

Angiotensin II stimulates expression of transcription factors c-Jun and c-Fos in cyclosporine induced human gingival fibroblasts

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ABSTRACT: The present study demonstrates that the expression of c-Jun and c-Fos are elevated in gingival fibroblast cells treated with angiotensin II and cyclosporine. The healthy human gingival tissues were collected and gingival fibroblasts were isolated and cultured. We used RT-PCR and Western blot analysis to identify the expression of c-Jun and c-Fos in cyclosporine and angiotensin II treated human gingival fibroblast cells. We found that angiotensin II in combination with cyclosporine induces c-Jun and c-Fos expressions significantly; however, the angiotensin II antagonist losartan inhibits the expression of c-Jun and c-Fos ($p < 0.01$). The data suggest that angiotensin II in combination with cyclosporine modulates the expression of c-Jun and c-Fos in human gingival fibroblast cells.

Introduction

Drug-induced gingival overgrowth is an unwanted outcome of systemic medication and is limited to gingiva. Drugs associated with gingival enlargement are anticonvulsants (phenytoin), calcium channel blockers (nifedipine) and immunosuppressant (cyclosporine-A). Although the pharmacological effect of these drugs are different and directed toward various primary target tissues, all of them seem to act similarly on a secondary target tissue, i.e. the gingiva, causing almost similar clinical and histopathological findings (Bullon *et al.*, 1994; Nakou *et al.*, 1998; Varga *et al.*, 1998). Recent evidence indicates that angiotensin II (Ang II),

the main effector peptide of the renin angiotensin system (RAS), may be an important mediator in drug-induced gingival overgrowth (Santos *et al.*, 2009). Intragingival RAS is upregulated in experimental gingival fibrosis, and Ang II levels are frequently elevated in gingival fibroblast cells treated with CsA (Subramani *et al.*, 2012). Moreover, a recent study raised the possibility that an Ang II receptor antagonist may be therapeutically efficacious for patients with liver and kidney fibrosis (Cernes *et al.*, 2011).

Transcription factors are important mediators involved in signal transduction that bind to specific DNA sequence in gene promoters, and regulated transcription activity. Emerging attention has been focused on the regulation and function of transcription factors, such as active protein-1 (AP-1) during gingival overgrowth (Eferl *et al.*, 2008; Lia *et al.*, 2007). c-Jun and c-Fos are the prototype AP-1 components. The related proteins, JunB, JunD, Fra1, Fra2, and FosB, show

expression patterns and transactivation potentials distinct from those of c-Jun or c-Fos. AP-1 is involved in cell proliferation, differentiation and apoptosis and other biological process (Eferl *et al.*, 2003). Ang II is pro-oxidant and fibrogenic cytokine that stimulates AP-1 in human fibroblasts and plays a critical role in fibrogenesis (Butaller *et al.*, 2003). To elucidate the molecular mechanisms implicated in drug-induced gingival overgrowth, the current study explored the influence and interactions of Ang II in CsA induced human gingival fibroblast cells towards the expression of transcription factors c-Jun and c-Fos.

Materials and methods

Human gingival fibroblasts (HGF) cultures

HGF were prepared as described in our previous study (Tamilselvan *et al.*, 2007). Briefly, the explants were cultured in a minimum essential medium supplemented with 10% fetal bovine serum in plastic culture dishes, with medium change every 3 days for 10 to 15 days until confluent cell monolayers were formed. After three to four subcultures, homogeneous, slim, spindle-shaped cells growing in characteristic swirls were obtained. The typical fibroblast cultures prepared were used as confluent monolayers at subculture levels 5 to 10. The explants of gingival fibroblast were cultured in 60-mm plastic tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 0.4 mg/ml amphotericin B in 5% CO₂ at 37°C until confluent cell monolayers were formed. When the cells became confluent, they were harvested and subcultured. Gingival fibroblast cultures were classified as i) CsA-treated gingival fibroblast culture (C-HGF); ii) Ang II treated gingival fibroblast culture (A-HGF); iii) CsA and Ang II (LAC-HGF); vii) healthy gingival fibroblast culture (CONT). HGF cells in cultures at the sixth

to eighth passages were seeded at a density of 3.5x10¹⁰ cells/60 mm plastic tissue culture well and maintained in 5ml of medium. After 8 days of culture, these cells were washed three times with phosphate buffered saline. CsA (1 mg) was dissolved in 100 ml ethanol and added to 900 ml of serum free culture medium (DMEM) to make a 10⁻³ mg/ml stock solution. Experimental medium was prepared by adding 25 ml of this stock to 50 ml of DMEM, yielding a final drug concentration of 500 ng/ml. This concentration approximates gingival tissue levels of CsA in clinical settings and was previously shown to induce evidence of toxicity in HGF. Ang II (40 pmol/single well) was added to the medium (500 µl/well). In experiments in which Ang II antagonist was used, it was added 15 min before Ang II. The cells were exposed up to 24 hours. Appropriate solvent controls were carried out, and no detectable effect on cell behavior was noted.

