American Journal of Pathology, Vol. 148, No. 1, January 1996

Absence of Exogenous Interleukin-4-Induced Apoptosis of Gingival Macrophages May Contribute to Chronic Inflammation in Periodontal Diseases

Masafumi Yamamoto,*Keiko Kawabata,* Kohtaro Fujihashi,* Jerry R. McGhee,*† Thomas E. Van Dyke,‡ Thaddeus V. Bamberg,* Takachika Hiroi,§ and Hiroshi Kiyono*§

From the Departments of Oral Biology and Microbiology,† Research Center in Oral Biology and Immunobiology Vaccine Center, University of Alabama at Birmingham Medical Center, Birmingham, Alabama; Graduate Periodontology, Boston University School of Graduate Dentistry, Boston. and the Department of Mucosal Immunology,§ Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan*

Inflamed gingival tissues are enriched in macrophages (MØs) and CD4-positive T cells; however, T helper-type cytokines such as interleukin (IL)-2 and IL-4 are absent. Therefore, we investigated whether a relationship exists between IL-4 receptor (IL-4R) expression and MØ persistence in the absence of exogenous IL-4. Gingival MØs, when compared with monocyte(MN)/MØs from peripheral blood mononuclear cells, expressed high levels of IL-4R mRNA. Furthermore,* in vitro *cultures of gingival MØs remained viable whereas identically treated peripheral blood MN/MØs rapidly lost viability. However, when gingival MØs were incubated with recombinant IL-4 (rIL-4), the cell viability was dramatically reduced. When the frequency of apoptotic cells was assessed in rIL-4-treated gingival MØ cultures, higher numbers of apoptotic cells were noted in rIL-4-treated* versus *control cultures. Furthermore, rIL-4-treated MØs from inflamed gingiva showed DNA fragmentation as assessed by electrophoresis. These findings clearly show that addition of exogenous rIL-4 to gingival MØ cultures leads to cell death by apoptosis. This finding would suggest that topical application of rIL-4 may inhibit the persistence of MØs in adult periodontitis, which could then lead to decreased inflammation. (Am J Pathol 1996, 148:331–339)

In recent years, immunological studies have focused on the elucidation of molecular mechanisms for the regulation of the life cycle of leukocytes in normal and diseased situations. It has now become clear that the mechanisms resulting in cell death are more complicated than had been appreciated. Two distinct pathways by which eukaryotic cells undergo cell death are recognized, ie, necrosis and programmed cell death, or apoptosis.[1] The former cell death pathway can be induced by direct (or indirect) damage to the cell membrane, such as exposure to poisons or physical injury. The latter form of cell death is essential for the maintenance of appropriate cell numbers in the host. The process of programmed cell death is characterized by chromatin condensation, DNA fragmentation, and membrane blebbing. Recently, it was shown that cultivation of monocytes (MNs) from peripheral blood mononuclear cells (PBMCs) without any stimulus resulted in the induction of rapid apoptosis.[2,3] However, the addition of microbial components, eg, lipopolysaccharide (LPS) or proinflammatory cytokines, eg, interleukin (IL)-1 and tumor necrosis factor-α to these MN cultures reduced the frequency of cells undergoing apoptosis.[3] On the other hand, IL-4 has been shown to induce apoptosis in peripheral blood MNs when incubated with IL-1, tumor necrosis factor-α, or LPS.[4]

Supported in part by U.S. Public Health Service grants DE 08228, DE 09837, DE 06436, AI 35544, AI 35932, DE 04217, and AI 18958 and contract AI 15128.

Accepted for publication October 6, 1995.

Address reprint requests to Dr. Hiroshi Kiyono, Department of Oral Biology, BBRB 761, The University of Alabama at Birmingham, Birmingham, AL 35294-2170.

IL-4 is a key cytokine for the growth and proliferation of B lymphocytes. This cytokine also possesses additional regulatory effects for MN/MØs. For example, IL-4 suppresses the ability of MN/MØs to produce cytokines, prostaglandin E_2, or H_2O_2.[5–12] On the other hand, IL-4 is capable of inducing major histocompatibility complex class II expression and adhesion molecules of MN/MØs.[5] Thus, it is clear that IL-4 is an important cytokine for regulation of MN/MØ function in localized inflammatory sites. In this context, our previous studies have shown that gingival mononuclear cells isolated from adult periodontitis (AP) patients produce the cytokines interferon (IFN)-γ, IL-5, and/or IL-6 but not IL-2 and IL-4.[13] Furthermore, it was also shown that high numbers of MØs occur in the inflamed gingival tissues of patients with AP and contribute to the localized inflammation.[14,15]

We have tested the hypothesis that the lack of IL-4 may in fact be related to the persistent occurrence of MØs at sites of inflammation such as in AP. To address this point, MØs isolated from inflamed periodontal tissue of AP patients were assessed for the expression of IL-4 receptor (IL-4R) by reverse transcription polymerase chain reaction (RT-PCR) using capillary electrophoresis. The isolated MØs were then incubated either in the presence or absence of human recombinant IL-4 (rIL-4) and assessed for apoptotic cell death by acridine orange and ethidium bromide staining and by DNA electrophoresis. Our results provide an explanation for the persistence of MØs in localized tissues of AP patients and suggest that cytokines such as IL-4 may be used in noninvasive therapy.

