Proteomic Analysis in Cyclosporin A-Induced Overgrowth of Human Gingival Fibroblasts

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Cyclosporin A (CsA) has been used as an immunosuppressive drug to prevent organ transplant rejection and to treat autoimmune diseases. CsA has a proliferative effect on human gingival fibroblasts (HGF) in vitro. However, the molecular mechanisms underlying CsA-induced proliferation in HGF remain to be elucidated. This study was aimed to investigate the CsA responsive proteins in HGF using systematic proteomic approach. Cell viability was determined by MTT assay and reactive oxygen species (ROS) was measured by fluorescent spectrometer. Proteins profiled by two-dimensional gel electrophoresis (2-DE) were identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and electrospray ionization quadrupole time-of-flight mass spectrometry (EQ-TOF MS). To confirm the expression changes of proteins by proteomics analysis, Western blot was performed using specific antibody. CsA increased the cell viability of HGF in a dose- and time-dependent manner. Significantly, seventeen proteins were overexpressed in the CsA-treated HGF, whereas three proteins were found to be expressed less than the untreated cells. The identified proteins were mainly related with cell proliferation, metabolism, and oxidation. The overexpression of peroxiredoxin 1 (Prx 1) confirmed by Western blotting and reduction of cytosolic reactive oxygen species (ROS) levels in the CsA-treated HGF demonstrated that Prx 1 may play a crucial role in the HGF proliferation induced by CsA. Upregulation of Galectin 3 in CsA-treated HGF indicated that it is related to CsA-induced proliferation. These proteomic analysis data will provide an efficient approach in understanding the mechanisms of HGF proliferation by CsA.

Key words proteomics; cyclosporin A; overgrowth; human gingival fibroblast

Cyclosporin A (CsA), a lipophilic cyclic polypeptide derived from the fungus Tolypocladium inflatum, has been widely used as an immunosuppressant to prevent organ transplantation rejection and to treat various immunological diseases such as rheumatoid arthritis atrophic dermatitis, and Behcet’s disease. 1,2) The clinical use of CsA is associated with a number of side effects including nephrotoxicity, hepatotoxicity, neurotoxicity, hypertension and gingival overgrowth (GO). 3,4) The GO occurrence in oral tissues after CsA therapy may interfere with the normal oral functions, also cause delayed eruption of teeth, impaired speech, an oral hygiene problem, and a psychological impact and may influence compliance with medical therapy. 5,6) The reported incidence of GO appears approximately 25% (range, 8—70%), 7) is highly variable depending on genetics, duration, drug dose, serum and salivary concentrations, oral hygiene, age, and gender. 5)

Although there are a number of histological and biochemical studies on CsA-induced gingival overgrowth (CsAGO), the precise mechanism underlying the pathogenesis of CsAGO is still unclear. There has been proposed mechanism underlying the pathogenesis of CsAGO in a number of studies. It is known that the expression levels for peptide growth factors such as transforming growth factor-β1 (TGF-β1), fibroblast growth factor-2 (FGF-2), insulin-like growth factor (IGF-1) and platelet derived growth factor-β (PDGF-β) are elevated in the CsAGO. 9,10) Increased growth factors in the CsAGO may be responsible for promoting fibroblast proliferation and production of extracellular matrix components in GO. 11) One prevailing hypothesis is that CsA induces GO by promoting proliferation of gingival cells, although there was one report that CsA suppressed proliferation. 5) Moreover, some previous studies have demonstrated that CsA stimulates the proliferation of human gingival fibroblasts (HGF) 3,12)

On the other hand, there has been still controversy over the pathology of CsAGO with conflicting reports as to whether it represents a true hyperplasia. It would appear that CsA has the potential to alter the metabolism HGF in several ways. 13) Furthermore, Jacobs and Hassel 14) reported that CsA reacted with a major metabolite OL-17, resulting in an increase in protein synthesis and promotion of proliferation in HGF. In contrast, some studies have suggested that CsAGO involves an increase in the amount of collagen and extracellular matrix formation rather than an increase in the number of cells. 15,16)

Oxidant stresses are known to induce tissue damage, cell death, and pathogenesis. In practice, free radicals such as reactive oxygen species (ROS) are involved in pathogenesis of various diseases including cancer, degenerative neuronal disease and stroke. There are, however, biological antioxidant of host defense mechanisms to neutralize such oxidant stresses, including DOD, peroxidase, catalase and thioredoxin. 17)

There are some report suggesting the relationship between CsA and oxidation. Imbert et al. 18) reported that CsA protects rat hepatocytes from lethal injury by oxidant chemicals, suggesting that CsA has a protective effect on oxidative stress. In addition, CsA contributes to allograft survival by maintenance of their peroxisomal function, β-oxidation activity. 19) Excessive ROS production followed by the mitochondria causes apoptosis and necrosis, contributes to hypoxia injury, can be attenuated at the mitochondrial level of ROS by

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CsA. These reports suggest another possibility that oxidative regulation may be involved in CsAGO.

