17β-estradiol and progesterone upregulate cyclooxygenase-2 expression in the human gingival fibroblasts

[1]Ostad S. N., [2]Motahhary P., [2]Beshkar M., [*1]Ghahremani M.H.

[1]Department of Toxicology and Pharmacology, Faculty of Pharmacy, [2]Department of Oral and Maxillofacial Pathology, Faculty of Dentisty, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Gingivitis is associated with 60-75% of all pregnancies and elevated levels of 17β-estradiol and progesterone is known to increase gingival inflammation and the proinflammatory prostaglandins in the human gingiva. Since cyclooxygenase-2 (COX-2) is an inducible enzyme responsible for the production of prostaglandins at the sites of inflammation, it is plausible to hypothesize that 17β- estradiol and progesterone could contribute to gingival inflammation by upregulation of COX-2 expression and subsequent prostaglandin formation. To examine this hypothesis, primary cultures of human gingival fibroblasts (HGFs) from either sex were established. The cells were treated with different concentrations (10^{-5}, 10^{-7}, and 10^{-9} M) of 17β-estradiol and progesterone, and expression of COX-2 protein was detected immunocytochemically. The growth potential and proliferation of these cells were studied using trypan blue exclusion method and MTT assay. The results show that both 17β-estradiol and progesterone upregulate COX-2 expression in the HGFs significantly. In addition, progesterone is more effective than 17β-estradiol to induce COX-2 expression at 10^{-5}M but not at lower concentration (10^{-9}M). Furthermore, cells prepared from either sex do not show any difference in COX-2 expression following hormone treatment and neither hormones show any changes in proliferation of these cells. In conclusion, the results of this investigation clearly illustrate significant regulatory effects of 17β-estradiol and progesterone on COX-2 expression in the cultured HGFs. Thus, one possible pathogenetic mechanism of the female sex hormone-associated gingivitis *in vivo* may be the synthesis of proinflammatory prostaglandins via upregulation of COX-2 expression by gingiva in response to elevated levels of circulating estrogens and progesterone.

Keywords: Gingivitis, Sex hormone, COX-2, Prostaglandin, Immunocytochemistry.

INTRODUCTION

Gingival inflammation is the most common oral manifestation of elevated levels of circulating estrogens and progesterone in conditions such as pregnancy and oral contraceptive usage (1-3). There are several lines of evidences elucidating various aspects of the relationship between these hormones and gingival inflammation (4, 5). Gingival inflammation has been characterized by an increase in formation of prostaglandins (PGs) within the gingival tissue (6). It has been reported that sex hormones increase PG synthesis in various cells (7-9). In endothelial cell, progesterone has upregulates PG production and expression of COX enzyme (7). Furthermore, in cultured human uterine myometrial cells, 17β-estradiol up-regulates prostacyclin production via an increase in expression of both COX-1 and COX-2(10). In this regard, data from in vitro investigations suggested that increase of PG in the human gingival tissue is mediated by the upregulation of cyclooxygenase-2 (COX-2) isoenzyme in the gingival fibroblasts and subsequent PG formation (11). On the other hand, PG synthesis has been shown to be stimulated by 17β-estradiol and progesterone in the human gingival tissue homogenate (12). However, it is unclear whether COX-2 is upregulated in the human gingival fibroblasts stimulated by estradiol and progesterone.

These evidences converge to formulate a hypothesis by which 17β-estradiol and progesterone give rise to gingival inflammation. Therefore, we have investigated the effect of 17β-estradiol and progesterone on human primary gingival fibroblasts and evaluated the expression of COX-2. Stimulaton of the human gingival fibroblasts (HGFs) with estradiol and progesterone may give

Corresponding: mhghahremani@tums.ac.ir

rise to upregulation of COX-2 expression and subsequent prostaglandin production and gingival inflammation.

MATERIALS AND METHODS

Chemicals and Materials

All cell culture materials were obtained from PAN Biotech (Aidenbach, Germany); FBS was purchased from Gibco (Paisley, UK) and all culture dishes were obtained from NUNC (Roskilde, Denmark). Amphotericin B, hematoxylin, MTT, bovine serum albumin, progesterone and 17β-estradiol were purchased from Sigma-Aldrich (Steiheim, Germany). Rabbit polyclonal anti COX-2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, USA) and immunocytochemical detection was performed using biotin-streptavidin-conjugated ABC kit from Dako Cytomation (Glostrup, Denmark).