Materials and Methods

Preparation of Gingival MØs and Peripheral Blood MNs

Gingival mononuclear cells (GMCs) were obtained from patients in the severe stage of AP previously described.[13,15–17] Briefly, GMCs were isolated from surgically extirpated gingival tissues by the Dispase digestion method. GMC or peripheral blood MN suspensions in serum free Dulbecco's minimal essential medium (DMEM; GIBCO, Grand Island, NY) were added to 24-well plates (Corning Glass Works, Corning, NY) and incubated for 1 hour in a humidified 37°C, 5% CO_2 atmosphere. After incubation, nonadherent cells were removed by extensive and vigorous washing of wells with serum-free DMEM. To harvest the adherent MN/MØs, the cell monolayers were incubated with ice-cold phosphate-buffered saline (PBS) containing 0.02% EDTA for 10 minutes. The MN/MØs were then removed by vigorous pipetting. The cell viability of MN/MØ preparations as determined by trypan blue dye exclusion was >98%. The adherent MN/MØ fraction was also examined by flow cytometry using the monoclonal antibody Leu M3. Greater than 95% of cells were Leu $M3^+$.

IL-4R-Specific RT-PCR

The RT-PCR for analysis of IL-4R mRNA was performed as previously described.[13,18] Briefly, for the isolation of RNA the acid guanidinium thiocyanate phenol chloroform extraction procedure was used. These RNA preparations from MN/MØs were then subjected to the IL-4R-specific RT-PCR. Total RNA from MN/MØs was added to a reaction mixture containing 5 mmol/L $MgC1_2$ (Perkin Elmer Cetus, Norwalk, CT), 1X PCR buffer II (Perkin Elmer Cetus), 1 mmol/L each of dNTPs (Perkin Elmer Cetus), 1 U/μl RNAse inhibitor (Promega, Madison, WI), 2.5 U/μl Moloney murine leukemia virus reverse transcriptase (GIBCO), and 2.5 μmol/L oligo $d(T)_{16}$ (Perkin Elmer Cetus). Samples were then placed on a thermal cycler (Perkin Elmer Cetus) and incubated for 15 minutes at 42°C, followed by 5 minutes at 99°C and then 5 minutes at 5°C. After reverse transcription, RT products were added to each tube with AmpliTaq DNA polymerase (1.25 U/50 μl), 5′ primer (0.15 μmol/L), 3′ primer (0.15 μmol/L), $MgC1_2$ (2 μmol/L) and 1X PCR buffer II (Perkin Elmer Cetus). Primer specific for IL-4R (5′ primer, TGAAGTCTGGGATTTCCTACAGGGCACG; 3′ primer, TCAAACAACTCCACACATCGCACCACGC) was obtained from Clontech Laboratories (Palo Alto, CA) and β-actin (5′ primer, ATGGATGATGATATCGCCGCG; 3′ primer, CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC) was prepared by the University of Alabama at Birmingham Oligonucleotide Core Facility. After heating at 95°C for 2 minutes for initial denaturation, cDNAs were amplified for 35 cycles (95°C for 1 minute and 60°C for 1 minute). At the end of 35 cycles, products were held at 60°C for 7 minutes and then stored at 4°C.

Capillary Electrophoresis Analysis

For the quantitative analysis of RT-PCR products, capillary electrophoresis with a laser-induced fluorescence detection system (LIF-P/ACE, Beckman Instruments, Fullerton, CA) was applied.[19,20] An argon ion laser source with excitation at 488 nm and emission at 530 nm was used for laser fluorescence

detection. Separations were carried out in the reversed polarity mode. Analysis of RT-PCR products was conducted by using a coated capillary tube (internal diameter, 100 μm; length, 37 cm; Beckman Instruments) in Tris-borate EDTA containing replaceable linear polyacrylamide and the fluorescent intercalator (0.4 μg/ml). A 10 μl volume of PCR products was diluted by 40 μl of double-distilled water and injected hydrodynamically for 10 seconds at 0.5 psi. RT-PCR products were run for 25 minutes at 200 V/cm. The level of IL-4R mRNA was normalized to the corresponding β-actin value.

Culture System

Gingival MØs and peripheral blood MNs were resuspended in DMEM containing 10% fetal calf serum and cultured at cell concentration of 2×10^5/ml in 12- × 75-mm polypropylene tubes (Falcon, Becton Dickinson, NJ) to avoid spontaneous activation by adherence during the incubation period.[21] Different concentrations (0 to 200 ng/ml) of rIL-4 (Genzyme, Cambridge, MA) were added to these MN/MØ cultures. In some cases, MN/MØs were cultured in the presence of *Escherichia coli* LPS (10 ng/ml; Sigma Chemical Co., St. Louis, MO) exactly as described by others.[3]

DNA Analysis by Flow Cytometry

To determine DNA content, nuclear double-stranded DNA in MN/MØs was disclosed by staining with propidium iodide and analyzed by flow cytometry as previously described.[18] Briefly, MN/MØs were fixed by 70% ethanol. The cells were then incubated for 30 minutes at 37°C in the presence of 250 μl of RNAse (500 U/ml; Sigma). A 250 μl aliquot of propidium iodide (50 μg/ml; Poly Sciences, Warrington, PA) was added to individual samples and incubated for 30 minutes at room temperature. All samples were then analyzed by flow cytometry to determine G_0 and G_1, S, and G_2 plus M phases of cell cycle in MN/MØ preparations.

Analysis of Apoptotic Cells

To evaluate the proportion of apoptotic *versus* necrotic cells induced by incubation with rIL-4, the acridine orange/ethidium bromide staining method was applied.[22] Cultures of MN/MØs (1×10^4 to 1×10^5 cells/25 μl) were mixed with a solution of 1 μl of acridine orange (100 μg/ml in PBS; Sigma) and ethidium bromide (100 μg/ml in PBS; Sigma) and a 10 μl aliquot was placed on a microscope slide (Corning Glass Works) and covered with a coverslip. Apoptotic cells were assessed with a 40× or 60× dry objective fluorescence microscope (Nikon, Tokyo, Japan).