Although there are a number of histological and biochemical studies focused on the mechanism of CsAGO, the progress of understanding its molecular mechanism has been limited. Recently, proteomic analysis has been highlighted as a technique that not only enables to find cellular proteins changed in response to internal stresses, external stimulations, or developmental changes, but also to assess the amount of the changes and posttranslational modification of proteins.

The present study was designed to investigate the changes in proteome of HGF induced by the CsA treatment using proteomic analysis, providing advanced understanding on mechanisms of CsAGO.

MATERIALS AND METHODS

Cell Culture and Cell Viability Assay  HGF was maintained in Dulbecco’s modified Eagle’s medium (DMEM) media supplemented with 10% fetal bovine serum (Gibco, U.S.A.) under 5% CO₂ at 37 °C. CsA (Sigma, U.S.A.) was dissolved in distilled RPMI 1680 and sterilized through 0.2 μm filter. Cell viability was determined using 3-(4,5-di- methylthiazol-2-yl)-2,5-diphenytertra zolium bromide (MTT) assay (Sigma, U.S.A.).

Proteomic Assay. 1) Two-Dimensional Electrophoresis After a lysis buffer containing 8 M urea, 4% 3-(cholamido-propyl)-dimethylammonio]propane sulfonate (CHAPS), 40 mM Tris base, 1% diithiothreitol (DTT) was added to 0.5% immobilized pH gradient (IPG) buffer, the sample suspension was sonicated in short bursts on ice. The lysate was centrifuged in a microcentrifuge at 30000 × g for 15 min at 4 °C. The supernatant was stored at −80 °C until used. The first dimension of 2-dimensional electrophoresis (2-DE) was performed on a IPG strip (Amersham Biosciences, U.K.). Linear pH 3—10 IPG strips (24 cm) were rehydrated overnight at room temperature in rehydrating buffer (8M urea, 1% DTT, 2% CHAPS, and 0.5% IPG buffer). Sample of 500 μg was applied during rehydration. The first dimension was run for 55500 V h at 20 °C using the following conditions: 500 V for 1 h, 1000 V for 1 h, and 8000 V for 6 h and 30 min. Next, gels were equilibrated for 30 min in an equilibration buffer I containing 50 mM Tris–Cl, 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS) and 0.1% DTT and subsequently equilibration buffer II containing 50 mM Tris–Cl, 6 M urea, 30% glycerol, 2% SDS and 0.25% iodoacetic acid. The second dimension was run on Ettn DALT II system (Amersham Biosciences, U.K.). A 12.5% SDS-polyacrylamide slab gel was used for the second dimensional gel electrophoresis.

The IPG strips were placed on the surface of the second dimensional gel, and then were sealed with 0.5% agarose in 1% SDS electrophoresis buffer. Gels were run overnight at 110 V until the dye front reached the bottom of the gel.

2) Silver Staining Silver staining of gels was performed using Silver Stain PlusOne kit (Amersham Biosciences, U.K.). The use of glutaraldehyde in the sensitization step and formaldehyde in the silver impregnation step was omitted. After electrophoresis, the gels were fixed with 40% methanol and 10% acetic acid for 30 min. The gels were sensitized by incubating in sensitizing solution (0.2% sodium thiosulphate, 30% methanol, sodium acetate, 68 g/l), and rinsed with three changes of distilled water for 5 min each. They were then incubated in 0.25% silver nitrate for 20 min, followed by rinsing, and the gels were rinsed twice with distilled water for 1 min and then developing in 0.15% formaldehyde in 2.5% sodium carbonate with intensive shaking. The development was terminated by incubating the gels with 1.46% ethylenediaminetetraacetic acid (EDTA).

