Oral Microbiology Immunology 2008: 23: 291–298
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY
AND IMMUNOLOGY

Profiling of inflammatory cytokines produced by gingival fibroblasts after human cytomegalovirus infection

J. E. Botero[1,2], A. Contreras[1,2], B. Parra[2,3]

[1]Periodontics Department, School of Dentristry, [2]Periodontal Medicine Group, School of Dentistry, [3]Viral Emerging Diseases Research Group, School of Basic Sciences, Universidad del Valle, Cali, Colombia

Botero JE, Contreras A, Parra B. Profiling of inflammatory cytokines produced by gingival fibroblasts after human cytomegalovirus infection.
Oral Microbiol Immunol 2008: 23: 291–298 © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard.

Introduction: The purpose of this study was to determine the profile of inflammatory cytokines that are produced after *in vitro* infection of gingival fibroblasts with human cytomegalovirus (HCMV).
Materials and methods: Gingival fibroblasts were infected with the Towne strain of HCMV and the cytokine profile in the supernatant was studied using a human inflammation antibody array. Expression of messenger RNA (mRNA) using reverse transcription–polymerase chain reaction for interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) was also analyzed in infected gingival fibroblasts and gingival specimens from subjects with and without periodontitis according to HCMV detection. HCMV was determined in subgingival samples by nested polymerase chain reaction.
Results: Gingival fibroblasts produced mainly IL-1α, IL-12p40, IL-12p70, IL-6, TNF-α, and IL-1β after HCMV infection. Expression of mRNA for IL-1β and TNF-α was increased after HCMV infection. Production of IL-1β and TNF-α was increased in HCMV-positive periodontitis specimens. In addition, infected gingival fibroblasts produced more IL-8, monocyte chemoattractant protein 1, macrophage inflammatory proteins 1α, and 1β over time postinfection in comparison to baseline. The lowest production of all cytokines studied corresponded to IL-2, IL-4, IL-13, and interferon-γ. A decreasing production pattern was observed for granulocyte–macrophage colony-stimulating factor, IL-7, and IL-17 while IL-11 and macrophage colony-stimulating factor were increased at 72 h postinfection.
Conclusions: HCMV infection in gingival fibroblasts upregulated the production of proinflammatory-related cytokines and chemokines. The expression of IL-1β and TNF-α was increased both *in vitro* and in specimens from HCMV-positive subjects with periodontitis. The overproduction of proinflammatory cytokines and chemokines as a result of viral infection should be considered an important pathogenic mechanism linking HCMV to periodontitis *in vivo*.

Key words: cytokines; gingival fibroblasts; human cytomegalovirus; periodontitis

Javier E. Botero, Calle 3 # 36-00 San Fernando, School of Dentistry, Universidad del Valle, Cali, Colombia
Tel./fax: (57) 2 558 1936;
e-mail: drjavo@yahoo.com
Accepted for publication November 6, 2007

Gingival connective tissue represents a complex environment where different cell types occur (23, 25, 27). After the permanent antigenic challenge induced by the bacterial biofilm, cells that are not obligatory residents start to infiltrate the tissue. The result is a diverse repertoire of cells including macrophages, neutrophils, T cells, and B cells (46, 55). The number of infiltrating cells depends on the degree of antigenic challenge. Before cells of the acquired immune response can be activated and become established in the tissues, cells such as epithelial and connective tissue cells are stimulated to produce the signals necessary to activate the immune response (7, 19, 22, 32, 43, 50). The main connective tissue cell of the gingiva is the gingival fibroblast, a cell that is not only capable of synthesizing connective tissue matrix components but

also able to respond to bacterial antigens and other stimuli like cytokines (38, 44). Studies have determined that the gingival fibroblasts express Toll-like receptors and CD14, which confirms their capacity to respond to lipopolysaccharide (3, 31, 40, 51, 53). Other studies have found that they express receptors for innate cytokines like interleukin-1α (IL-1α), IL-1β, and tumor necrosis factor-α (TNF-α) (11, 34, 35, 48). Moreover, the gingival fibroblasts play an important role in inflammatory responses in the periodontal connective tissue because they also produce cytokines (5, 24). Many of the immune processes implicated in the periodontal bone and connective tissue loss observed in periodontitis could be, in part, regulated by gingival fibroblasts.

As discussed above, gingival fibroblasts respond to bacterial challenge and to stimulus from the host by producing cytokines. As investigations have advanced, some herpes viruses have been pointed out as potential pathogens in periodontal disease. The human cytomegalovirus (HCMV) is perhaps the most important virus implicated (42). Studies have found that infection with CMV (human and murine) results in cytokine production by microglias and macrophages (9, 39). Considering that the gingival fibroblast is a susceptible cell for infection with HCMV, this study determined the profile of the inflammatory cytokines produced after *in vitro* infection of gingival fibroblasts with HCMV.

Materials and methods

The study protocol was revised and approved by the Institutional Review Board for Human Research of the Universidad del Valle (Cali, Colombia) according to the Helsinki Declaration of 1975, as revised in 2000. When human gingival biopsies were used, previous authorization and signature of a written informed consent was obtained from patients with periodontitis referred to the dental clinics of the School of Dentistry at the Universidad del Valle between January 2006 and April 2007.

Gingival fibroblasts

Gingival fibroblasts were obtained from connective tissue from a periodontally healthy donor during a crown-lengthening procedure. A 3-mm sample of connective tissue was cut into smaller pieces using a scalpel blade and immediately placed in a 25-cm^2 culture flask (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) with 3 ml α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS). The flask was incubated at 37°C in 5% CO_2. After 1 week, the medium was replaced with 5 ml fresh medium and the culture flask was observed daily for cell growth around the tissue. When fibroblasts were growing out of the connective tissue, the pieces of connective tissue were carefully removed from the flask and incubated until cell confluency was reached. Medium was replaced every 3 days during this time. When the cells reached 90% confluency, they were detached by trypsin treatment (0.25%), washed with α-MEM supplemented with 10% FBS, and split apart into 25 cm^2 culture flasks. Gingival fibroblasts were cultured by repeated passages and every 2 weeks, a stock of gingival fibroblasts was frozen in liquid nitrogen for future experiments. Cells between passages 10 and 13 were used in the experiments.