Fibroblast cultures

Primary cultures of human gingival fibroblasts (HGFs) were established from gingival biopsies obtained from six healthy individuals (three males and three females), age of 19-26 years with no clinical signs of periodontal disease. All biopsies were obtained from retromolar gingival tissue during surgical extraction of impacted third molars. Informed consent was obtained from all donors prior to the surgical procedure. HGFs were obtained from tissue explants using a slight modification of a previously established protocol (13). Briefly, explants were rinsed in phosphate buffered solution (PBS) and finely chopped into pieces of about 1-2 mm^3. Tissues were then placed in 6-well plates and carefully layered with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1μg/ml amphotericin B. The explants were incubated undisturbed at 37°C in a humidified atmosphere with 5% CO_2. After 10 days of incubation, the cells were visible as outgrowth from explants. The medium was changed every three days until HGFs outgrowth reached 70-80% confluence. The cells were then harvested using trypsin (0.05%), EDTA (0.53 mM) and transferred to a 75cm^2 tissue culture flask. In all experiments, cells with the passage number of 3-10 were used.

Growth potential study

The growth potential was studied for 120 hours on cells obtained from individuals. The cells were seeded in 24-well plates at 1×10^4 /well in triplicate. At the indicated time intervals, cells were harvested and counted using trypan blue exclusion method. The effect of various concentrations of progesterone and 17β-estradiol on prolife-ration of these cells was studied using MTT assay. Briefly, cells were plated at 6000/well in 96-well plates and 24 hours after plating, the drugs were added to the media and incubated for 24 hours. Then, cells were incubated for 3 hours with MTT at final concentration of 0.5mg/ml. After incubation, medium was removed and the formazan crystals were dissolved in DMSO and the color was measured at 570 nm in a microplate reader.

Drug treatment

The drug treatment of the HGF cells was performed as follows: PBS (as negative control or vehicle group), 10^{-5}M 17β-estradiol, 10^{-9}M 17β-estradiol, 10^{-5}M progesterone, 10^{-9}M progesterone, and a combination of 10^{-7}M 17β-estradiol and 10^{-7}M progesterone. The drug treatment was for 24 hours. Progesterone and 17β-estradiol were the water soluble forms specially prepared for tissue culture (Sigma-Aldrich, Germany) and were prepared in PBS at 100x stock solution.

Immunocytochemical procedure

HGFs were seeded at 5×10^4 cells/well in 4-well chamber slides using DMEM, penicillin/ streptomycin (100 U/ml and 100 mg/ml, respectively) and 10% FBS, and were allowed to adhere overnight. The following day, cells were washed with PBS and rendered quiescent by culturing for 24 hours in fresh medium containing 1% FBS. After the drug treatment period, the cells were fixed with 95% ethanol: 5% acetic acid at 4°C for 5 min. The fixed cells were washed and incubated overnight at 4°C with 1% bovine serum albumin to block nonspecific binding. Following the blocking period, slides were incubated overnight at 4°C with polyclonal rabbit anti COX-2 antibody. Then, the primary antibody was washed by Tris buffer and the slides were incubated for 30 min at room temperature (RT) with 3% H_2O_2 solution to inactivate endogenous peroxidase activity. This step was followed by incubation for 30 min at RT with biotinylated secondary antibody. COX-2 expression was visualized by addition of streptavidin-conjugated horse raddish peroxidase and diaminobenzidine tetrahydrochloride (DAB) as chromogen. Finally, slides were counterstained with hematoxylin and mounted in an aqueous mounting medium. Negative control staining consisted of the same procedure without primary antibody. After immunocytochemical staining, slides were examined under microscope and analyzed using the Olysia Bioreport software (Olympus, Japan). Digital image analysis was performed on the basis of cytoplasmic immunostaining intensity. For each chamber, at least five random fields were digitized and in each fields, DAB signal were measured in 100 cells