The percentage of apoptotic and necrotic cells were calculated as follows: VN, viable cells with normal nuclei; VA, viable cells with apoptotic nuclei; NVN, nonviable cells with normal nuclei; NVA, nonviable cells with apoptotic nuclei. The percentage of apoptotic cells = (VA + NVA/VN + VA + NVN + NVA) × 100; the percentage of necrotic cells = (NVN/VN + VA + NVN + NVA) × 100; and the percentage of dead cells = (NVN + NVA/VN + VA + NVN + NVA) × 100.

Assessment of DNA Fragmentation

As an additional parameter of apoptosis, DNA fragmentation was assessed by electrophoresis according to the method previously described.[23] Briefly, MN/MØs (2×10^6 cells) were centrifuged after incubation with or without rIL-4. The cells were then washed twice in ice-cold PBS and lysed with Triton X-100/Tris buffer. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1). After the 1.1 mol/L ammonium acetate and 50% isopropanol treatment, DNA was mixed with 1 μg/ml DNAse-free RNAse (Boehringer Mannheim, Indianapolis, IN) and 0.1% sodium dodecyl sulfate (SDS). The sample was re-extracted and re-precipitated by the same procedure. The DNA samples were then resuspended in Tris-EDTA buffer and loading buffer containing 50% glycerol, 15 mmol/L EDTA, 2% SDS, and 0.05% bromphenol blue. Individual samples were next incubated for 10 minutes at 60°C and then electrophoresed in 1% agarose containing 1 μg/ml ethidium bromide in a 40 mmol/L Tris-acetate and 1 mmol/L EDTA buffer, pH 8.0, for 60 minutes at 80 V. The bands of DNA were visualized by ultraviolet light and photographed with a Polaroid camera.

Statistics

The results were analyzed statistically by Student's *t*-test.

Results

Viability of Gingival and PBMC MN/MØs in Vitro

Inasmuch as previous studies showed that unstimulated peripheral blood MNs *in vitro* die by apoptosis

Table 1. *MN/MØs Isolated from Inflamed Gingival Tissues Are in Higher Stages of Activation when Compared with PBMC MNs*

MN/MØs	Cell cycle analysis (%)		Cell viability (%)	
	G_0/G_1	S + G_2/M	24 hours	48 hours
Inflamed gingiva	82.6 ± 2.5	17.3 ± 2.5	84.6 ± 1.8	72.4 ± 2.1
Peripheral blood	96.3 ± 0.6	3.7 ± 0.6	54.2 ± 3.6	38.1 ± 2.8

Freshly isolated gingival and peripheral blood MN/MØs from the same patients were incubated with propidium iodide for the analysis of cell cycle stage by flow cytometry. Cell viability was examined after incubation for 24 or 48 hours. Freshly isolated MN/MØs from inflamed gingiva and peripheral blood of patients possessed greater than 98% cell viability.

within 24 to 48 hours,[2,3] it was important to determine whether MN/MØs isolated from inflamed gingival tissues would follow the same route of cell death without exogenous stimulation *in vitro.* In the first study, MN/MØs were isolated from inflamed gingival tissues and were also separated from PBMCs. The purified MN/MØs were cultured for 48 hours without exogenous stimulation and were stained with ethidium bromide for the assessment of cell viability. After 48 hours of incubation, MØs isolated from inflamed gingiva maintained a good cell viability (70 to 80%). In contrast, more than 60% of peripheral blood MNs died during this 48 hour period (Table 1). As it has been shown that viability of peripheral blood MNs is improved when cultured on polystyrene plates or in the presence of LPS and IL-1,[3] our results suggest that MN/MØs isolated from inflamed gingiva could be in a higher stage of activation when compared with MN/MØs from peripheral blood. In support of this, cell cycle analysis revealed that approximately 17% of gingival MØs were in S plus G_2/M phase of the cell cycle, whereas less than 4% of peripheral blood MN/MØs were in S plus G_2/M phase (Table 1). Furthermore, our separate study showed that gingival MN/MØs from inflamed tissues expressed higher levels of inflammatory cytokine-specific (eg, IL-6) mRNA in comparison with peripheral blood MN/MØs (data not shown). Taken together, these findings demonstrated that MØs in inflamed gingiva are activated.

Expression of IL-4R by Gingival MØs

To study the effect of IL-4 on MØs isolated from inflamed gingival tissues, it was important to determine whether isolated MØs express IL-4R. For this purpose, RNA samples were obtained from MN/MØs of inflamed gingiva or the PBMCs of AP patients for the examination by IL-4R-specific RT-PCR using capillary electrophoresis. The levels of IL-4R-specific message were significantly higher in RNA isolated from gingival MØs when compared with MN/MØs from PBMCs of the same patient (Figure 1). However, when MN/MØs from the PBMCs were incubated with an optimal concentration of LPS, the levels of IL-4R increased and were comparable to or, in some cases, higher than those of gingival MØs. These findings suggested that the environment of chronic inflammation caused by the ubiquitous bacterial microflora most likely induces IL-4R expression on gingival MØs.

