive cells located in the most superficial portion of the JE (area B in panel A). The staining pattern that represents detection of DNA damage is located in the nucleus. Note the absence of positive cells in the SE. (C) Presence of p53-positive cells, preferentially located in the deeper layers of the JE (area C in panel A). (D) Bcl-2 staining pattern of the mononuclear cells present in the ICT (area D in panel A). Magnification for panels B to D, ×250.

5193
under the clinically normal conditions evaluated in this study would speak against a direct bacterial effect leading to apoptosis within the inflammatory infiltrate. Possible hypotheses on the molecular regulation of this phenomenon come from in vitro experiments indicating that when deprived of certain cytokines or bacterial challenges, different leukocytes undergo apoptosis. In general, exposure to proinflammatory cytokines, such as interleukin-1β and tumor necrosis factor alpha, or bacterial lipopolysaccharide seems to prevent apoptosis (24) and to be associated with Bcl-2 expression (4). Conversely, other cytokines such as interleukin-4 and transforming growth factor β have been associated with an increase in p53 expression and apoptotic changes (22). A variety of ex vivo investigations have determined that the gingival mononuclear cell infiltrate expresses both apoptosis-preventing (proinflammatory) and apoptosis-inducing (anti-inflammatory) cytokines (25, 50, 6). The detection of both Bcl-2- and p53-positive cells within the inflammatory infiltrate is therefore not unexpected and suggests the presence of a finely regulated cytokine network, the balance of which may determine onset or inhibition of the apoptotic process and thus both the fate of individual cells and eventual variations in the size and cellularity of the infiltrate. Further investigations are needed in this area.

High numbers of DNA damage-positive cells were also found in the superficial layers of the junctional epithelium. This stratified epithelium is particular since it provides both a seal to restore mucosal continuity around erupted teeth and a compartment for peripheral defense (34). It is constantly exposed to a mixed bacterial flora which includes gram-negative anaerobes, and it has an exceptionally high rate of turnover. According to one estimation, epithelial cell desquamation in this tissue is 50 to 100 times faster than in the adjacent oral mucosa (21). High levels of DNA damage are presently considered to be a characteristic of the uppermost layers of rapidly renewing epithelia such as the intestinal mucosa or epidermal epithelium (7, 39), while wild-type p53 expression has been detected in the parabasal cells of these epithelia (30). In the present study, cells showing DNA damage and cells expressing the p53 apoptosis-inducing protein were found in topographically distinct regions of the junctional epithelium. These localizations are consistent with the sequential development of the apoptotic process, i.e., the initial expression of the p53 apoptosis-inducing protein in the parabasal layers, and consequent detection of DNA damage in the more superficial layers (Fig. 1B and C). These observations may be interpreted as the result of several, possibly correlated phenomena: (i) the interaction between specific bacteria present in dental plaque and junctional epithelium keratinocytes; (ii) the production of specific intraepithelial autocrine and paracrine stimuli; and/or (iii) the effect of paracrine stimuli generated by the subepithelial connective tissue infiltrate. It has been shown that a variety of bacterial pathogens are able to induce apoptosis in the infected cells: the leukotoxin of a periodontal pathogen, Actinobacillus actinomycetemcomitans, has been shown to induce apoptosis on human T cells (23); similarly, the intestinal pathogen Shigella flexneri induces apoptosis in infected macrophages (51). In this respect, it is generally agreed that some dental plaque bacteria are able to penetrate within the junctional epithelium, and they have been detected in an intracellular location (32, 33). Further investigations are in progress in this area. Also of interest is the recent recognition that junctional epithelium, like most body epithelia, plays an active role in the maintenance of surface integrity. Gingival keratinocytes, in fact, have been shown to produce, and respond to, a variety of cytokines and other inflammatory molecules (40) that may play a pivotal role in the homeostasis of this epithelium, possibly through induction and prevention of programmed cell death.

In summary, the results of this study indicate that apoptosis-associated cell damage is a prevalent phenomenon at sites of chronic bacterially induced inflammation in human gingiva and may play a role in the regulation of mucosal inflammation.

ACKNOWLEDGMENTS

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REFERENCES


### TABLE 3. Regression model showing that density of cells with DNA breaks = β₁ density of Bcl-2-positive cells + β₂ density of p53-positive cells

<table>
<thead>
<tr>
<th>Parameter (β₃)</th>
<th>Estimate</th>
<th>t for H₀⁻⁻</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2-positive cells</td>
<td>0.021</td>
<td>0.288</td>
<td>0.781</td>
</tr>
<tr>
<td>p53-positive cells</td>
<td>0.919</td>
<td>3.008</td>
<td>0.017</td>
</tr>
</tbody>
</table>

*Model (error): df, 2 (8); sum of squares, 1,903,299.294 (360,690.706); mean square, 951,649.647 (45,013.338); F value, 21.142; P = 0.001; r² = 0.841. *Parameter estimate = 0.
In situ detection of apoptosis in chronic inflammation


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