3) Image Analysis The silver-stained 2-DE gels were scanned with LabScan software on Imagescanner (Amersham Biosciences, U.K.), digitized and analyzed using Image-Master 2D (Amersham Biosciences, U.K.). Matching of the spots was performed by use of a reference gel prepared from five gels. Spot standardization was carried out for all matched spots.

4) Destaining Silver-stained proteins were destained using chemical reducers to remove the silver as described previously with the following critical modifications. Two stock solutions of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate were prepared in water. A working solution was prepared by mixing them at a 1 : 1 ratio prior to use. After interesting protein spots were excised from the gel, they were incubated in the working solution until the brownish color disappeared. The reaction was stopped by rinsing the gels in water and 200 mM ammonium bicarbonate. Subsequently, the gel was cut into small pieces, washed with water, and dehydrated repeatedly with changes of acetoni trile until the gel pieces turned into opaque white color. The gel pieces were dried in a vacuum centrifuge for 30 min.

Trypsin digestion of proteins in-gel enzymatic digestion was performed as previously described. Briefly, the digestion was performed by incubating the proteins in 5—10 ng/μl of trypsin and 50 mM ammonium bicarbonate and incubated overnight at 37 °C. The resultant peptides were extracted three times with 10—20 μl of 5% trifluoroacetic acid in 50% acetonitrile and dried using a vacuum centrifuge for 30 min.

5) Identification of Proteins The dried samples were analyzed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometer (Voyager-DE PRO) for peptide mass fingerprinting, and by electrospray ionization quadrupole time of flight (ESI-Q-TOF) mass spectrometry analysis for peptide sequencing. The database searches were carried out using MS-Fit, which has an access to world wide web at http://kr.expasy.org or http://www.ncbi.nlm.nih.gov.

Detection of ROS Production ROS production was monitored by fluorescence spectrometer (Hitachi F-4500, Japan) using 2',7'-dichlorofluorescein diacetate (DCF-DA). Cells were plated on 96-well plate and treated with N-acetyl-cysteine (NAC; Sigma, U.S.A.) and CsA. DCF-DA (25 μM) was added into the media for further 10 min at 37 °C. Excitation was measured at 480 nm and emission was measured at 530 nm.

Western Blotting Cells were washed twice with PBS and proteins were solubilized in a lysis buffer (1% NP-40, 500 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Benzamid, 1 μg/ml Trypsin inhibitor) containing a cocktail of protease inhibitor (Complete, Germany). Lysates were in-
cubated for 30 min at 4 °C, and centrifuged at 11000 g for 20 min. Protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Pierce, IL, U.S.A.). Protein extracts (100—300 μg) were boiled with SDS-sample buffer for 5 min and then subjected to electrophoresis on 12% polyacrylamide gel. They were electrobotted onto nitrocellulose membrane (Amersham Pharmacia Biotech, U.K.) and blocked with 5% skim milk (Becton Dickinson, U.S.A.) in Tris-buffered saline–0.1% Tween 20 (TBS-T). Primary antibodies used were a rat monoclonal anti-peroxiredoxin (Prx) (Pharmingen, CA, U.S.A.), Galectin 3 (Santa Cruz, U.S.A.) and anti-β-Actin (Santa Cruz, U.S.A.) as a control marker. Blots were subsequently washed three times in TBS-T for 5 min and incubated with specific peroxidase-coupled secondary antibodies (Sigma, U.S.A.). Bound antibodies were visualized using an enhanced chemiluminescent detection system (Amersham Pharmacia Biotech, U.K.).

RESULTS AND DISCUSSION

CsA, is an immunosuppressive drug used in organ transplanted patients to prevent graft rejection and to treat various immunological diseases. The prominent side-effect of CsA therapy in oral tissue is gingival overgrowth, which is basically characterized by accumulation of extracellular matrix within the connective tissues and promotion of HGF proliferation. Despite of extensive studies over the decades focused on CsAGO, the precise mechanism underlying its pathogenesis is still unclear. The side effects of CsA may be a significant clinical interest and therefore, their mechanisms deserve further comprehensive investigations.

Previous studies demonstrated that CsA has a stimulatory effect on HGF. In contrast, other reports showed that CsA has no effect on proliferation or an inhibitory effect in gingival fibroblasts. Nevertheless, the present study showed that CsA promoted the proliferation of HGF over 0.1—10 μM in a dose- and time-dependent manner (Fig. 1). CsA increased HGF viability about 120—150% compared to the control and showed maximal survival rate at 10 μM. These results show that CsA has a stimulating effect on HGF, which was consistent with report that CsA increases the proliferation of human gingival fibroblasts in vitro.

