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Over-expression and potential role of cyclophilin A in human periodontitis

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Background and Objective: We previously demostrated that EMMPRIN participates in the periodontitis and its interaction with Cyclophilin A possibility exists in animal periodontitis models. This study is aimed to address the expression and potential role of cyclophilin A (CypA) in human periodontitis.

Material and Methods: Gingival tissues and peripheral blood were collected from patients with moderate to severe periodontitis or from healthy donors. Western blotting and immunohistochemistry were performed to detect the expression and distribution of CypA in the gingival tissues. Peripheral blood mononuclear cells (PBMCs) and neutrophils were isolated from the peripheral blood by Ficoll–Paque density-gradient centrifugation. Chemotaxis assays were applied to evaluate the effects of different concentrations of CypA (100, 300 and 500 ng/mL) on the migration of PBMCs and neutrophils. Supernatants of human THP-1 cells were collected after treatment with 200 ng/mL of CypA for different periods of time (1, 3, 6, 12 and 24 h) to detect the levels of interleukin (IL)-1β, IL-8 and tumor necrosis factor alpha (TNF-α) by ELISA.

Results: Western blot analyses revealed an increase of CypA expression in inflamed gingival tissues compared with healthy tissues. Immunohistochemistry identified that the over-expressed CypA was localized in the infiltrating cells and/or in the extracellular matrix in the inflamed gingival connective tissues. The positive infiltrating cells contained mononuclear cells and lobulated-nuclei neutrophils. Chemotactic assays showed that 300 ng/mL of CypA apparently facilitated the chemotaxis of PBMCs/neutrophils from healthy donors, compared with the no-treatment control ($p < 0.01$ for PBMCs, $p < 0.05$ for neutrophils), whereas 100 and 500 ng/mL of CypA only weakly enhanced the chemotaxis of PBMCs/neutrophils ($p > 0.05$ for PBMCs/neutrophils, not significant). The PBMCs/neutrophils from patients with periodontitis exhibited a stronger ability to migrate when stimulated with 300 ng/mL of CypA than did PBMCs/neutrophils from healthy donors ($p < 0.05$ for PBMCs, $p < 0.01$ for neutrophils). ELISA revealed that the level of TNF-α secreted by THP-1 cells was elevated after treatment with 200 ng/mL of CypA for 12 h compared with the no-treatment 0-h control ($p < 0.05$). The IL-8 level was sharply raised after 3 h of stimulation with 200 ng/mL of CypA ($p < 0.01$ compared with 0 h), but no significant change was observed at the other time points ($p > 0.05$). There was no statistical difference at any of the treatment time points for the secretion of IL-1β ($p > 0.05$ for 1, 3, 6, 12 and 24 h compared with 0 h).

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Conclusions: CypA participates in the pathogenesis of human periodontitis. It may be involved in the inflammatory response of periodontal tissues through inducing the chemotaxis of PBMCs/neutrophils and the secretion of TNF-α/IL-8.

Periodontitis is a very common infective disease characterized by the inflammation and destruction of periodontal tissues. The inflammatory response represents the migration of leukocytes from the general circulation into gingival connective tissues (1). Infiltrating neutrophils, lymphocytes and monocytes/macrophages secrete various mediators under inflammatory conditions (2). The inflammatory response is a key step in initiating the synthesis of MMPs and the subsequent imbalance between activated MMPs and their host-derived endogenous inhibitors, which then leads to pathological periodontal tissue destruction during periodontitis (3).

Cyclophilin A (CypA), a member of the immunophilin family, is a peptidylprolyl *cis-trans*-isomerase, an important component in the process of protein folding. CypA was originally identified as an intracellular receptor for cyclosporine A (4,5) and also can be released extracellularly by cells in response to inflammatory stimuli (6). The secreted CypA induces chemotaxis via interaction with its cellular receptor, extracellular MMP inducer (EMMPRIN), and thus contributes to the inflammatory response (7). The CypA–EMMPRIN interaction is also related to the production of matrix-degrading enzymes and to the expression of cytokines (8,9). The potential roles of the CypA–EMMPRIN interaction have been identified in several diseases, such as rheumatoid arthritis and inflammatory cardiomyopathy (7). EMMPRIN is a plasma membrane protein best known for its ability to function as an extracellular MMP inducer, and it is also a regulator of inflammatory cell chemotaxis (10). We previously demonstrated the roles of EMMPRIN in human periodontitis (11–15) and the possible existence of the CypA–EMMPRIN interaction within an animal model of periodontitis (16). However, to our knowledge, it has still not been determined whether and how CypA is involved in the pathogenesis of human periodontitis.