Virus

HCMV Towne strain was used in all *in vitro* experiments. To produce a stock of virus, gingival fibroblasts grown in a 25-cm^2 culture flask were infected with a dilution of 10^{-6} of the original virus and incubated with α-MEM supplemented with 10% FBS at 37°C and 5% CO_2. Supernatant was replaced with fresh medium once a week and the infected cells were maintained until a 100% cytopathic effect was observed (approximately 2 weeks). At this time, supernatant was collected and stored at −70°C as the viral stock to be used in all the experiments. Cells were detached by trypsin treatment, washed with α-MEM supplemented with 10% FBS, and stored in liquid nitrogen for future experiments.

To determine the concentration of virus in the stock, three aliquots of the supernatant were quantified by real-time polymerase chain reaction (PCR) using a commercial kit (LC CMV kit, Roche LightCycler version 2.0; Roche Diagnostics, Indianapolis, IN). The final viral stock contained 710,000 copies of HCMV/μl and was used for the experiments.

In vitro infection of gingival fibroblasts

Gingival fibroblasts (100,000 cells/ml) were seeded in six-well plates (Falcon, Becton Dickinson Labware) and maintained in α-MEM supplemented with 10% FBS, at 37°C and 5% CO_2 until 100% confluency was observed. At this point, medium was replaced with α-MEM supplemented with 2% FBS and the plates were incubated for 24 h; medium was then changed for fresh α-MEM supplemented with 2% FBS and 50 μg/ml ascorbic acid (Sigma, St Louis, MO) and the plates were incubated for another 24 h (baseline).

For infection, 10-fold serial dilutions of the viral stock were prepared in α-MEM. Supernatants were removed from the cell culture plates and 500 μl of each virus dilution containing either 35.5×10^5 (71,000 copies/μl), 35.5×10^4 (7100 copies/μl), 35.5×10^3 (710 copies/μl), or 35.5×10^2 (71 copies/μl) viral copies were added per well. The final density of gingival fibroblasts before infection was 750,000 cells. Plates were incubated at 37°C in 5% CO_2 for virus adsorption for 1 h and then 2.5 ml α-MEM supplemented with 2% FBS was added to each well and the plates were incubated for 12, 24, and 72 h. As a non-infected control, gingival fibroblasts were detached and collected at baseline and total RNA was purified immediately and used for messenger RNA (mRNA) analysis. Supernatant was collected at the indicated times and used for cytokine profiling. An additional control included cells that were inoculated with ultraviolet (UV)-inactivated HCMV (71,000 copies/μl, 35.5×10^5). For UV inactivation, 2 ml of the viral dilution was exposed to UV light (there was a 3 cm distance between the UV lamp and the virus) in a 10-mm sterile plate dish, for 20 min. The inactivated virus was then used to infect gingival fibroblasts, which were incubated for 72 h. Exposure to UV light affects the infectivity of the virus. Viral infection was verified by immunofluorescence assay of infected cells (anti-HCMV pp72) (52) at the various experimental time-points (0, 12, 24, and 72 h postinfection). All experiments were performed three times.

Cytokine profiling

To determine the profile of cytokine production from gingival fibroblasts infected with HCMV, a human inflammation antibody array was used (RayBiotech, Norcross, GA) according to the instruction manual. Supernatant from cultured gingival fibroblasts was collected at baseline (0 h, non-infected gingival fibroblasts) and at 24 and 72 h postinfection with 71,000 copies of HCMV/μl (explained above). The list of cytokines studied using this technique is presented in Table 1. The principle of the method comprised a membrane that was coated with specific antibodies for each cytokine forming an array. After blocking the membrane, the

Table 1. List of human inflammatory cytokines studied by antibody array (RayBiotech)

Cytokine	Name	Cytokine group
G-CSF	Granulocyte colony-stimulating factor	Growth factors
GM-CSF	Granulocyte–macrophage colony-stimulating factor	Growth factors
ICAM1	Intercellular adhesion molecule 1	Integrins
IFN-γ	Interferon-γ	Acquired
I-309	Immune-associated nucleotide	Chemokine
IL-1α	Interleukin-1α	Innate
IL-1β	Interleukin-1β	Innate
IL-2	Interleukin-2	Acquired
IL-3	Interleukin-3	Growth factors
IL-4	Interleukin-4	Acquired
IL-6	Interleukin-6	Innate
IL-6sR	Interleukin-6 soluble receptor	Innate
IL-7	Interleukin-7	Growth factors
IL-8	Interleukin-8	Chemokine
IL-10	Interleukin-10	Acquired
IL-11	Interleukin-11	Growth factors
IL-12p40	Interleukin-12 subunit 40	Innate
IL-12p70	Interleukin-12 subunit 70	Innate
IL-13	Interleukin-13	Acquired
IL-15	Interleukin-15	Innate
IL-16	Interleukin-16	Chemokine
IL-17	Interleukin-17	Growth factors
IP-10	Interferon-inducible protein	Chemokine
MCP-1	Monocyte chemoattractant protein-1	Chemokine
MCP-2	Monocyte chemoattractant protein-2	Chemokine
M-CSF	Macrophage colony-stimulating factor	Growth factors
MIG	Monokine induced by interferon-γ	Chemokine
MIP-1α	Macrophage inflammatory protein-1α	Chemokine
MIP-1β	Macrophage inflammatory protein-1β	Chemokine
MIP-1δ	Macrophage inflammatory protein-1δ	Chemokine
RANTES	Regulated on activated normal T-cell expressed and secreted	Chemokine
TGF-β1	Transforming growth factor-β1	Acquired
TNF-α	Tumor necrosis factor-α	Innate
TNF-β	Tumor necrosis factor-β	Innate
sTNF-RI	Tumor necrosis factor soluble receptor I	Innate
sTNF-RII	Tumor necrosis factor soluble receptor II	Innate
PDGF-BB	Platelet-derived growth factor	Growth factors
TIMP2	Tissue inhibitor of metalloproteinases 2	Anticollagenase

sample was added and incubated at room temperature. Cytokine detection was completed by incubation with a biotinylated antibody followed by horseradish peroxidase-conjugated streptavidin. Signals were detected by exposing the membrane to an autoradiography film for 10 s (KODAK X-Omat LS Film; Kodak, Rochester, NY). The exposed film was developed in an automated radiography film processor (Gendex, Lake Zurich, IL). Developed membranes were scanned and the intensity of the spot signals was analyzed using an open-source public software according to the instruction manual (ImageJ; National Institutes of Health, Bethesda, MD).