Proteomics analysis has emerged as most sensitive detector of gene alterations and aberrant gene expression, protein modification as results of physio-pathogenesis. In the present study, proteomic method was employed to detect possible changes at a protein level after CsA treatment in HGF. The concentration 10 μM of CsA treatment for 48 h in HGF for proteomics was selected in similar range of the plasma concentration (100—200 μg/ml), because CsA could induce
obviously proliferation of HGF in vitro and in vivo.\textsuperscript{12)} The present study is the first report identified the significantly changed proteins in the CsA-induced HGF proliferation using proteomics analysis. After HGF were exposed to 10 \( \mu \text{M} \) CsA for 48 h, total proteins were extracted and separated by 2-DE using pH 3—10 IPI strips in HGF, and then the profiles were stained with silver. A representation of one set among them is shown in Fig. 2. Six hundred to 800 spots of protein of approximately 500 \( \mu \text{g} \) were observed on the gel.

The initial matching process was done by software program. Accuracy of the matches was confirmed by manually comparing each gel to the reference and other individual gels for three times. The differential image analysis showed changed spots in the CsA-treated HGF. Spot numbers on 2-DE image indicate the protein spots, whose expression was differential between the CsA-treated HGF and the control (Fig. 2). Twenty proteins were consistently different between the CsA-treated group and the control on 2-DE gels. Seventeen proteins among them were upregulated in the CsA-treated HGF, whereas 3 proteins were downregulated. Based on the cut-off value of over two-fold increase or decrease, 9 out of 17 spots were significantly increased (Fig. 3) and 2 out of 3 spots were significantly decreased (Fig. 4). Only proteins with significantly altered levels were excised for identification by mass spectrometry. The spots were then excised from the profile and destained. These proteins were digested overnight by trypsin and they were analyzed by MALDI-TOF MS and by ESI-TOF MS. The results of the MALDI-TOF MS and ESI Q-TOF MS unequivocally indicated that the significantly upregulated 9 proteins and downregulated 2 proteins were identified. Tables 1 and 2 summarized the results of protein identification showing protein definition. The analyzed protein spots were all those which displayed the differential changes over two-fold of protein, suggesting that those proteins were closely related to the effects of CsA treatment in HGF. The identified proteins in the present study were fallen into three functional categories: [1] proliferation associated proteins, including eEF, Rho GDI\( \alpha \), galectin-3 and Cathepsin D, which were upregulated, whereas ANXA 2 was downregulated; [2] the metabolism associated protein, including b-phosphogluconolactonase, acetyl-CoA C-acetyltransferase, aldolase A protein and translation elongation factor 1, which were upregulated, whereas Esterase D was downregulated; [3] oxidation associated proteins, including prx 1 and glutathione-S-transferase omega were upregulated.

Peroxiredoxins (Prxs) are a family of intracellular antioxidant proteins that protect cells against the damaging effects of reactive oxygen intermediates such as H\(_2\)O\(_2\).\textsuperscript{24)} In addition to their antioxidant role, Prxs have implicated in cellular functions such as regulation of gene expression, cell proliferation and differentiation.\textsuperscript{25)} In addition, there is a possibility that apoptotic signals and regulators may be involved in the pathogenesis of CsAGO, based on that apoptosis plays an important role in the maintenance of tissue homeostasis. This hypothesis was confirmed by a recent study that up-regula-
tion of Bcl-2, an antiapoptotic molecule, and inhibition of apoptosis were involved in pathogenesis of CsAGO.26) It was also reported that Prx proteins could protect cells from apoptosis through the inhibition of c-Abl, c-Myc, NF-κB and TNF-α, and with a mechanism similar to that of Bcl-2.27) The present study showed that Prx 1 protein was significantly upregulated in the cells were treated with 1 μM and 10 μM CsA for 48 h, which was confirmed by Western blot. The protein levels of Prx 1 in the CsA-treated HGF were increased about 2 folds, compared to the control (Fig. 5A). The result was consistent with that of the 2-DE gel. Furthermore, cytosolic ROS levels were reduced clearly by CsA treatment in HGF. To determine whether CsA reduces cytosolic ROS level in HGF or not, cytosolic ROS levels were measured using DCF-DA in cells treated with 10 μM CsA for 48 h. As shown in Fig. 5B, cytosolic ROS levels were significantly reduced by CsA treatment in HGF. The previous studies and the present results suggest the possibility that Prx 1 may play a role in promoting proliferation in the CsA-treated HGF, with reducing cytosolic ROS levels as an antioxidant action, even if its underlying mechanisms were still unclear.