In order to address these issues, we examined the expression and distribution of CypA in human gingiva, and investigated the effects of CypA on the chemotaxis of neutrophils and mononuclear cells, as well as on the secretion of some proinflammatory cytokines by monocytes.

Material and methods

Patients

Gingival tissues and peripheral blood were obtained from seven patients with moderate to severe periodontitis and from six healthy donors without systematic diseases. All patients with periodontitis met the revised diagnostic criteria of periodontitis. There were no significant age or gender differences between the healthy donors and the patients with periodontitis. The Ethics and Investigation Committee of the School & Hospital of Stomatology, Wuhan University, granted ethical approval for this study and all subjects provided their informed consent.

Gingival tissue collection

Inflamed gingival tissues were excised during either periodontal-flap surgery or extractions of teeth with a poor periodontal prognosis. Healthy gingival tissues were collected during crown-lengthening procedures or extractions for orthodontic purposes. Each specimen was divided into two parts of approximately equal size. One part was immediately fixed in 4% paraformaldehyde solution and then processed using routine procedures to create 5-μm-thick paraffin sections for immunohistochemistry analyses. The other part was stored at −70°C until used for western blotting.

Cell isolation

A 6-mL sample of peripheral blood was collected from each healthy donor and each periodontitis patient. The red blood cells of fresh peripheral blood were induced to aggregate by the addition of 600 ml of 3% dextran solution and incubation for 30 min. Peripheral blood mononuclear cells (PBMCs) and/or neutrophils were isolated by Ficoll–Paque (LTS1077; TBD, Tianjin, China) density-gradient centrifugation. The PBMCs were extracted from the second layer. Neutrophils were enriched from the third layer after lysis of red cells in cold 0.83% NH_4Cl solution for 3 min. The cell suspension was spread onto a slide and examined by microscopy after Giemsa staining. The numbers of PBMCs and/or neutrophils on slides were counted and the isolation rate was calculated. The isolated PBMCs and/or neutrophils were maintained in RPMI-1640 containing 1% fetal bovine serum (FBS). The viability rate was measured using a nucleocounter™ before the chemotaxis assay.

Western blot

Total protein was isolated from the stored gingiva and the protein concentration was measured according to our previous methods (12,14). The PageRuler™ Prestained Protein Ladder (Fermentas AB) was used for molecular-weight determinations. Protein samples (40 μg of protein per lane) were separated by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel (at 70 V for 2 h) and were then transferred onto Hybond™ poly(vinylidene difluoride) transfer membrane (Amersham Biosciences) using a humidified blotter. The membranes were washed five times with Tris-Buffered Saline and Tween 20 (TBST) buffer for 10 min each wash and were then blocked by incubation in 5% (weight by volume) skim milk

for 2 h at 37°C. Then, the membranes were incubated with rabbit antibody against β-actin (1 : 1000 dilution; Pierce, Rockford, IL, USA) and CypA (1 : 1000 dilution; Abcam, Cambridge, UK) overnight at 4°C. After another five, 10-min washes in TBST buffer, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1 : 15,000 dilution; Pierce) for 2 h at 37°C. The membranes were washed for another five, 10-min washes in TBST buffer. The ECL western blotting system (Santa Cruz, CA, USA) was used for chemiluminescence according to the manufacturer's instructions to visualize the protein bound. The blots were exposed to photographic films (KODAK). All antibodies were diluted in TBST.

Immunohistochemistry

Sections were deparaffinized and immersed for 10 min in methanol containing 0.3% hydrogen peroxide to inactivate endogenous peroxidase. The sections were rinsed in phosphate-buffered saline (three times, for 5 min each rinse) before being covered with 10% normal rabbit serum for 15 min to block nonspecific binding. Next, the sections were incubated with rabbit anti-CypA (1 : 300 dilution; Abcam), overnight at 4°C. After another rinse, the sections were treated with goat anti-rabbit IgG for 10 min and then reacted for 10 min with the avidin–biotin–peroxidase complex. The reaction products were visualized by immersing the sections in 0.03% diaminobenzidine solution. Finally, the sections were counterstained with hematoxylin. As a negative control, phosphate-buffered saline was used instead of anti-CypA.