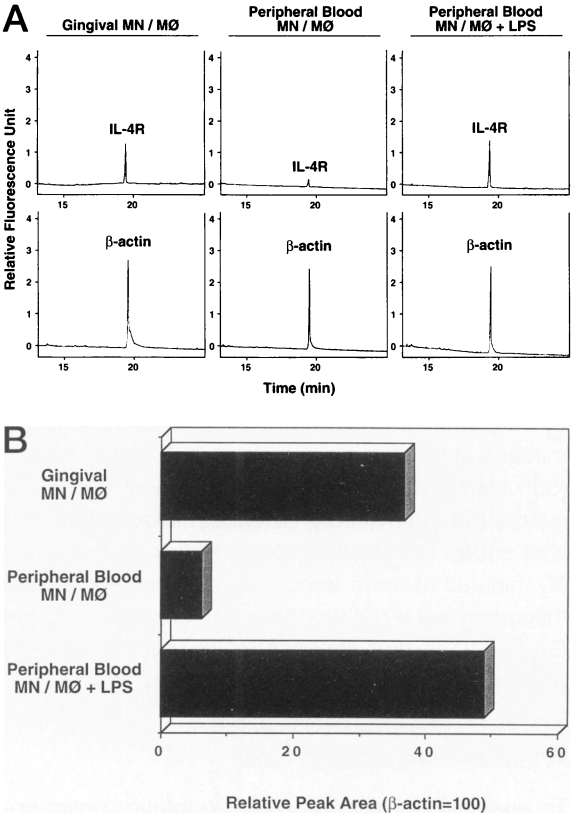

Figure 1. *Characterization of IL-4R-specific message from gingival MN/MØs by cytokine-specific RT-PCR using capillary electrophoresis. MN/MØs were isolated from inflamed gingiva or PBMCs of the same patient. An aliquot of peripheral blood MN/MØs was stimulated with 10 ng of E. coli LPS for 48 hours. The RNA samples were obtained from gingival and peripheral blood MN/MØs for IL-4R-specific RT-PCR. The level of specific message was determined by the analysis of PCR-amplified products using capillary electrophoresis* (**A**). *The level of IL-4R was expressed as relative peak area based on the value of β-actin* (*β-actin = 100;* **B**).

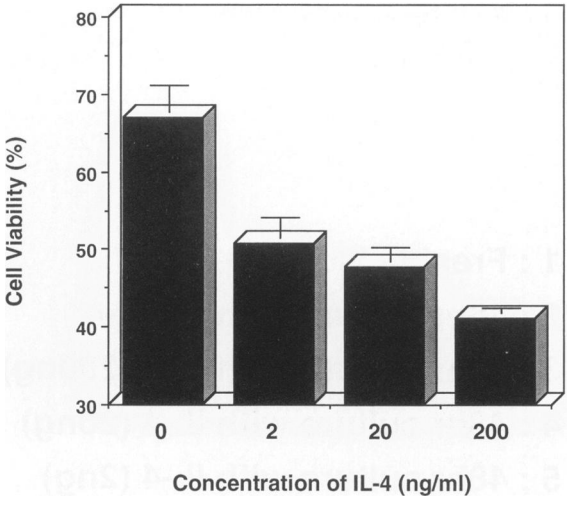

Figure 2. *Cell viability in IL-4-treated MØs isolated from inflamed gingival tissues. MØs were incubated with different concentrations of rIL-4 for 48 hours and cells were then stained with ethidium bromide. The values are the mean cell viability ± SD (n = 3). P < 0.01.*

Lethal Effects of IL-4 for Gingival MØs

As IL-4 has been shown to induce programmed cell death (apoptosis) in stimulated peripheral blood MNs,[4] the effect of IL-4 on IL-4R$^+$ MØs isolated from inflamed gingival tissues was next examined. When different concentrations of IL-4 (0 to 200 ng/ml) were added to gingival MØ cultures, the percentage of cell viability decreased in a dose-dependent fashion (Figure 2). Thus, a decline in cell viability was already noted in cultures containing as little as 2 ng/ml rIL-4. Furthermore, 200 ng/ml rIL-4 reduced gingival MØ viability from 70 to 80% down to 40%.

Induction of Apoptosis in Gingival MØs by rIL-4

From the results described above, exogenous IL-4 induced cell death in gingival MØ cultures. As the death of eukaryotic cells occur by either necrosis or apoptosis, these rIL-4-treated gingival MØs were examined for the appearance of necrotic or apoptotic cells by acridine orange and ethidium bromide staining. The frequency of apoptotic cells with characteristics of chromatin condensation were increased in cultures containing gingival MØs and rIL-4. As little as 2 ng/ml rIL-4 induced apoptotic cells in gingival MØs (Figure 3). Furthermore, approximately 60% of rIL-4-treated cells became apoptotic when a 200 ng/ml concentration of the cytokine was used. In contrast, the frequency of necrotic cells did not change (Figure 3). Thus, approximately 10% of cultured cells died by necrosis regardless of whether they were treated with IL-4 or not.

To further confirm these results at the moleculer level, DNA electrophoretic analysis was performed, because a particular feature of apoptosis is the occurrence of fragmentation of nuclear DNA. Freshly isolated MØs from inflamed periodontal tissue did not show any DNA fragmentation. On the other hand, strong cleavage of DNA into 180- to 200-bp fragments was seen in gingival MØs cultured with rIL-4. Untreated MØs exhibited a low frequency of DNA fragmentaion when compared with gingival MØs cultured with rIL-4 (Figure 4). These results indicated that exogenous IL-4 induced programmed cell death in IL-4R$^+$ MØs isolated from inflamed gingival tissues.