Isolation of total RNA and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA was isolated from the treated HGF cells by single step, acid guanidium thiocyanate phenol chloroform extraction method (Chomczynski and Sacchi, 1987). The total RNA was transcribed to cDNA using First Strand cDNA synthesis Kit (Qbiogene, Chandigarh, India) for reversetranscriptionpolymerasechainreaction(RT-PCR) according to manufacturer instructions. The primers were designed from the known sequences as: c-Jun 5'-AGCTCTGCTTCACAGCGC-3' (sense) and 5'-GGCCTCCTGTCATGGTCTT-3' (antisense); c-Fos 5'- -3' and 5'-GAACCCCTCCTGCTCATCTGCACGTTCTT-3' (antisense). As a positive control β-actin primers designed as 5'-AAGGATTCCTATGTGCCG-3' (sense) and 5'-CATCTCTTGCTCGAAGTC -3'. Primers were from Biocorporals, Chennai, India and other laboratory chemicals were from Merck, Chennai, India. The amplification profile was as follows: denaturing at 94°C for 1 min; annealing at

optimal temp (55°C for c-Jun, 63°C for c-Fos and 53°C for β -actin) for 2 min, extension at 72°C for 2 min. The cDNA was amplified in Perkins Elmer PCR analyzer for 36 cycles followed by a step of 10 min at 72°C to extend the partially amplified products. These cycling conditions were established empirically to give linear increase in product intensity proportional to the amount of template. The PCR products were electrophoresed using Bang Genei electrophoresis apparatus on 1.2% agarose gel (Hi Media, Chennai, India) and visualized by ethidium bromide (Sigma, New Delhi, India) staining. The gels were photographed and their image data were analyzed by Bio-Rad image analyzer (1D analysis). The relative amount of each gene expression was calculated as the ratio of the individual c-Jun and c-Fos to the intensity of β -actin gene products as the control. The relative expression of each gene such as c-Jun and c-Fos from control and treated HGF were compared.

Western blot analysis

Western blot analysis of CsA treated HGF cells were performed after standard procedure. Briefly, the treated HGF cells were homogenized in an ice-cold RIPA lysis buffer (Santa Cruz, USA). The lysates were centrifuged and the supernatant was collected. The protein concentration from HGF cell lines was determined. Protein samples (50 μ g of fibroblasts cell lysates) were separated with 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. After the transfer, membranes were blocked at room temperature for 2 h with 5% bovine serum albumin in TTBS (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20). The membranes were then incubated with anti-c-Jun or anti-c-Fos (1:2000) in 0.5% bovine serum albumin in TTBS overnight at room temperature. Corresponding secondary antibody conjugated to horseradish peroxidase (Santa Cruz, USA) were used. The membranes were developed with an enhanced chemiluminescence detection system (Thermo Scientific, IL) and exposed on X-ray films. Densitometry was performed using a documentation program and the data were

expressed as mean \pm SEM.

Statistical methods

The difference in the levels of expression of the c-Jun and c-Fos genes between control and treated HGF was analyzed by the Student's t-test. Results are presented as mean \pm SEM. P values <0.05 were considered statistically significant.

Results

Ang II-stimulated gingival fibroblast cells were assessed by analyzing western blot technique. Ang II increased c-Jun and c-Fos proteins in HGF in a time dependent manner. The maximum level of incorporation was 2.6 fold increase compared with control. Ang II stimulated protein synthesis in HGF was inhibited by AT₁ receptor antagonist losartan (1 μ M, Fig 1a, 1b). On the other hand, CsA stimulated AP-1 expressions was assessed by analyzing c-Jun and c-Fos expressions.