Chemotaxis assay

Chemotaxis assays were performed using 24-well transwell units with the two compartments separated by a 5-μm polycarbonate membrane (Costar, Cambridge, NY, USA). One-hundred-microlitre cell suspensions of PBMCs and/or neutrophils were added to the upper compartments. Five-hundred microlitres of RPMI-1640 containing 1% FBS and different dilutions of CypA (100, 300 and 500 ng/mL) was added to the lower compartments. *N*-formyl-methionine-leucine-phenylalanine was used as a positive control and RPMI-1640 containing 1% FBS was used as a negative (no-treatment blank) control. The chambers were incubated at 37°C in 5% CO_2 for either 90 min (PBMCs) or 45 min (neutrophils), then taken out, fixed and stained with Giemsa reagent. The number of cells appearing on the lower surface of the filter was counted in four fields using a microscope (Olympus Optical Co Ltd, Tokyo, Japan) fitted with a × 40 objective lens. The counts obtained for the four fields were averaged to provide a mean count for each well. After the final neutrophil count, the filters were recovered from the transwell and sealed with gum. Digital photographs were obtained of neutrophils in the sealed filters using a microscope equipped with a video camera (AxioCam HRc; Zeiss, Hallbergmoss, Germany).

ELISA

The human monocyte cell line, THP-1, was obtained from the China Center for Type Culture Collection (Wuhan, China). THP-1 cells were seeded in 24-well plates, at a density of 1×10^6 cells/mL, in RPMI-1640 containing 10% FBS. THP-1 cells were treated with 200 ng/mL of CypA and the cell-culture supernatants were prepared for ELISA after 1, 3, 6, 12 and 24 h of incubation. The 0-h blank control received no treatment. Interleukin (IL)-1β, IL-8 and tumor necrosis factor alpha (TNF-α) were severally measured using corresponding ELISA kits (Boster, Wuhan, China) according to the manufacturer's instructions.

Statistical analyses

All results were expressed as mean ± standard deviation. SPSS 13.0 was used for data analysis. Analysis of variance and Tukey's test were used to compare the means. The null hypothesis was rejected at $p < 0.05$.

Results

Over-expression and distribution of CypA in inflamed gingival tissues

CypA was detected in all specimens by western blotting. The CypA protein band of the inflamed gingiva from patients with periodontitis was visibly wider than that of the normal gingiva from healthy donors (Fig. 1).

Immunostaining of CypA was negative or weakly positive in healthy gingiva. Two specimens were negative for CypA immunostaining and four were weakly positive, mainly in the extracellular matrix. Stronger and wider (distributed in a larger area) staining was observed in the connective tissues of the inflamed gingiva from patients with periodontitis. Gingival epithelium was always negative for CypA. The increased staining was principally distributed in the infiltrating cells and in the extracellular matrix (Fig. 1): two samples in the extracellular matrix, three in the infiltrating cells and two in both the matrix and the infiltrating cells. The infiltrating cells that stained positive for CypA were identified as lobulated-nuclei neutrophils and mononuclear cells from their overall morphology by microscopy under a high-power lens. Endothelial cells were occasionally moderately positive for CypA in inflamed gingiva.

Effects of CypA on the chemotaxis of PBMCs/neutrophils

PBMCs and/or neutrophils were effectively isolated from human peripheral blood by Ficoll–Paque density-gradient centrifugation. Cell counting was performed on the smear slides and showed that the number of PBMCs/neutrophils was more than 96% of the total cells (Fig. 2). Furthermore, the rate of viable cells was more than 97%.

The chemotaxis assays showed that 100, 300 and 500 ng/mL of CypA induced more PBMCs/neutrophils from healthy donors to migrate across the transwell filter. Compared with the no-treatment control the numbers of PBMCs/neutrophils were significantly higher when induced with 300 ng/mL of CypA ($p < 0.01$ for PBMCs,