Total RNA purification

Gingival fibroblasts were detached at the indicated times: 0, 12, 24, and 72 h postinfection and total RNA was immediately isolated from the cell pellet. A commercially available kit for RNA purification was used according to the manufacturer's instruction protocol (QIAmp RNA blood mini kit; Qiagen, Valencia, CA). To avoid DNA carryover, DNase treatment was included in the protocol following the instructions of the manufacturer (RNase-free DNase set; Qiagen, Valencia, CA). Total RNA was quantified using a fluorometer (Qubit fluorometer; Invitrogen Corp., Carlsbad, CA, USA) and an RNA quantification kit (Quant-iT RNA Assay kit, Invitrogen Corp.). RNA was stored at −70°C for reverse transcription–PCR (RT-PCR).

RT-PCR

To determine the levels of mRNA expression of IL-1β and TNF-α in HCMV-infected fibroblasts, semi-quantitative RT-PCR was performed. Primer sequence and amplification temperature are depicted in Table 1. Primer sequences (Table 2) were used from previous publications (16, 54) based on GenBank accessions spanning exon–exon conjunctions and were non-reactive to genomic DNA.

Complementary DNA (cDNA) was synthesized using 3 ng total RNA through a reverse transcription reaction. The first strand was synthesized using 1 μl oligo(dT) primer (Promega, Madison, WI), 2.75 μl Diethylene Pyrocarbonate (DEPC) water, and 3 ng total RNA and was incubated at 72°C for 5 min and immediately chilled on ice. A reverse transcription master mix was prepared using 4 μl buffer 5×, 1 μl 10 mM dNTPs, 0.25 μl RNasin, and 1 μl AMV reverse transcriptase (all Promega, Madison, WI). After primer annealing, 6.25 μl of the master mix was added to each vial and programmed in a thermocycler (Peltier Thermal cycler, MJ Research, BioRad, Hercules, CA) at 42°C for 1 h and 95°C for 5 min. After reverse transcription, each cDNA was adjusted to 100 μl using DEPC-treated water and stored at −20°C for PCR analysis.

A 45-μl PCR mix per tube was prepared as follows: 34.75 μl PCR grade water, 5 μl buffer 10× (Promega), 2.5 μl of 2.5 mM dNTPs (Promega), 0.5 μl primer mix, 2 μl of 25 mM $MgCl_2$ (1.0 mM), 0.25 μl of 5 U/μl *Taq* polymerase (Promega), and 5 μl cDNA. $MgCl_2$ concentration was optimized for each PCR product and varied as shown in Table 1. Standard PCR conditions were set to: 95°C for 1 min, 35 cycles corresponding to 94°C for 45 s, 58°C for 45 s (annealing temperature varied according to product, Table 1), and 72°C for 1 min; a final step of 72°C for 5 min.

Amplification products were electrophoresed in 2% agarose gel stained with ethidium bromide, and were observed and photographed in a UV transilluminator. PCR product band intensity was analyzed using the ImageJ software.

Table 2. Primer sequence and polymerase chain reaction amplification conditions for interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α)

Product/cytokine	Primer sequence 5′–3′	Amplicon size (bp)	Annealing temp. (°C)	$MgCl_2$ (mM)
IL-1β	F-ATAAGCCCACTCTACAGCT R-ATTGGCCCTGAAAGGAGAGA	443	50	1.5
TNF-α	F-TCGGGCCAATGCCCTCCTGGCCAA R-GTAGACCTGCCCAGACTCGGCAAA	468	50	1.5
GAPDH	F-CGTCTTCACCACCATGGAGA R-CGGCCATCACGCCACAGTTT	220	58	2

Gingival specimens

To measure the expression level of mRNA expression of IL-1β and TNF-α in healthy and diseased periodontal tissues, RT-PCR was performed in gingival biopsies from periodontitis patients and healthy donors. Determination of HCMV in subgingival samples from each patient was performed according to Parra and Slots (37) and Contreras and Slots (12).

In total, gingival biopsies were obtained at baseline during clinical examination from 14 patients with periodontitis (HCMV-positive = 10, HCMV-negative = 4) and three non-periodontitis patients (HCMV-negative = 3). Subject and clinical information for gingival specimens were as follows: for HCMV-positive samples: mean age 40.3 years, probing depth 7.26 mm, clinical attachment level 7.50 mm; HCMV-negative samples: mean age 43.3 years, probing depth 7.06 mm, clinical attachment level 7.01 mm; and non-periodontitis samples: mean age 30.8 years, probing depth 2.03 mm, clinical attachment level 1.15 mm.

Biopsies from periodontitis patients included a 3-mm bevelled incision in the palatal side of the maxillary teeth at a location that was intensively inflamed and had pocket depth >5 mm. Specimens from non-periodontitis subjects, were collected during mandatory periodontal surgery for prosthetic reasons. The difference from periodontitis patients was that gingival tissues had no apparent signs of inflammation and no periodontitis was diagnosed. All specimens were collected in microcentrifuge vials and immediately flash frozen in liquid nitrogen and before being stored at –70°C for analysis.

Total RNA was purified using a commercially available kit (RNeasy Fibrous Tissue Mini kit; Qiagen). To avoid DNA carryover, DNase treatment was included in the protocol and the instructions of the manufacturer were followed (RNase-Free DNase set, Qiagen). Total RNA was quantified, stored and analyzed as explained above.

Data analysis

The signal intensity of each spot was adjusted to the corresponding internal control provided in each membrane by the manufacturer (cytokine profiling). The ratio of expression was calculated by dividing the signal intensity of each cytokine by the signal intensity of the internal control of each sample. Signal intensity was analyzed using the IMAGEJ software. The final value was expressed as the ratio of expression (y axis) ± SD from two experiments. Data was analyzed with the Kruskall–Wallis and Mann–Whitney tests ($P < 0.05$).

Band intensity of each PCR product for IL-1β and TNF-α was analyzed using the IMAGEJ software. Values were adjusted relative to the expression of GAPDH in the same sample and were expressed as the ratio of expression. Mean ratio of expression (±SD) was calculated from three experiments. Differences between infection times and HCMV concentration were assessed with the Kruskall–Wallis and Mann–Whitney tests ($P < 0.05$). Differences for the expression of the mRNA of IL-1β and TNF-α between groups (gingival biopsies) were evaluated with Mann–Whitney test ($P < 0.05$).