Kinetics of Apoptosis in IL-4-Treated Gingival MØs

As IL-4 was shown to be capable of inducing programmed cell death in gingival MØs, it was important to evaluate the kinetics of the rIL-4 effect on gingival MØs. Thus, gingival MØs were cultured with 200 ng/ml rIL-4 for periods of up to 48 hours (Figures 5 and 6). After only 6 hours of incubation with rIL-4, the frequency of dead cells with characteristics of apoptosis was increased when compared with untreated MØ cultures (Figure 5). Furthermore, the per-

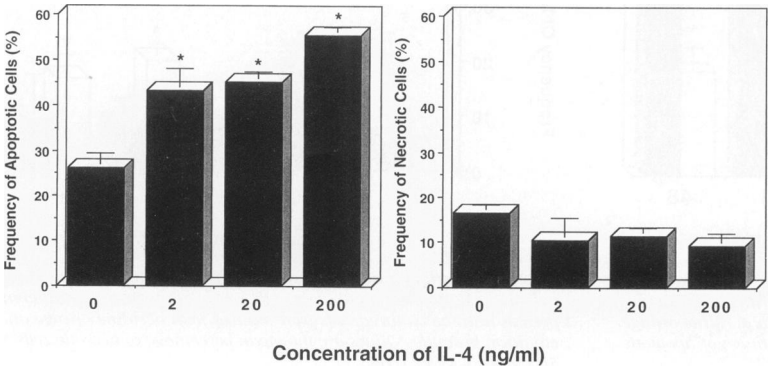

Figure 3. *Comparison of the frequency of IL-4-induced apoptotic or necrotic cells in inflamed gingival MØs. MØs were incubated with different concentrations of rIL-4 for 48 hours. After incubation, the cells were stained with acridine orange and ethidium bromide for the analysis of apoptotic and necrotic cells. Values are the mean percentage of apoptotic (or necrotic) cells ± SD (n = 3). *P < 0.01.*

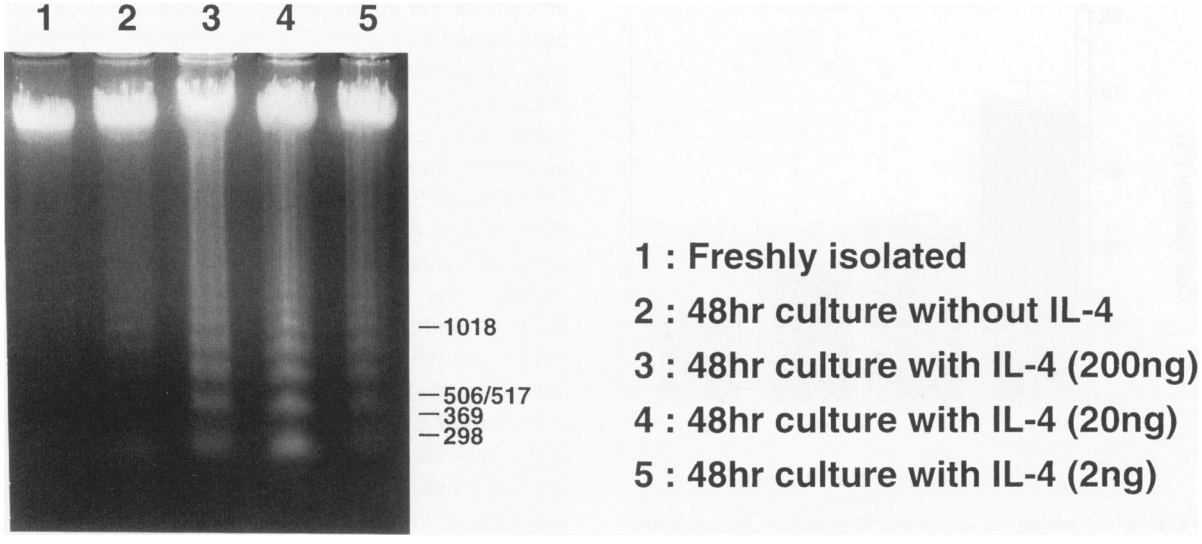

Figure 4. *Electrophoretic analysis of DNA isolated from inflamed gingival MØs. MØs were incubated with different concentrations of rIL-4 for 48 hours. After incubation, DNA was isolated from 2 × 10^6 cells and electrophoresed in a 1% agarose gel.*

centage of apoptotic cells consistently increased after 48 hours of incubation. Thus, the frequency of apoptotic cells reached 60 to 70% by 48 hours of incubation with rIL-4 (Figure 5). In contrast, the frequency of apoptotic cells did not change after 48 hours of incubation in cultures that did not contain rIL-4 (Figure 5). It was also important to note that the percentage of necrotic cells did not increase in IL-4-treated MØ cultures (Figure 6). However, a higher frequency of necrotic cells was observed in untreated cultures after 24 to 48 hours of incubation in comparison with cultures containing rIL-4 (Figure 6).

These results suggest that the effect of IL-4 for the induction of apoptosis in gingival MØs occurred as early as 6 hours, and a higher frequency of apoptotic cells was seen at 48 hours of incubation.