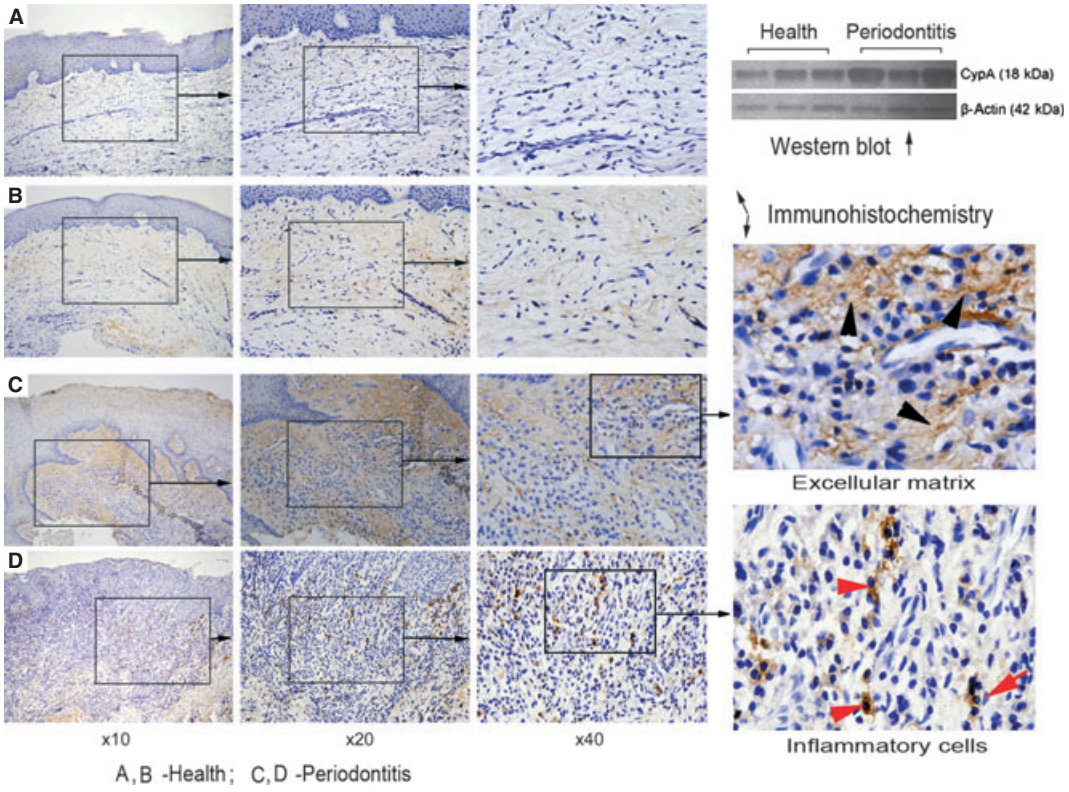

Fig. 1. Over-expression and distribution of cyclophilin A (CypA) in the inflamed gingiva. The western blotting results are shown at the top right of the figure: the CypA protein bands from patients with periodontitis were wider than those from healthy donors. (A–D) Immunostaining for CypA in healthy gingiva (A, B) and in inflamed gingiva from patients with periodontitis (C, D). Immunostaining was negative or only weakly positive in healthy gingiva (A, B) but was strongly positive in inflamed gingiva (C, D); the increased staining was mainly distributed in the extracellular matrix (black arrowheads) and/or infiltrating cells, including mononuclear cells (red arrowheads) and lobulated-nuclei neutrophils (red arrow).