Data were analyzed using statistical software (STATA, statistical software for WINDOWS STATACORP LP, College station, TX, USA).

Results

Gingival fibroblasts were infected with HCMV at a concentration of 71,000 copies/μl and supernatant was collected at 24 and 72 h postinfection. Supernatant collected at baseline from uninfected gingival fibroblasts was considered as the starting point for comparisons with time postinfection. Cytokines were split into four groups (Table 1) and the results are presented accordingly. The mRNA expression for IL-1β and TNF-α from infected gingival fibroblasts and gingival specimens was evaluated.

Effect of HCMV infection on innate cytokines production by gingival fibroblasts

An increase in the production of IL-1α, IL-12p40, IL-12p70, and IL-6 (Fig. 1) was observed at 72 h postinfection. A threefold increase of the above mentioned cytokines was observed in infected gingival fibroblasts as compared to baseline, uninfected cells. Levels of IL-1β were not as high as levels of other proinflammatory cytokines and IL-1β was only detected 24 h postinfection with a maximum increase at 72 h. In contrast, TNF-α was high at baseline and had increased further 72 h postinfection (Fig. 1).

The expression of mRNA for IL-1β and TNF-α was analyzed in infected gingival fibroblasts (Figs 2, 3) and in gingival specimens from patients with periodontitis (Fig. 4). Gene expression for IL-1β increased over time and was higher at 72 h ($P \leq 0.05$) while expression of the TNF-α gene peaked at 24 h (Fig. 2). Nonetheless, expression of IL-1β and TNF-α ($P \leq 0.05$) was higher than baseline, uninfected gingival fibroblasts, and UV-inactivated HCMV-infected gingival fibroblasts at

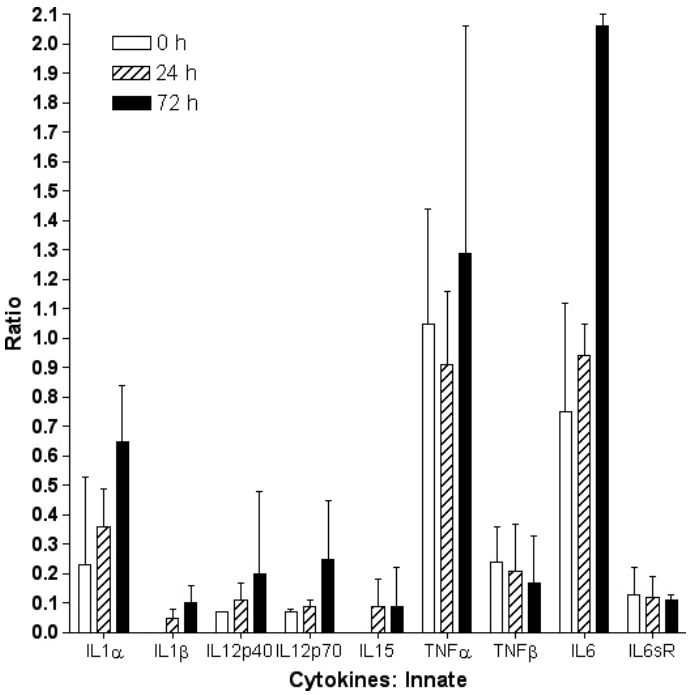

Fig. 1. Innate cytokine production after 24–72 h HCMV infection. Bars represent the mean ratio ± SD from 2 experiments.

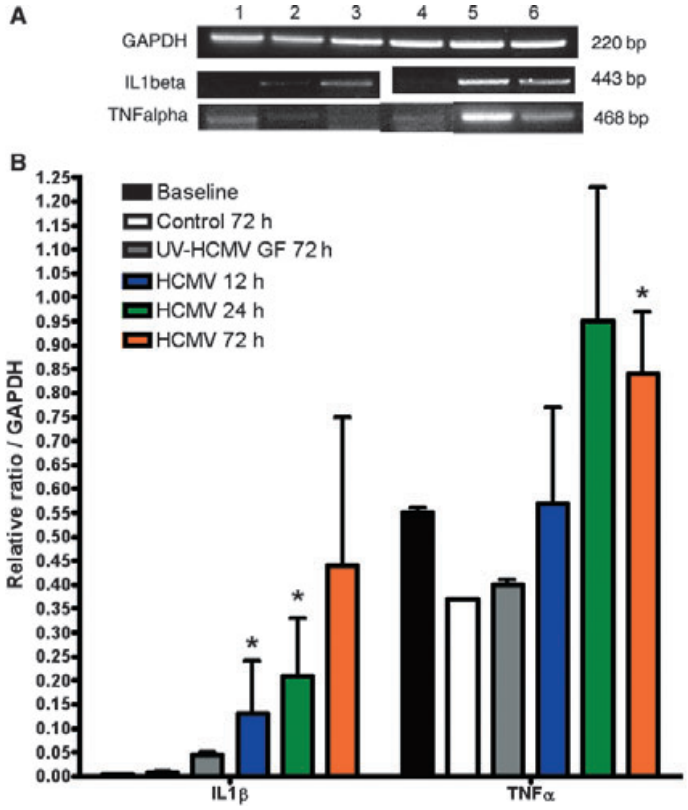

Fig. 2. mRNA expression of IL1β and TNFα after HCMV infection (71,000 c/μl). (A) PCR amplicons corresponding to GAPDH, IL1β and TNFα as follows: lane 1 Baseline, lane 2 Control non-infected GF 72 h, lane 3 UV-HCMV infected GF at 72 h, lane 4 HCMV infected GF at 12 h p.i, lane 5 HCMV infected GF at 24 h p.i. and lane 6 HCMV infected GF at 72 h p.i. (B) Relative ratio of expression of mRNA at the indicated time for IL1β and TNFα. Data is presented as the mean of three experiments ± SD. * Statistical differences as compared to baseline, control 72 h and UV-HCMV infected GF 72 h (Mann Whitney test $P \leq 0.05$).