Discussion

A major characteristic of chronically inflamed lesions is the presence of MNs and especially activated MØs. MNs recruited from the blood circulation into these lesions mature into MØs upon exposure to various stimuli including cytokines or microorgan-

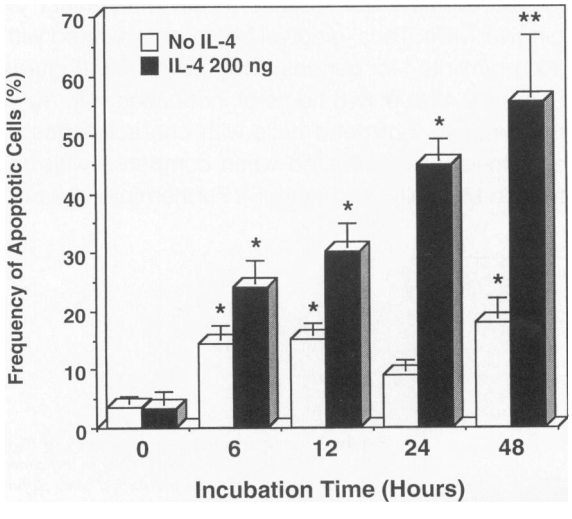

Figure 5. *Kinetics for development of apoptotic cells in inflamed gingival MØs. MØs were incubated with or without rIL-4 for different periods and, after culture, the cells were stained with acridine orange and ethidium bromide. Values are the mean percentage of apoptotic cells ± SD (n = 3). *P < 0.05, **P < 0.01.*

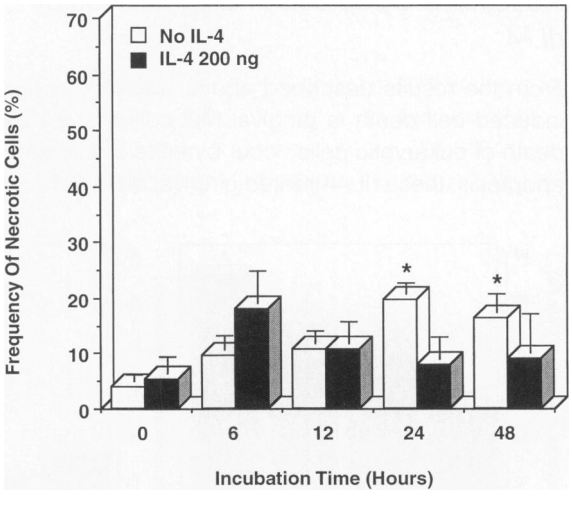

Figure 6. *Kinetics for development of necrotic cells in inflamed gingival MØs. MØs were incubated with or without rIL-4 for different periods and the cultured cells were stained with acridine orange and ethidium bromide. Values are the mean percentage of necrotic cells ± SD (n = 3). *P < 0.05.*

isms and their degradation products (eg, LPS).[24] Our present study provides new evidence that the resident MØs in inflamed gingiva, which are exposed to abundant microorganisms, express high levels of IL-4R (Figure 1). On the other hand, circulating MN/MØs in peripheral blood exhibit only low to undetectable levels of IL-4R. Although gingival MØs function in the removal of foreign products including pathogenic bacteria at the site of infection, the persistent occurrence of activated MØs also exacerbates the destruction of host tissues. As the inflamed gingival lesion (eg, AP) is characterized by damage of connective tissue and resorption of alveolar bone caused in part by activated MØs,[25] we have examined the molecular environment (eg, cytokines) that provides optimal conditions for the accumulation and survival of MØs in these disease sites.

Our previous studies have shown that GMCs isolated from inflamed gingiva of patients with AP expressed a unique profile of T helper (Th)1 and Th2 cytokine mRNA.[13] Thus, messages for IL-5 and IL-6 were noted, although mRNA for IL-2 or IL-4 was not detected by cytokine-specific dot-blot hybridization or by RT-PCR. Nevertheless, immunohistochemical analysis provided evidence for the occurrence of IL-4-producing cells.[26] It should be indicated that secreted IL-4 was not assessed in that study.[26] Furthermore, IL-4 appears to be lacking in other chronic inflammatory lesions such as rheumatoid synovitis.[27] These findings clearly suggest that a lack of IL-4 (or reduced IL-4 synthesis) occurs in the local disease site. As IL-4 has been shown to down-regulate MØs and MNs,[5–12] a lack of IL-4 may contribute to the persistent accumulation of MØs in chronically inflamed gingival tissue.

Our present findings clearly support a relationship between persistence of gingival IL-4R$^+$ MØs in the absence of IL-4. For example, incubation of MØs isolated from inflamed gingiva without any co-stimuli resulted in the maintenance of good cell viability whereas identically treated PBMC-derived MNs underwent cell death (Table 1). This finding supports studies by others that MØs isolated from the inflamed tissues are in an activated stage. For example, it was shown that MØs in the inflamed gingiva expressed high levels of major histocompatibility complex class II (eg, HLA-DR)[28] and are actively producing cytokines such as IL-1.[29] In addition, our present study demonstrated that gingival MØs isolated from inflamed tissues expressed a high message level for IL-4R (Figure 1). When these activated IL-4R$^+$ MØs were isolated from inflamed gingival tissues and then treated with rIL-4, the frequency of dead cells was increased (Figure 2). This was a result of the induction of apoptosis by rIL-4 as the number of apoptotic cells was increased in rIL-4-treated gingival MØ cultures (Figure 3). Furthermore, DNA fragmentation was also noted in these rIL-4-treated cells (Figure 4). These findings suggest that although MØs that reside in the local inflammatory lesion are expressing IL-4R and are sensitive to the effect of IL-4, the lack of this cytokine prevents the activated IL-4R$^+$ MØs from becoming apoptotic.