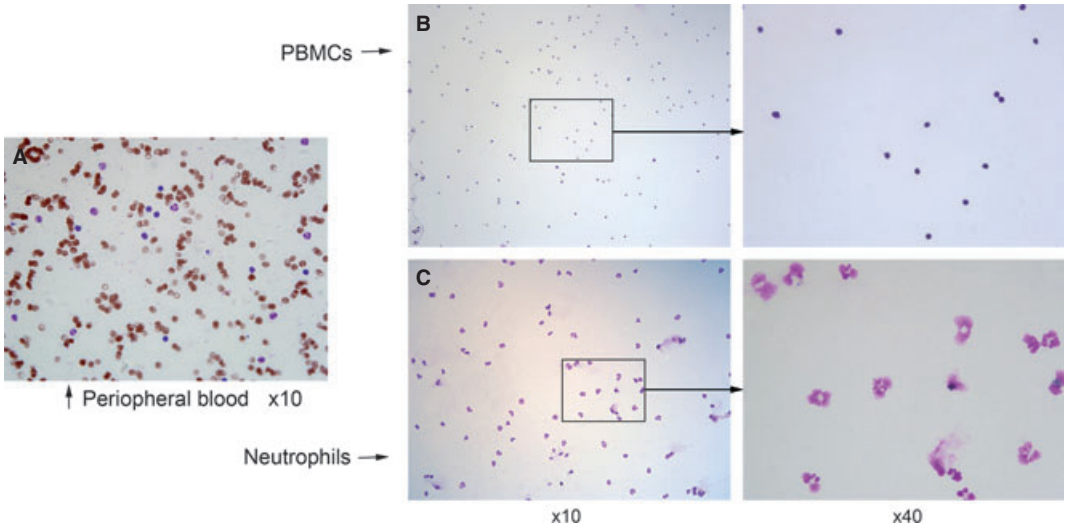

Fig. 2. Isolation of peripheral blood mononuclear cells (PBMCs) and neutrophils from peripheral blood. Giemsa staining shows PBMCs and neutrophils on smear slides of the cell suspension. (A) Unprocessed peripheral blood containing red cells, platelets and various types of leucocytes. (B) Mononuclear cells (blue/purple nucleus). (C) Neutrophils (typical purple lobulated nuclei). After effective isolation more than 96% of the cells were mononuclear cells or neutrophils.

$p < 0.05$ for neutrophils), but not when induced with 100 or 500 ng/mL of CypA ($p > 0.05$ for PBMCs/neutrophils). Likewise, PBMCs/neutrophils from patients with periodontitis also showed a distinctly enhanced invasion ability when stimulated with 300 ng/mL of CypA compared with the no-treatment control ($p < 0.01$). Importantly, the effects of CypA on PBMCs/neutrophils from patients with periodontitis were stronger than the effects of CypA on PBMCs/neutrophils from healthy donors ($p < 0.05$ for PBMCs, $p < 0.01$ for neutrophils) (Fig. 3).