72 h (Fig. 2). Uninfected and UV-inactivated HCMV-exposed gingival fibroblasts showed lower gene expression. Expression of mRNA for IL-1β was higher with increasing copy numbers of HCMV than with low copy numbers (Fig. 3). In contrast, expression of TNF-α was higher than expression of IL-1β even with a low number of HCMV copies, and different HCMV doses induced different expression levels of mRNA (Fig. 3). In addition, gene expression in gingival biopsies was higher for IL-1β and TNF-α in HCMV-positive periodontitis than HCMV-negative periodontitis samples and healthy tissues, but the difference was not statistically significant (Fig. 4).

Effect of HCMV infection on acquired cytokine production by gingival fibroblasts

The lowest production of all cytokines studied corresponded to IL-2, IL-4, IL-13, and interferon-γ (Fig. 5) The primary function of gingival fibroblasts is not the specialized production of cytokines, in contrast to CD4 T cells. However, upon infection with HCMV, the production of IL-2, IL-4, and IL-13 was increased at 24 h and was very low or not detectable at 72 h. In contrast, IL-10 production was higher at 72 h but the difference from baseline was minimal. The major change in production was observed for transforming growth factor-β1, this was increased at 24 h and although it had

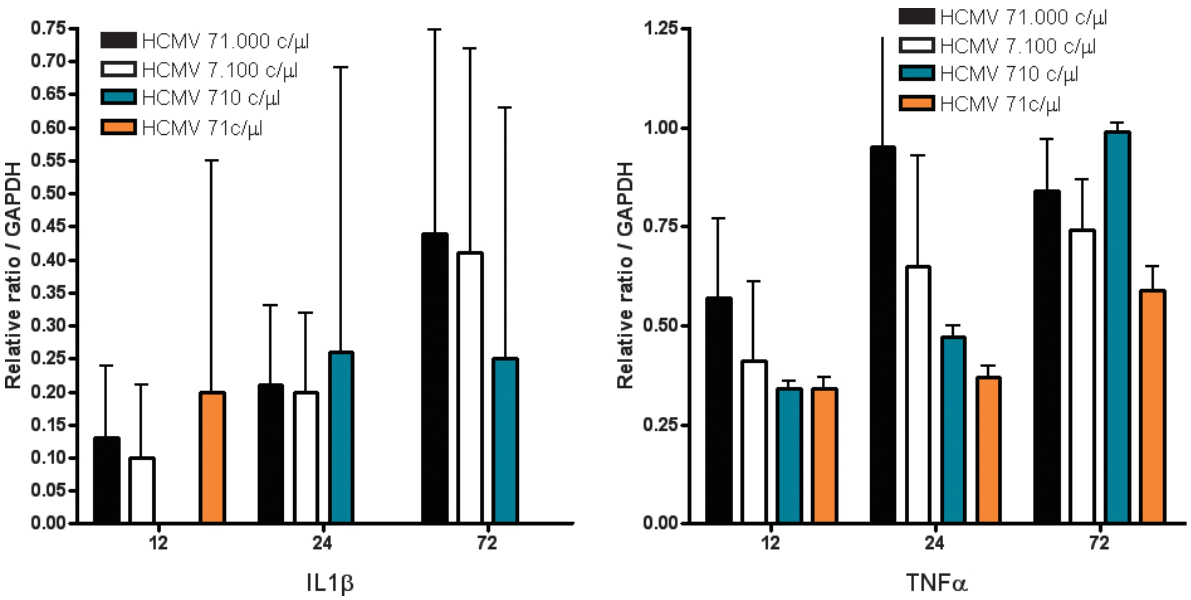

Fig. 3. Comparison of different HCMV infection doses on mRNA expression of and IL1β (A) and TNFα (B) at the indicated time. Data is presented as the mean of three experiments ± SD.

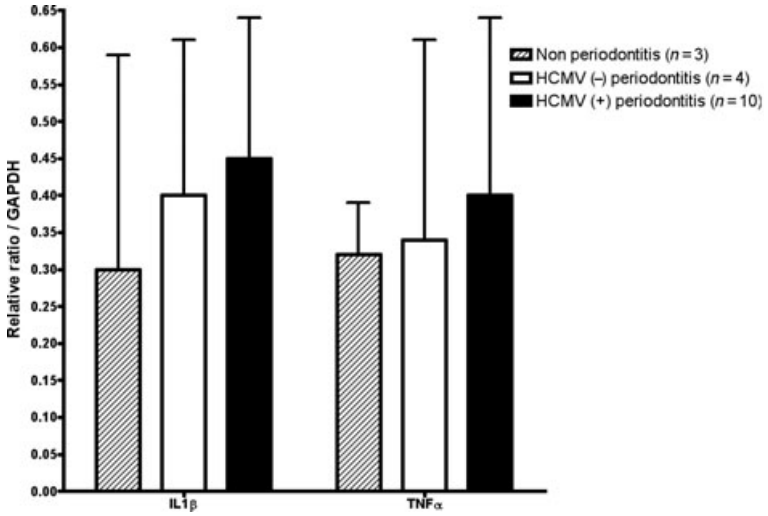

Fig. 4. Analysis of mRNA expression for IL1β and TNFα in gingival specimens from non-periodontitis, HCMV(-) periodontitis and HCMV(+) patients. Data is presented as the mean ± SD.

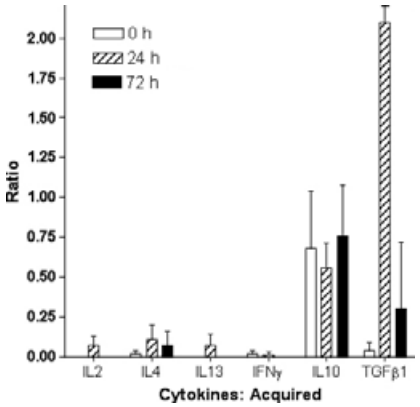

Fig. 5. Acquired cytokines production after 24–72 h HCMV infection. Bars represent the mean ratio ± SD from 2 experiments.

decreased at 72 h, it was still higher than baseline (Fig. 5).

Effect of HCMV infection on chemokines production by gingival fibroblasts

Overall, the production of IL-8, monocyte chemoattractant protein 1 (MCP1), macrophage inhibitory protein (MIP) 1α, and MIP-1β was progressive at 72 h in comparison to baseline (Fig. 6). The production of RANTES was increased at 24 h and although it had decreased by 72 h it was still higher than baseline.