Our present study has provided the first evidence that MØs isolated from inflamed gingiva express significant mRNA for IL-4R. This finding would further support the concept that activated MØs in the local disease site express IL-4R and are responsive to IL-4 normally provided by adjacent T cells, ie, Th2-type cells. Nevertheless, although Th2-type cells producing IL-5 and IL-6 are present, these do not make IL-4 in inflamed gingival tissues. In this regard, our separate study showed that CD4$^+$ T cells isolated from inflamed gingival tissues did not express mRNA for IL-4.[30] However, other Th2-type cytokines such as IL-5, IL-6, and IL-13 were consistently found. In addition, strong message for IFN-γ was always detected in RNA isolated from gingival CD4$^+$ T cells. Others have also shown that IFN-γ message was expressed by GMCs from patients with AP.[31] As IFN-γ has been shown to up-regulate MØs,[32,33] the continuous production of this cytokine by Th1-type cells may create a suitable environment for accumulation of activated MØs in periodontal tissue inflammation. Furthermore, it has also been demonstrated that IFN-γ can inhibit MN/MØ cell death and DNA fragmentation.[4] Likewise, the absence of IL-4 at the local disease site could be an additional contributing factor for the continuous occurrence of MØs in inflamed disease. Taken together, a lack of IL-4 together with enhanced IFN-γ production by Th1-type gingival CD4$^+$ T cells may lead to destructive pathways through activation of MØs and release of soluble mediators.

Our present study demonstrated that IL-4 could induce programmed cell death in *ex vivo* MØ cultures from inflamed periodontal tissues. To this end, it was previously shown that IL-4 induced apoptosis in LPS- or IL-1-stimulated human MNs and MØs from peripheral blood.[4] In this regard, one can postulate that MNs recruited from the blood circulation to the disease site become activated IL-4R$^+$ MØs upon exposure to LPS from periodontal disease-associated microorganisms (eg, *Porphyromonas gingivalis*). Dysregulated Th1- and Th2-type cytokine synthesis, increased IFN-γ production, and lack of IL-4 synthesis create a pathological

environment for MØs. Thus, it is possible that exogenous local application of IL-4 might be beneficial for resolution of this diseased tissue. To this end, it has been shown that systemic administration of IL-4 inhibits development of inflammation in experimental arthritis.[34] As IL-4 appears to provide beneficial effects for prevention or reduction of pathology inflammation, one could consider treatments that reverse dysregulated Th1- to Th2-type cytokine (IL-4) responses by localized $CD4^+$ T cells. This would provide a novel treatment regimen to correct inflamed gingival tissues. Furthermore, it is equally feasible to consider the topical application of rIL-4 to the diseased tissue sites.

Acknowledgments

The authors express their thanks to Drs. James E. Roberts, Alvin W. Stevens, and Donald W. Thompson for providing gingival tissues from their periodontal surgery and Ms. Sheila D. Shaw for her excellent secretarial help in the preparation of the manuscript.

References

1. Kerr JFR, Wyllie AH, Currie AR: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972, 26:239–257
2. Mangan DF, Wahl SM: Differential regulation of human monocyte programmed cell death (apoptosis) by chemotactic factors and pro-inflammatory cytokines. J Immunol 1991, 147:3408–3412
3. Mangan DF, Welch GR, Wahl SM: Lipopolysaccharide, tumor necrosis factor-α, and IL-1β prevent programmed cell death (apoptosis) in human peripheral blood monocytes. J Immunol 1991, 146:1541–1546
4. Mangan DF, Robertson B, Wahl SM: IL-4 enhances programmed cell death (apoptosis) in stimulated human monocytes. J Immunol 1992, 148:1812–1816
5. te Velde AA, Klomp JPG, Yard BA, de Vries JE, Figdor CG: Modulation of phenotypic and functional properties of human peripheral blood monocytes by IL-4. J Immunol 1988, 140:1548–1554
6. Donnelly RP, Fenton MJ, Finbloom DS, Gerrard TL: Differential regulation of IL-1 production in human monocytes by IFN-γ and IL-4. J Immunol 1990, 145: 569–575
7. Essner R, Rhoades K, McBride WH, Morton DL, Economou JS: IL-4 down-regulates IL-1 and TNF gene expression in human monocytes. J Immunol 1989, 142: 3857–3861
8. Hart PH, Vitti GF, Burgess DR, Whitty GA, Piccoli DS, Hamilton JA: Potential antiinflammatory effects of interleukin 4: suppression of human monocytes tumor necrosis factor α, interleukin 1, and prostaglandin E2. Proc Natl Acad Sci USA 1989, 86:3803–3807
9. te Velde AA, Hujibens RJF, Heije K, de Vries JE, Figdor CG: Interleukin-4 (IL-4) inhibits secretion of IL-1β, tumor necrosis factor α, and IL-6 by human monocytes. Blood 1990, 76:1392–1397
10. Lee JD, Swisher SG, Minehart EH, McBride WH, Economou JS: Interleukin-4 down-regulates interleukin-6 production in human peripheral blood mononuclear cells. J Leukocyte Biol 1990, 47:475–479
11. Standiford TJ, Strieter RM, Chensue SW, Westwick J, Kasahara K, Kunkel SL: IL-4 inhibits the expression of IL-8 from stimulated human monocytes. J Immunol 1990, 145:1435–1439
12. Miossec P, Briolay J, Dechaniet J, Wijdenes J, Martinez-Valdez H, Banchereau J: Inhibition of the production of proinflammatory cytokines and immunoglobulins by interleukin-4 in an *ex vivo* model of rheumatoid synovitis. Arthritis Rheum 1992, 35:874–883
13. Fujihashi K, Beagley KW, Kono Y, Aicher WK, Yamamoto M, DiFabio S, Xu-Amano J, McGhee JR, Kiyono H: Gingival mononuclear cells from chronic inflammatory periodontal tissues produce interleukin (IL)-5 and IL-6 but not IL-2 and IL-4. Am J Pathol 1993, 142:1239–1250
14. Zappa U, Reinking-Zappa M, Graf H, Espeland M: Cell populations and episodic periodontal attachment loss in humans. J Clin Periodontol 1991, 18:508–515
15. McGhee ML, Ogawa T, Pitts AM, Moldoveanu Z, Mestecky J, McGhee JR, Kiyono H: Cellular analysis of functional mononuclear cells from chronically inflamed gingiva tissue. Reg Immunol 1989, 2:103–110
16. Ogawa T, Tarkowski A, McGhee ML, Moldoveanu Z, Mestecky J, Hirsch HZ, Koopman WJ, Hamada S, McGhee JR, Kiyono H: Analysis of Human IgG and IgA subclass antibody-secreting cells from localized chronic inflammatory tissue. J Immunol 1989, 142: 1150–1158
17. Kono Y, Beagley KW, Fujihashi K, McGhee JR, Taga T, Hirano T, Kishimoto T, Kiyono H: Cytokine regulation of localized inflammation: induction of activated B cells and IL-6 mediated polyclonal IgG and IgA synthesis in inflamed human gingiva. J Immunol 1991, 146:1812–1821
18. Yamamoto M, Fujihashi K, Beagley KW, McGhee JR, Kiyono H: Cytokine synthesis by intestinal intraepithelial lymphocytes: both γ/δ T cell receptor-positive and α/β T cell receptor-positive T cells in the G_1 phase of cell cycle produce IFN-γ and IL-5. J Immunol 1993, 150:106–114
19. Srinivasan K, Girard JE, Williams P, Roby RK, Weedn VW, Morris SC, Kline MC, Reeder DJ: Electrophoretic separations of polymerase chain reaction-amplified DNA fragments in DNA typing using a capillary electrophoresis-laser induced fluorescence system. J Chromatogr 1993, 652:83–91