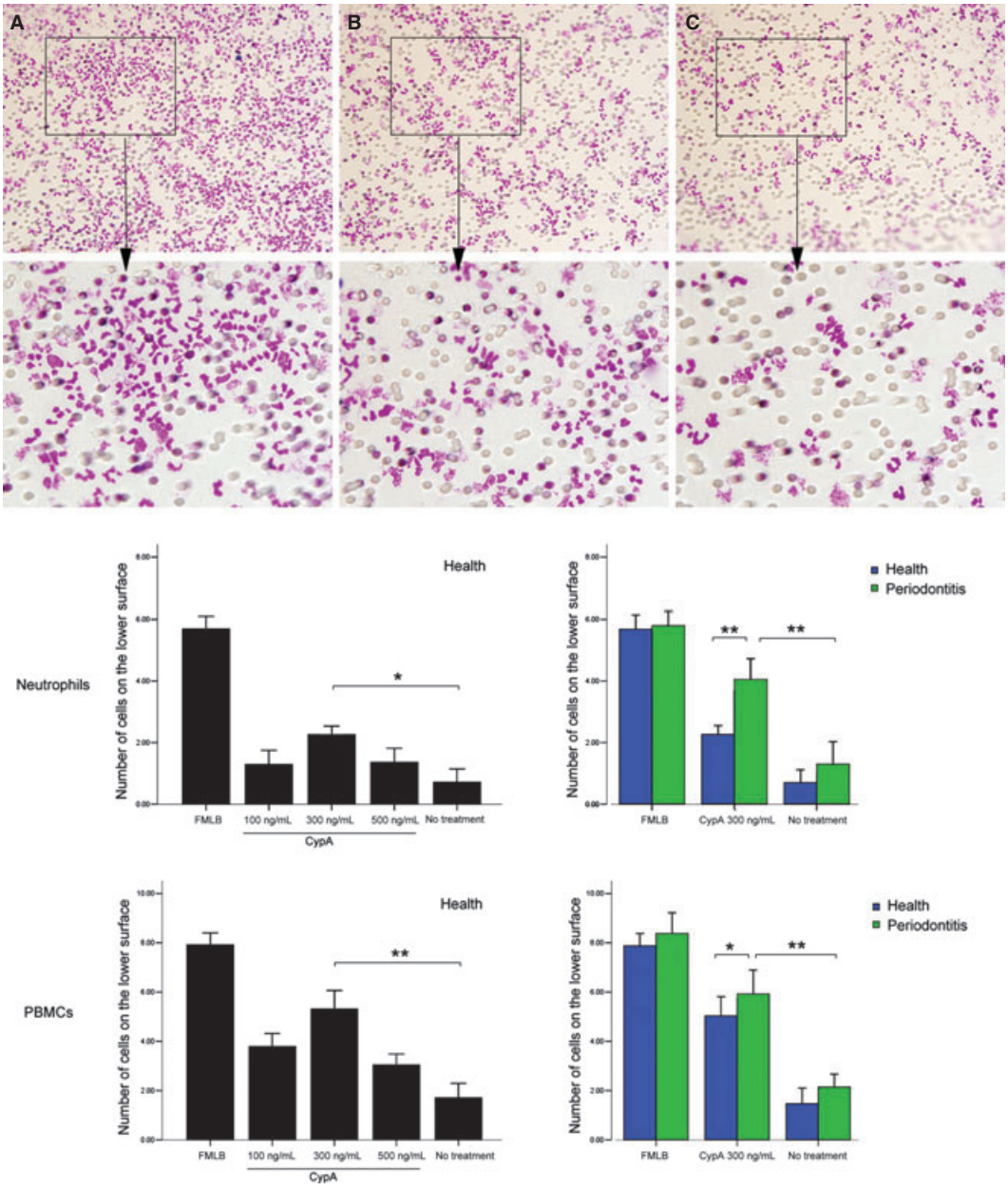

Fig. 3. Effects of cyclophilin A (CypA) on the chemotaxis of neutrophils/peripheral blood mononuclear cells (PBMCs). The panels at the top of the figure show the microscopic appearance of neutrophils on the lower surface of the transwell filter after treatment with (A) *N*-formyl-methionine-leucine-phenylalanine (FMLP) (positive control) or (B) 300 ng/mL of CypA, or (C) with no treatment (blank control). Quantitative results for chemotaxis assays are shown as bar charts in the lower panel: 100, 300 and 500 ng/mL of CypA all induced the migration of neutrophils/PBMCs from healthy donors; the number of neutrophils/PBMCs under the filter of the transwell was statistically different between cells treated with 300 ng/mL of CypA and the no-treatment control ($p < 0.05$ for neutrophils, $p < 0.01$ for PBMCs); 300 ng/mL of CypA also enhanced the migration of neutrophils/PBMCs from patients with periodontitis ($p < 0.01$ compared with the no-treatment control), and the effect was stronger than that for neutrophils/PBMCs from healthy donors ($p < 0.01$ for neutrophils; $p < 0.05$ for PBMCs); *$p < 0.05$; **$p < 0.01$.

Effects of CypA on cytokine secretion by THP-1 cells

ELISA analyses revealed that 200 ng/mL of CypA slightly promoted the secretion of IL-1β from THP-1 cells after stimulation for 1, 3, 6, 12 and 24 h; however, the differences were not statistically significant ($p > 0.05$). The TNF-α level was higher at the 12-h time-point than in the 0-h blank control ($p < 0.05$). The secretion of IL-8 was sharply increased after stimulation for 3 h compared with the 0-h blank control ($p < 0.01$). Although a slight increase or decrease of TNF-α or IL-8 was observed at the other time-points, these differences were not statistically significant compared with the 0-h blank control ($p > 0.05$ for TNF-α and IL-8) (Fig. 4).

Discussion

In this study, we examined the expression and the distribution of CypA in inflamed and healthy gingiva. We also investigated the influence of CypA on the chemotaxis of PBMCs and/or neutrophils from patients with periodontitis and from healthy donors, as well as the effect of CypA on the secretion of IL-1β, IL-8 and TNF-α by THP-1 cells.

Both western blotting and immunohistochemistry detected increased expression of CypA in inflamed gingiva compared with healthy gingiva. The increased immunostaining was mainly distributed in the infiltrating cells and the extracellular matrix under inflamed conditions. The CypA-positive infiltrating cells comprised mononuclear cells and lobulated-nuclei neutrophils. They were considered to be the target cells of CypA-mediated chemotaxis. In other words, CypA may participate in human periodontitis through inducing the chemotaxis of mononuclear cells and neutrophils, as subsequently proved in the chemotaxis assays *in vitro*.

Interestingly, a seemingly converse immunohistochemistry finding was reported in one of our previous studies, in that lobulated-nuclei neutrophils were rarely positive for CypA in rat experimental periodontitis (16). Therefore, we deemed that CypA was irrelevant to the migration of neutrophils during the inflammation of rat periodontitis. It was not difficult to understand such a different result between rat and human in view of the distinct immunobiology between the