Effect of HCMV infection on growth factor production by gingival fibroblasts

Production of growth factors was very irregular over time (Fig. 7). A decreasing pattern was observed for granulocyte–macrophage colony-stimulating factor, IL-7, and IL-17 while IL-11 and macrophage colony-stimulating factor were increased at 72 h postinfection.

Discussion

Gingival fibroblasts were infected with HCMV to study the production of cytokines after infection. It has been observed that growth factors respond to different stimuli, such as bacterial antigens and prostaglandin E2, to produce a wide range of cytokines (1, 8, 28, 49). It was interesting to find that infection of gingival fibroblasts with HCMV increases the production of different cytokines. Periodontitis is an inflammatory process that is primarily initiated by bacterial challenge and connective and bone tissue loss occurs after the host immune response is activated. Associations of representative cells like T cells and macrophages are key players in defining how much of the periodontal tissues are degraded. However, the role of other infectious agents, such as HCMV, is not clear; the possibility of HCMV-infected gingival fibroblasts in inflamed periodontal tissues could be plausible. This study presents the experimental infection results for a profile of human inflammatory cytokines. It was observed that gingival fibroblasts mainly produced innate proinflammatory cytokines and chemokines. The same observation has been observed for herpes simplex virus 1 (HSV-1) and murine CMV (18, 21, 30, 36). One study found that HSV induces IL-6 in gingival fibroblasts but not in keratinocytes (47). Overproduction within periodontal connective tissues of potent osteoclast activators, proinflammatory cytokines, and chemokines sets up the perfect microenvironment for periodontal destruction. IL-1β is considered a key cytokine in periodontal disease because of

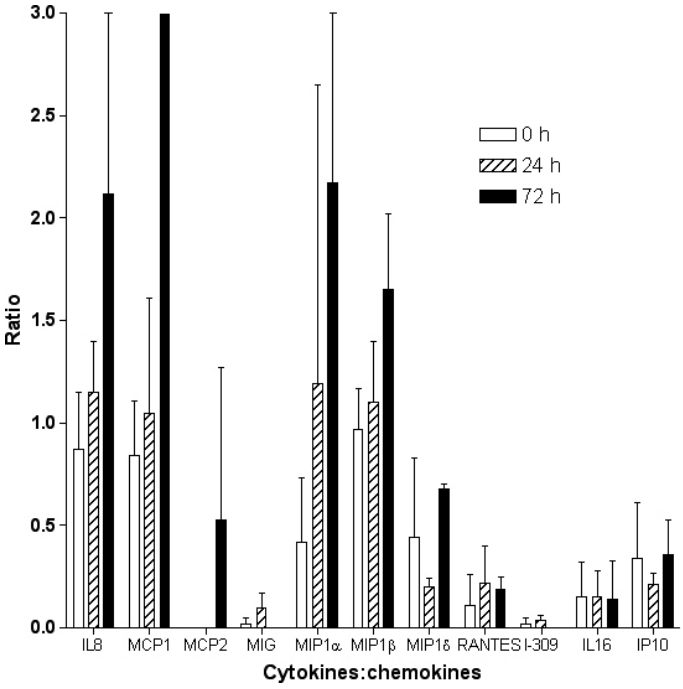

Fig. 6. Chemokine production after 24–72 h HCMV infection. Bars represent the mean ratio ± SD from 2 experiments.

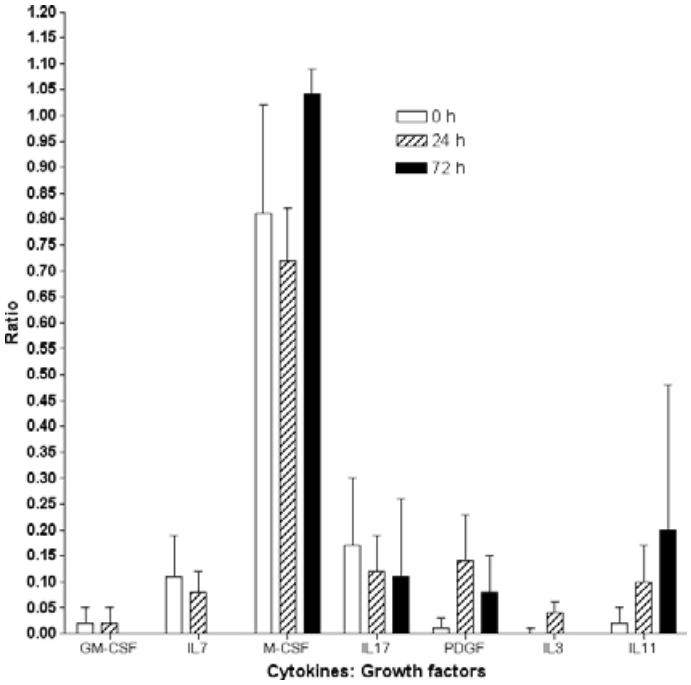

Fig. 7. Growth factors production after 24–72 h of HCMV infection. Bars represent the mean ratio ± SD from 2 experiments.

its potent inflammatory activity and osteoclast activation (10, 13, 26, 45). It has been observed in high quantities in gingival crevicular fluid from periodontitis lesions in comparison to healthy sites (4, 14, 20). TNF-α is another important cytokine involved in osteoclast differentiation (2, 6, 33) and regulating bone loss in periodontitis (17, 29, 53). The overproduction of these cytokines by gingival fibroblasts in inflamed periodontal tissues results in increased collagenolytic activity and proinflammatory action (15, 41).

The *in vitro* infection of gingival fibroblasts with HCMV resulted in increased production of inflammatory cytokines such as IL-1α, TNF-α, IL-6, IL-8, MCP-1, MIP, macrophage colony-stimulating factor, and IL-1β in comparison to non-infected gingival fibroblasts. Additionally, the increased expression of IL-1β and TNF-α in HCMV-positive periodontitis specimens is an important finding that contributes to an explanation of the relation of HCMV in periodontitis.

HCMV infection in gingival fibroblasts upregulated the production of proinflammatory related cytokines and chemokines. The expression of IL-1β and TNF-α was increased both *in vitro* and in specimens from subjects with HCMV-positive periodontitis. The overproduction of proinflammatory cytokines and chemokines as a result of viral infection should be considered as an important pathogenic mechanism linking HCMV to periodontitis *in vivo*.