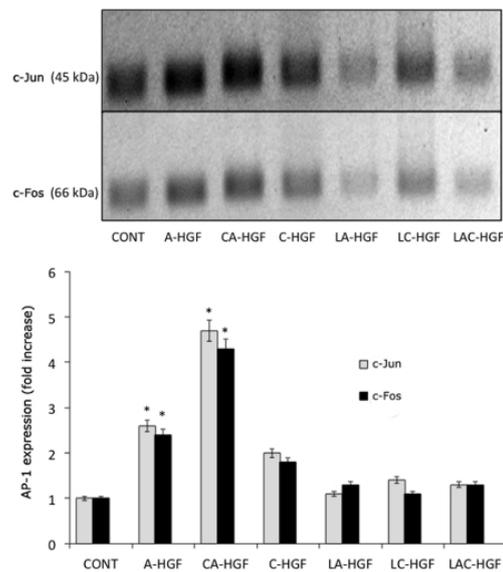


FIGURE 1: (a) Representative immunoblots of different treatments showed single bands of c-Jun and c-Fos. (b) Messenger RNA expression (c-Jun and c-Fos, relative to β -actin) after different treatments (mean \pm SEM n=3). Abbreviations: CONT, untreated HGFs; A-HGF, Ang II treated HGFs; CA-HGF, CsA-Ang II treated HGFs; C-HGF, CsA treated HGFs; LA-HGF, losartan-Ang II treated HGFs; LC-HGF, losartan-CsA treated HGFs; LAC-HGF, losartan-Ang II-CsA treated HGFs. Stars indicate statistically significant differences from CONT.

After the incorporation of CsA for 0-10 hrs at 37°C produced a significant increase in c-Jun and c-Fos expressions (Fig 1a, 1b). The maximum level of expression was 2-fold increase compared with the control. The treatment of gingival fibroblast cells with CsA-Ang II for 0-10 hours at 37°C produced a significant increase in c-Jun and c-Fos expressions compared to either individual CsA/Ang II or control HGF cells (4.7 fold increase vs control; 2.3 fold increase vs CsA; 1.7 fold increase vs Ang II, Fig.1b).

This effect of was inhibited considerably by pretreatment with losartan (Fig. 1b). These data suggest the possible role of Ang II as an autocrine growth factor for the proliferation of gingival fibroblasts under CsA stimulation. To further determine whether the increased protein levels of c-Jun and c-Fos were due to increased gene expression, we examined the mRNA levels of c-Jun and c-Fos in HGF treated with Ang II and CsA by relative RT-PCR. The results showed statistically significant elevation 4.8 fold increase of c-Jun and c-Fos mRNA in the CA-HGF ($p < 0.0002$, Fig 2) and thus confirmed the established elevation of c-Jun and c-Fos protein levels. The effect of AT1 receptor blocker losartan on the Ang II-increased c-Jun and c-Fos activity was also investigated. HGF cells were pretreated with losartan (1 μ M) for 1 hr and subsequently stimulated with Ang II and CsA for 10 hrs. The Ang II induced c-Jun and c-Fos gene expression was inhibited by losartan (Fig 2). These data show Ang II directly induces c-Jun and c-Fos expression in HGF.

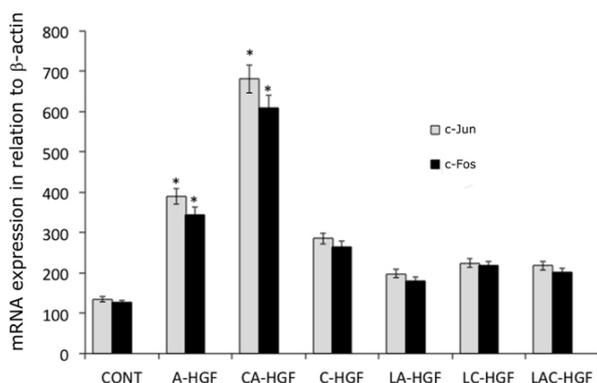


FIGURE 2: Angiotensin II increased c-Jun and c-Fos mRNA expression in HGF cells. The total RNA was isolated, and RT-PCR was performed using gene-specific primers. β -actin was used as the internal control for PCR. The results are shown as mean \pm SEM ($n=3$ per group). Stars indicate statistically significant differences from CONT.