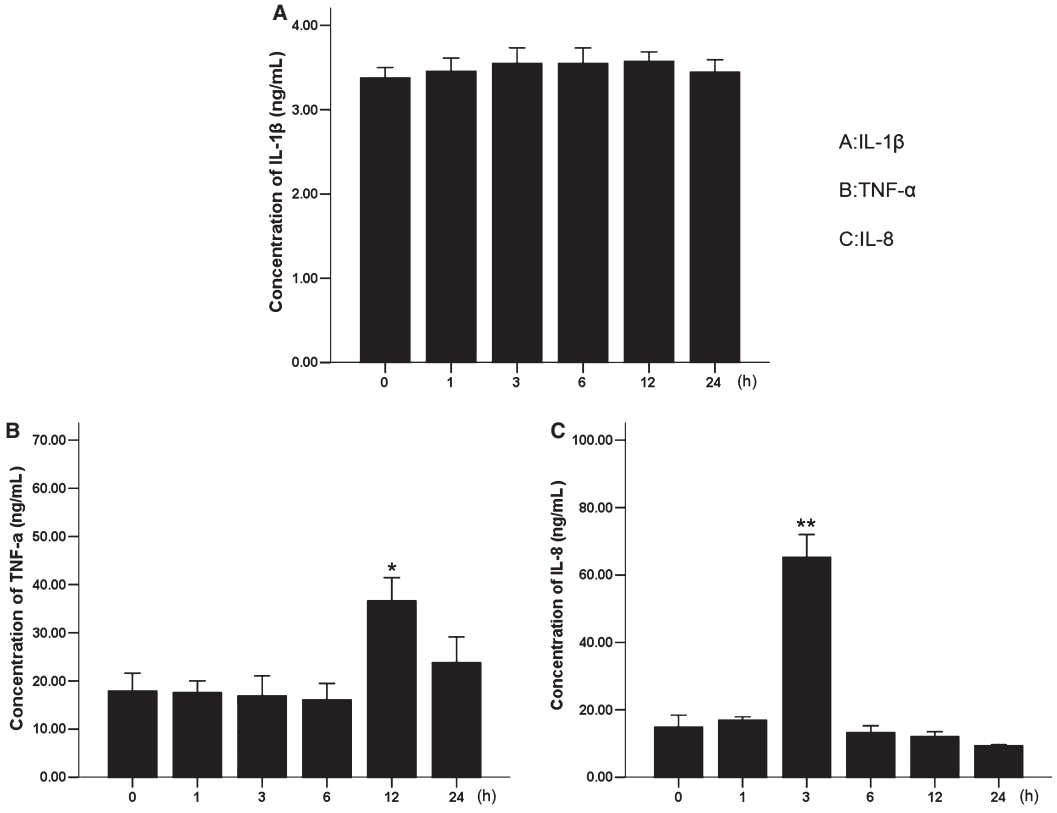

Fig. 4. Effects of cyclophilin A (CypA) on the secretion of cytokines by THP-1 cells. (A) There was no obvious difference in the secretion of interleukin (IL)-1β at the five different treatment time-points ($p > 0.05$). (B) Secretion of tumor necrosis factor alpha (TNF-α) was much higher at the 12-h treatment time-point ($p < 0.05$) but was not significantly different from the control at the other treatment time-points ($p > 0.05$). (C) The secretion of IL-8 showed a sharp increase at the 3-h time-point ($p < 0.01$) but no significant difference from the control at the other time-points ($p > 0.05$). *$p < 0.05$; **$p < 0.01$ compared with the 0-h blank control.

species. The neutrophil-related first defense against periodontal pathogens is more efficient in humans than in rats (17).

The chemotaxis activity of CypA has already been well documented (9,10,18–23). In the current study, 300 ng/mL of CypA induced a sharp increase in the migration of PBMCs/neutrophils from healthy donors. Moreover, 300 ng/mL of CypA showed a stronger ability to facilitate the chemotaxis of PBMCs/neutrophils from patients with periodontitis than of PBMCs/neutrophils from healthy donors. It was reasonable to draw the conclusion that CypA might participate in the pathogenesis of human periodontitis via facilitating the chemotaxis of PBMCs/neutrophils.

Regretfully, how (or even whether) CypA influences the chemotaxis of monocytes and macrophages in periodontitis was not precisely determined because PBMCs are a mixture of different types of cells, including monocytes, macrophages and lymphocytes. In fact, there are some discrepancies in the chemotaxis activity of CypA on monocytes/macrophages. Yang *et al.* (9) found that CypA could enhance the invasion of human macrophages, but not monocytes, in rheumatoid arthritis. Damsker *et al.* (10) reported that CypA was involved in the chemotaxis of monocytes in collagen-induced arthritis of mouse. Wang *et al.* (18) demonstrated that CypA induced the chemotaxis of the human monocyte cell line, THP-1. It is still necessary to clarify the actual effects of CypA on the chemotaxis of monocytes/macrophages.