Acknowledgments

This study was supported by a grant from COLCIENCIAS (Colombian institute for development of science and technology Francisco José de Caldas) and the Universidad del Valle (312-2004, 1106-05-16316). Javier E. Botero holds a scholarship from COLCIENCIAS. The HCMV Towne strain was a kind gift from Dr Mark Stinsky and Phil Lashmit at the University of Iowa (USA).

References

1. Almasri A, Wisithphrom K, Windsor LJ, Olson B. Nicotine and lipopolysaccharide affect cytokine expression from gingival fibroblasts. J Periodontol 2007: **78**: 533–541.
2. Asagiri M, Takayanagi H. The molecular understanding of osteoclast differentiation. Bone 2007: **40**: 251–264.
3. Bainbridge BW, Darveau RP. *Porphyromonas gingivalis* lipopolysaccharide: an unusual pattern recognition receptor ligand for the innate host defense system. Acta Odontol Scand 2001: **59**: 131–138.
4. Beklen A, Ainola M, Hukkanen M, Gürgan C, Sorsa T, Konttinen YT. MMPs, IL-1, and TNF are regulated by IL-17 in periodontitis. J Dent Res 2007: **86**: 347–351.
5. Belibasakis GN, Johansson A, Wang Y et al. Cytokine responses of human gingival fibroblasts to *Actinobacillus actinomycetemcomitans* cytolethal distending toxin. Cytokine 2005: **30**: 56–63.
6. Blair HC, Zaidi M. Osteoclastic differentiation and function regulated by old and new pathways. Rev Endocr Metab Disord 2006: **7**: 23–32.
7. Bosshardt DD, Lang NP. The junctional epithelium: from health to disease. J Dent Res 2005: **84**: 9–20.
8. Chae HJ, Byun JO, Chae SW et al. p38 MAPK and NF-kappaB on IL-6 release in human gingival fibroblasts. Immunopharmacol Immunotoxicol 2005: **27**: 631–646.
9. Cheeran MC, Hu S, Yager SL, Gekker G, Peterson PK, Lokensgard JR. Cytomegalovirus induces cytokine and chemokine production differentially in microglia and astrocytes: antiviral implications. J Neurovirol 2001: **7**: 135–147.
10. Chole RA, Tinling SP, Faddis BT. Human recombinant interleukin-1 receptor antagonist blocks bone resorption induced by interleukin-1 beta but not interleukin-1 alpha. Calcif Tissue Int 1994: **55**: 12–15.
11. Chou HH, Takashiba S, Maeda H et al. Induction of intracellular interleukin-1 beta signals via type II interleukin-1 receptor in human gingival fibroblasts. J Dent Res 2000: **79**: 1683–1688.
12. Contreras A, Slots J. Mammalian viruses in human periodontitis. Oral Microbiol Immunol 1996: **11**: 381–386.
13. Delima AJ, Oates T, Assuma R et al. Soluble antagonists to interleukin-1 (IL-1) and tumor necrosis factor (TNF) inhibits loss of tissue attachment in experimental periodontitis. J Clin Periodontol 2001: **28**: 233–240.
14. Duarte PM, de Oliveira MC, Tambeli CH, Parada CA, Casati MZ, Nociti FH. Overexpression of interleukin-1beta and interleukin-6 may play an important role in periodontal breakdown in type 2 diabetic patients. J Periodontal Res 2007: **42**: 377–381.
15. Ejeil AL, Gaultier F, Igondjo-Tchen S et al. Are cytokines linked to collagen breakdown during periodontal disease progression? J Periodontol 2003: **74**: 196–201.
16. Garlet GP, Martins W, Fonseca BA, Ferreira BR, Silva JS. Matrix metalloproteinases, their physiological inhibitors and osteoclast factors are differentially regulated by the cytokine profile in human periodontal disease. J Clin Periodontol 2004: **31**: 671–679.
17. Graves DT, Cochran D. The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. J Periodontol 2003: **74**: 391–401.
18. Haagmans BL, van den Eertwegh AJ, Claassen E, Horzinek MC, Schijns VE. Tumour necrosis factor-alpha production during cytomegalovirus infection in immunosuppressed rats. J Gen Virol 1994: **75**: 779–787.
19. Hillmann G, Hillmann B, Geurtsen W. Immunohistological determination of interleukin-1 beta in inflamed human gingival epithelium. Arch Oral Biol 1995: **40**: 353–359.
20. Holmlund A, Hänström L, Lerner UH. Bone resorbing activity and cytokine levels in gingival crevicular fluid before and after treatment of periodontal disease. J Clin Periodontol 2004: **31**: 475–482.