20. Lu W, Han D-S, Yuan J, Andrieu J-M: Multi-target PCR analysis by capillary electrophoresis and laser-induced fluorescence. Nature 1994, 368:269–271
21. Kelley JL, Rozek MM, Suenram CA, Schwartz CJ: Activation of human blood monocytes by adherence to tissue culture plastic surfaces. Exp Mol Pathol 1987, 46:266–278
22. Duke RC, Cohen JJ: Morphological assays of apoptosis. Current Protocols in Immunology, vol 1. Edited by JE Coligan, AM Kruisbeek, DH Margulies, EM Shevach, W Strober. New York, 1992, pp 3.17.1–3.17.3
23. Yamamoto M, Fujihashi K, Amano M, McGhee JR, Beagley KW, Kiyono H: Cytokine synthesis and apoptosis by intestinal intraepithelial lymphocytes: signaling of high density $\alpha\beta$ T cell receptor$^+$ and $\gamma\delta$ T cell receptor$^+$ T cells via T cell receptor-CD3 complex resulted in interferon-γ and interleukin-5 production, while low density T cells undergo DNA fragmentation. Eur J Immunol 1994, 24:1301–1306
24. Adams DO, Hamilton TA: The cell biology of macrophage activation. Annu Rev Immunol 1984, 2:283–318
25. Williams RC: Periodontal diseases. N Engl J Med 1990, 322:373–382
26. Yamazaki K, Nakajima T, Gemmell E, Polak B, Seymour GJ, Hara K: IL-4 and IL-6 producing cells in human periodontal tissue. J Oral Pathol Med 1994, 23:347–353
27. Miossec P, Naviliat M, D' Angeac AD, Sany J, Banchereau J: Low levels of interleukin-4 and high levels of transforming growth factor β in rheumatoid synovitis. Arthritis Rheum 1990, 33:1180–1187
28. Marie J, Christine B, Martin G, Faure G: Immunohistological identification of cell subsets in human gingiva after local treatment for gingivitis or periodontitis. J Clin Periodontol 1986, 13:223–227
29. Matsuki Y, Yamamoto T, Hara K: Interleukin-1 mRNA-expressing macrophages in human chronically inflamed gingival tissues. Am J Pathol 1991, 138:1299–1305
30. Fujihashi K, Yamamoto M, Hiroi T, Bamberg TV, McGhee JR, Kiyono H: Selected Th1 and Th2 cytokine mRNA expression by CD4$^+$ T cells isolated from inflamed human gingival tissues. Clin Exp Immunol 1996 (in press)
31. Taubman MA, Eastcott JW, Shimauchi H, Takeichi O, Smith DJ: Modulatory role of T lymphocytes in periodontal inflammation. Molecular Pathogenesis of Periodontal Diseases. Edited by R Genco, S Hamada, T Lehner, JR McGhee, S Mergenhagen. Washington DC, 1994, pp 147–157
32. Sztein MB, Steeg PS, Johnson HM, Oppenheim JJ: Regulation of human peripheral blood monocyte DR antigen expression *in vitro* by lymphokines and recombinant interferon. J Clin Invest 1984, 73:556–565
33. Fischer DG, Golightly MG, Koren HS: Potentiation of the cytolytic activity of peripheral blood monocytes by lymphokines and interferon. J Immunol 1983, 130: 1220–1225
34. Allen JB, Wang HL, Cost GL, Bienkowski M, Wahl SM: Suppression of monocyte function and differential regulation of IL-1 and IL-1ra by IL-4 contribute to resolution of experimental arthritis. J Immunol 1983, 151:4344–4351