Discussion

Our study showed that Ang II modulates the expression of transcription factors c-Jun, c-Fos in relation to CsA in gingival fibroblasts cells. The previous reports from our group have shown the existence of intrinsic gingival renin angiotensin system (RAS) components as well as augmented Ang II expression in gingival fibroblast cells treated with CsA (Tamilselvan *et al.*, 2012). These data suggested that CsA stimulates Ang II expression in gingival fibroblast cells. Ang II induces constriction, hypertrophy, and proliferation of vascular smooth muscle via the AT1 receptor (Peach, 1977; Leung, 2004; Suzuku *et al.*, 2003). It is generally assumed that the therapeutic effects of AT1 antagonists are due to direct receptor blockade; however, a secondary effect of these agents is increased plasma levels of Ang II, which may then activate AT2 receptors (Dandona *et al.*, 2007). This receptor bears only 34% sequence homology to the AT1 receptor and mediates growth inhibition and apoptosis of vascular smooth muscle. Accumulating *in vitro* and *in vivo* evidence indicates that Ang II, via the AT1 receptor, plays a central role in the onset and development of drug-induced gingival overgrowth (Ohuchi *et al.*, 2002). However, the investigations on molecular mechanisms of Ang II-mediated gingival hypertrophy are almost limited to *in vitro* studies on cultured gingival fibroblasts, and the *in vivo* mechanism of Ang II-mediated pathological gingival hypertrophy remains poorly understood.

Ang II is potentially induces AP-1 that regulate the processes such as proliferation, differentiation, apoptosis and transformation (Tharaux *et al.*, 2000; Fiebeler *et al.*, 2001; Schoder *et al.*, 2006). Nonetheless, the signaling mechanism underlying effects of Ang II on AP-1 pathway in gingival fibroblast remains to be fully elucidated. To investigate further, we analyzed AP-1 activity in HGF cell lines treated with Ang II and CsA. Our findings demonstrated the signal transduction mechanism underlying effects of CsA, Ang II in relation to c-Jun and c-Fos activity in gingival fibroblast cells. The induction of c-Jun protein could be detected after 2 hours following Ang II-CsA addition and reached a maximum 2-8 h after Ang II-CsA stimulation, whereas the induction of c-Fos was detected as soon as 1 hour and achieved a maximum 4 hours after Ang II-CsA

stimulation. These findings suggest that c-Fos may play a major role in the earlier course and c-Jun may play a major role in the later course of the biological activity of gingival fibroblast. Interestingly, various gingival fibrosis related genes, such as transforming growth factor- β 1, collagen, whose expressions are enhanced in gingival hypertrophy, have the AP-1 consensus sequence in their promoter regions. Previously, Izumi *et al.*, 2000 reported that losartan significantly inhibits the cardiac hypertrophy related gene expressions in stroke-prone spontaneously hypertensive rats. Furthermore, in another study found that the activation of cardiac JNK in rats by Ang II infusion is followed by a significant increase in cardiac AP-1 activity (Kim *et al.*, 1998). Found that the activation of cardiac JNK in rats by Ang II infusion is followed by a significant increase in cardiac AP-1 activity (Kim *et al.*, 1998). muscle via the AT1 receptor (Peach, 1977; Leung, 2004; Suzuki *et al.*, 2003). It is generally assumed that the therapeutic effects of AT1 antagonists are due to direct receptor blockade; however, a secondary effect of these agents is increased plasma levels of Ang II, which may then activate AT2 receptors (Dandona *et al.*, 2007). This receptor bears only 34% sequence homology to the AT1 receptor and mediates growth inhibition and apoptosis of vascular smooth muscle. Accumulating *in vitro* and *in vivo* evidence indicates that Ang II, via the AT1 receptor, plays a central role in the onset and development of drug-induced gingival overgrowth (Ohuchi *et al.*, 2002). However, the investigations on molecular mechanisms of Ang II-mediated gingival hypertrophy are almost limited to *in vitro* studies on cultured gingival fibroblasts, and the *in vivo* mechanism of Ang II-mediated pathological gingival hypertrophy remains poorly understood.

These findings led to examine the effect of losartan on c-Jun and c-Fos expression in gingival fibroblasts treated with CsA either alone or combination with Ang II. Notably, losartan was significantly decreased AP-1 expression in gingival fibroblasts. Further experiments are needed to determine whether Ang II and CsA modulate AP-1 activity directly or via known AP-1 regulators, such as MAPK, will provide further insight into the mechanism of development of drug-induced gingival overgrowth. In conclusion, our study provides

the first evidence that Ang II, either alone or in combination with CsA, modulates the expression of c-Jun and c-Fos, and suggests that AP-1 may in part be responsible for gingival tissue remodeling.

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