21. Iwamoto GK, Konicek SA. Cytomegalovirus immediate early genes upregulate interleukin-6 gene expression. J Investig Med 1997: **45**: 175–182.
22. Jarnbring F, Gustafsson A, Klinge B. Immunolocalization of interleukin-8 and proliferating cell nuclear antigen in gingival keratinocytes in patients with periodontitis. Acta Odontol Scand 2000: **58**: 249–254.
23. Kantarci A, Van Dyke TE. Resolution of inflammation in periodontitis. J Periodontol 2005: **76**: 2168–2174.
24. Kida Y, Kobayashi M, Suzuki T et al. Interleukin-1 stimulates cytokines, prostaglandin E2 and matrix metalloproteinase-1 production via activation of MAPK/AP-1 and NF-kappaB in human gingival fibroblasts. Cytokine 2005: **29**: 159–168.
25. Kinane DF, Lappin DF. Immune processes in periodontal disease: a review. Ann Periodontol 2002: **7**: 62–71.
26. Koide M, Suda S, Saitoh S et al. *In vivo* administration of IL-1 beta accelerates silk ligature-induced alveolar bone resorption in rats. J Oral Pathol Med 1995: **24**: 420–434.
27. Kornman KS, Page RC, Tonetti MS. The host response to the microbial challenge in periodontitis: assembling the players. Periodontol 2000 1997: **14**: 33–53.
28. Kubota K, Sakaki H, Imaizumi T et al. Retinoic acid-inducible gene-I is induced in gingival fibroblasts by lipopolysaccharide or poly IC: possible roles in interleukin-1beta, −6 and −8 expression. Oral Microbiol Immunol 2006: **21**: 399–406.
29. Lima V, Vidal FD, Rocha FA, Brito GA, Ribeiro RA. Effects of tumor necrosis factor-alpha inhibitors pentoxifylline and thalidomide on alveolar bone loss in short-term experimental periodontal disease in rats. J Periodontol 2004: **75**: 162–168.
30. Lokensgard JR, Hu S, Sheng W et al. Robust expression of TNF-alpha, IL-1beta, RANTES, and IP-10 by human microglial cells during nonproductive infection with herpes simplex virus. J Neurovirol 2001: **7**: 208–219.
31. Mochizuki S, Kobayashi M, Suzuki T et al. Gamma-interferon enhances expression of CD14/MyD88 and subsequent responsiveness to lipopolysaccharide from *Actinobacillus actinomycetemcomitans* in human gingival fibroblasts. J Periodontal Res 2004: **39**: 333–343.
32. Morimoto T, Nishihira J, Kohgo T. Immunohistochemical localization of macrophage migration inhibitory factor (MIF) in human gingival tissue and its pathophysiological functions. Histochem Cell Biol 2003: **120**: 293–298.
33. Nakao A, Fukushima H, Kajiya H, Ozeki S, Okabe K. RANKL-stimulated TNFalpha production in osteoclast precursor cells promotes osteoclastogenesis by modulating RANK signaling pathways. Biochem Biophys Res Commun 2007: **357**: 945–950.
34. Noguchi K, Iwasaki K, Shitashige M, Endo H, Kondo H, Ishikawa I. Cyclooxygenase-2-dependent prostaglandin E2 down-regulates intercellular adhesion molecule-1 expression via EP2/EP4 receptors in interleukin-1beta-stimulated human gingival fibroblasts. J Dent Res 2000: **79**: 1955–1961.
35. Ohe H, Takashiba S, Naruishi K et al. Tumor necrosis factor-alpha (TNF-alpha) - induced and interleukin-1 beta (IL-1 beta) - induced shedding of TNF receptors from gingival fibroblasts. J Interferon Cytokine Res 2000: **20**: 1077–1082.
36. Oshima M, Azuma H, Suzutani T, Ikeda H, Okuno A. Direct and mononuclear cell mediated effects on interleukin 6 production by glioma cells in infection with herpes simplex virus type 1. J Med Virol 2001: **63**: 252–258.
37. Parra B, Slots J. Detection of human viruses in human periodontal pockets using polymerase chain reaction. Oral Microbiol Immunol 1996: **5**: 289–293.
38. Patil C, Rossa C Jr, Kirkwood KL. *Actinobacillus actinomycetemcomitans* lipopolysaccharide induces interleukin-6 expression through multiple mitogen-activated protein kinase pathways in periodontal ligament fibroblasts. Oral Microbiol Immunol 2006: **21**: 392–398.
39. Pulliam L, Moore D, West DC. Human cytomegalovirus induces IL-6 and TNF alpha from macrophages and microglial cells: possible role in neurotoxicity. J Neurovirol 1995: **1**: 219–227.
40. Ren L, Leung WK, Loo TW, Jin L. Lipopolysaccharide-binding protein down-regulates the expression of interleukin-6 by human gingival fibroblast. J Periodontal Res 2005: **40**: 407–416.
41. Richards D, Rutherford RB. The effects of interleukin 1 on collagenolytic activity and prostaglandin-E secretion by human periodontal-ligament and gingival fibroblast. Arch Oral Biol 1988: **33**: 237–243.
42. Slots J. Update on human cytomegalovirus in destructive periodontal disease. Oral Microbiol Immunol 2004: **19**: 217–223.
43. Taguchi Y, Imai H. Expression of beta-defensin-2 in human gingival epithelial cells in response to challenge with *Porphyromonas gingivalis in vitro*. J Periodontal Res 2006: **41**: 334–339.
44. Takashiba S, Naruishi K, Murayama Y. Perspective of cytokine regulation for periodontal treatment: fibroblast biology. J Periodontol 2003: **74**: 103–110.
45. Tani-Ishii N, Tsunoda A, Teranaka T, Umemoto T. Autocrine regulation of osteoclast formation and bone resorption by IL-1 alpha and TNF alpha. J Dent Res 1999: **78**: 1617–1623.
46. Teng YT. The role of acquired immunity and periodontal disease progression. Crit Rev Oral Biol Med 2003: **14**: 237–252.
47. Teng YC, Chen YT, Cheng YY, Hung SL. Requirements for the upregulation of interleukin-6 by herpes simplex virus-infected gingival fibroblasts. Viral Immunol 2005: **18**: 170–178.
48. Tervahartiala T, Koski H, Xu JW et al. Tumor necrosis factor-alpha and its receptors, p55 and p75, in gingiva of adult periodontitis. J Dent Res 2001: **80**: 1535–1539.
49. Tipton DA, Gay DC, DeCoster VA. Effect of a cyclooxygenase-2 inhibitor on interleukin-1beta-stimulated activation of the transcription factor nuclear factor-kappa B in human gingival fibroblasts. J Periodontol 2007: **78**: 542–549.
50. Tonetti MS, Imboden MA, Lang NP. Neutrophil migration into the gingival sulcus is associated with transepithelial gradients of interleukin-8 and ICAM-1. J Periodontol 1998: **69**: 1139–1147.
51. Uehara A, Takada H. Functional TLRs and NODs in human gingival fibroblasts. J Dent Res 2007: **86**: 249–254.
52. von Muller L, Klemm A, Weiss M et al. Active cytomegalovirus infection in patients with septic shock. Emerg Infect Dis. 2006: **12**: 1517–1522.
53. Wang PL, Ohura K. *Porphyromonas gingivalis* lipopolysaccharide signaling in gingival fibroblasts – CD14 and Toll-like receptors. Crit Rev Oral Biol Med 2002: **13**: 132–142.
54. Yamamoto T, Kita M, Oseko F, Nakamura T, Imanishi J, Kanamura N. Cytokine production in human periodontal ligament cells stimulated with *Porphyromonas gingivalis*. J Periodontal Res 2006: **41**: 554–559.
55. Yamazaki K, Yoshie H, Seymour GJ. T cell regulation of the immune response to infection in periodontal diseases. Histol Histopathol 2003: **18**: 889–896.