Galectin-3 was upregulated in CsA-treated HGF in the present study. Galectin-3 is a group of lectin and is known as eBP (Ig E binding protein), Mac-2 and CBP-30, which has a variety of biological function, including growth regulator in proliferating cell, an intermediator in cell-extracellular matrix adhesion, and inhibitor of apoptosis interacting with Bcl-2.28) The recent finding showed that galectin-3 is elevated in tumor and therefore expected as a reliable tumor marker.29) It thus has the potential that one of the many functions of galectin-3 may be related to the CsA-induced HGF proliferation. In the present study, to confirm the change of Galectin 3 levels in cells treated with 1 μM and 10 μM CsA for 48 h, Western blot was performed. In the CsA-treated HGF, the protein levels of galectin-3 were increased about 2 folds, compared to the control (Fig. 6). This result shows that the

Table 1. Identities of Significantly Upregulated Proteins in the CsA-Treated HGF

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein name</th>
<th>Accession No.</th>
<th>Measured pI/mass</th>
<th>pI/Mr (in database)</th>
<th>Folds&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>ref</td>
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<td>287</td>
<td>Rho GDP dissociation inhibitor (GDI) alpha (Rho GDI α)</td>
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<td>7557768</td>
<td>ref</td>
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<tr>
<td>302</td>
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<td>pdb</td>
<td>LYAB</td>
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<td>4504983</td>
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</tr>
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<td>86728</td>
<td>pir</td>
<td>JH0255</td>
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<td>28614</td>
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<td>12654663</td>
<td>gb</td>
<td>AAH01169.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>) Among 17 spots upregulated significantly in the CsA-treated group compared to the control, nine spots which showed over 2 folds difference, were identified by MALDI-TOF. <sup>b</sup>) Increased folds in the normalized volume of a given protein spot in the CsA-treated HGF group vs. the control.

Table 2. Identities of Significantly Downregulated Proteins in the CsA-Treated HGF

<table>
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<tr>
<th>Spot No.</th>
<th>Protein name</th>
<th>Accession No.</th>
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<th>pI/Mr (in database)</th>
<th>%&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
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<sup>a</sup>) Among 3 spots downregulated significantly in CsA-treated group compared to the control, two spots which showed over 50% difference, was identified by MALDI-TOF in this study. <sup>b</sup>) Decreased percentages (%) in the normalized volume of a given protein spot in the CsA-treated HGF group vs. the control.

Fig. 5. Expression of Peroxiredoxine 1 (Prx 1) Was Increased in the CsA-Treated HGF

After incubation of HGF with 1 and 10 μM CsA for 48 h, protein levels of Prx 1 were determined by Western blotting and β-actin served as a loading control (A). CsA reduced intracellular ROS levels in HGF. DCF-DA-loaded cells were incubated with 10 μM CsA or 5 mM NAC, a ROS scavenger. The cytosolic ROS levels were detected by measuring the DCF fluorescence. Data are mean S.D. from five independent experiments. ∗p<0.05 vs. the corresponding control value (B).
protein might attribute to the proliferation of HGF by CsA, which has not been previously reported. However, to clarify the exact role of galectin-3 in CsAGO, further studies are needed.

The other proteins significantly different between the CsA-treated group and the control in HGF might be involved in the pathogenesis of the CsA-induced gingival overgrowth. However, there is no direct evidence showing the relationship between the pathogenesis of CsAGO and 11 proteins differently were expressed in the CsA-treated HGF. This is the first report describing the relationship between these proteins and CsAGO. Further quantitative confirmation of expression changes by Western blotting may be needed to understand the pathophysiological roles of these protein in CsAGO and also comparative investigations of these proteins expression with in vivo study would be required.

In conclusion, although only a limited number of proteins were identified by proteomic analysis, the data support the notion that downregulated or upregulated proteins containing Prx 1 may play a relevant role in CsA-induced proliferation of HGF. The information obtained with this proteomic analysis provides efficient approach in understanding mechanisms of the CsA-induced proliferation of HGF.

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