Regarding lymphocytes, $CD4^+$ T cells were chemotactic to CypA in collagen-induced mouse arthritis (10) and in asthma-mediated mouse lung inflammation (20). Likewise, previous studies were in complete agreement on the ability of CypA to induce the chemotaxis of neutrophils (10,21,22).

Other than the target-cell population, the optimal concentration of CypA for CypA-induced chemotaxis is also still under debate. Arora *et al.* (21) observed that 100 ng/mL of CypA was much more effective for inducing the chemotaxis of mouse neutrophils than was 50 ng/mL of CypA. Wang *et al.* (18) demonstrated that 200 ng/mL of CypA was the most effective dose for inducing the chemotaxis of monocytes. In this study, the optimal concentration was not proven, although 300 ng/mL of CypA was found to be more effective than either 100 ng/mL or 500 ng/mL of CypA for inducing the chemotaxis of PBMCs/neutrophils. Further, detailed, evidence is therefore required to determine the dose-dependent effects of CypA on chemotaxis.

To date, the mechanisms of CypA-mediated chemotaxis have been principally considered to involve the interaction between extracellular CypA and membrane-type EMMPRIN. Recently, Heine *et al.* (22) found that CypA had the capacity to interact with classical chemokines – macrophage inflammatory protein-2/chemokine (C-X-C motif) ligand 2 and keratinocyte chemoattractant/chemokine (C-X-C motif) ligand 1 – resulting in an increase of leukocyte recruitment in mouse lung inflammation. It will be of much interest and importance if CypA cooperates with other chemokines leading to an augmented inflammatory response in periodontitis.

In order to investigate the proinflammatory activity of CypA, we tested the effects of 200 ng/mL of CypA on the secretion of IL-1β, IL-8 and TNF-α by THP-1 cells. The levels of TNF-α and IL-8 were markedly increased at 12-h and 3-h time-points, respectively, whereas the level of IL-1β did not show an obvious change at any time-point. Wang *et al.* (18) found that 200 ng/mL of CypA up-regulated the secretion of IL-8 by THP-1 cells after 24 h of stimulation but did not affect the levels of TNF-α and IL-1β. Kim *et al.* (8) reported that THP-1 cells secreted increased amounts of TNF-α, IL-1β and IL-8 after stimulation with 1 μM CypA for 24 h. These results consistently indicate that CypA induces the secretion of IL-8 by THP-1 cells, but are conflicting on the ability of CypA to induce the secretion of TNF-α and IL-1β, which still awaits more in-depth experimentations. Regardless, our results suggest that CypA is able to stimulate the secretion of IL-8 and TNF-α by THP-1 cells. This was regarded as a way for CypA to contribute to the pathogenesis of human periodontitis.

Several investigators reported that CypA could induce endothelial activation and thus participate in the proinflammatory response (23,24). In this study, CypA-positive endothelial cells were frequently seen in the inflamed gingiva but were rarely observed in the healthy gingiva. Such a finding correctly supported their opinion on the CypA-induced endothelial activation.

CypA-induced chemotaxis and proinflammatory activities may also be associated with the physical inflammation found in periodontitis because only weak expression of CypA was detected in healthy gingiva. In our previous animal study, normal inflammatory infiltration was observed under the interdental gingival epithelium in rats, and CypA was weakly positive in the infiltrating cells (16).The infiltration was less frequently seen in human gingiva than in rat gingiva, but CypA was still weakly positive in extracellular matrix.

In addition to its chemotatic and proinflammatory activities, CypA is also able to stimulate the production of MMPs in some diseases (18,25,26). Using gelatin zymography, we examined the effects of 200 ng/mL of CypA on the production and activation of MMP-2 and MMP-9 by THP-1 cells; we found that the production of MMP-2 and MMP-9 was increased but their activation showed no obvious change (results not shown). Conversely, Yang *et al.* (9) did not detect an increase in MMP-2 production by THP-1 cells when treated with the same concentration CypA. Whether CypA contributes to periodontitis through promoting the production of MMPs is still uncertain. This is an interesting and relevant point because of the existing interaction between CypA and EMMPRIN.

In summary, our findings indicate that CypA is involved in the pathogenesis of periodontitis. CypA may regulate the inflammatory response of periodontal tissue through inducing

the chemotaxis of leukocytes (PBMCs/neutrophils) and the secretion of proinflammatory cytokines (IL-8 and TNF-α). However, further research is needed to determine the mechanistic role of CypA during the pathogenesis of periodontitis.

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