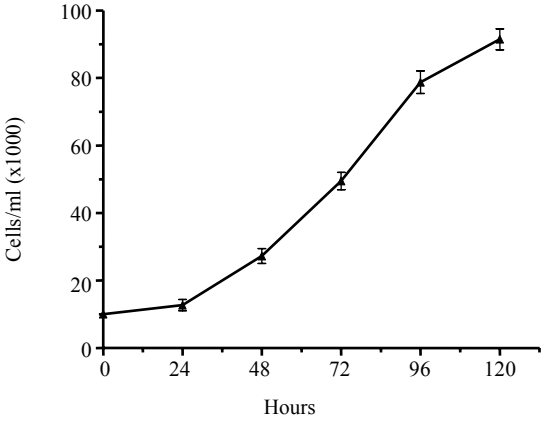

Figure 1. Growth potential of cultured human gingival fibroblasts. The cells were seeded at $1x10^4$ /ml in triplicate and counted by trypan blue exclusion method.

based on the pixel and was normalized to the background intensity (at ×200 magnification) (14, 15). The intensity of COX-2 expression obtained for each experiment was divided to the basal intensity in PBS treated group and reported as fold increase.

Statistical analysis

Each experiment was repeated in triplicate and the result from three independent experiments was reported as Mean ± SEM. The results were analyzed by general linear modeling and Mean from each independent experiment was analyzed using one-way analysis of variance (ANOVA) with Tukey test as *post hoc* multiple comparisons of specific groups and $p < 0.05$ was considered significant.

RESULTS

Primary Culture of HGF cells

Primary outgrowth of cells derived from human gingival tissue samples was observed after 10 to 15 days. As examined by inverted phase-contrast microscopy, almost all the cells which grew up from the gingival tissue explants had the morphology of gingival fibroblasts. Figure 1 shows the growth potential of one of the cultured human gingival fibroblasts obtained from healthy individuals. The HGF cells, seeded at 1×10^4, were doubled after 36 hours and were reached to 4×10^4 cell at 64 hours after incubation. The cells started to reach plateau phase in 120 hours after plating. The growth potential test was performed for all the HGF cells obtained from individuals. There was no difference in growth potential of HGF cells among the individuals. Furthermore, there was no significant difference in growth potential of cells obtained from either sexes (data not shown). It has been recognized that HGFs are heterogeneous *in vivo* (16), and the *in vitro* maintenance of these cells tends to reduce that heterogeneity by the process of selection. Thus, the length of time in culture was limited to 10 passages to preserve natural heterogeneity.

Immunocytochemical staining of COX-2 protein

Results demonstrate that HGFs resting in growth medium containing 1% FBS, as negative controls, do not express statistically significant amounts of COX-2 protein. However, when stimulated by 17β-estradiol and/or progesterone, HGFs significantly express higher levels of COX-2 protein (Fig. 2 a,b,c). The COX-2 expression has been disperse and mainly cytoplasmic. No nuclear or membrane accumulation of the protein expression was detected (Fig. 2). The result from quantitative analysis of the COX-2 expression is shown in figure 3. Compared to the negative controls, cells treated with 10^{-5}M and 10^{-9}M of 17β-estradiol, have increased COX-2 expression by 4.44 and 3.13 folds, respectively (*P<0.01*, compared to no treatment). When stimulated with 10^{-5}M and 10^{-9}M of progesterone, the cells have expressed 5.64 and 3.53 fold increases in COX-2 expression, respectively (Fig.3, *P<0.01*, compared to no treatment). These results also indicate that progesterone at 10^{-5} M has a more potent effect on induction of COX-2 expression than 17β-estradiol at equal doses, although this effect is not significant at 10^{-9}M .In HGF cells treated with a combination of 10^{-7}M 17β-estradiol and 10^{-7}M progesterone, the COX-2 expression compared to control cells increased by 5.16 fold. This result indicates that the combination of these hormones (10^{-7}M of each) has greater induction of COX-2 expression than 17β-estradiol at 10^{-5} M but slightly smaller that progesterone at 10^{-5}M. Furthermore, when the data was analyzed based on the sex of the individuals (Figure 4.), statistical analysis revealed that the induction of COX-2 expression was not sex related with neither of the hormones.

The effect of Progesterone and 17β-estradiol on growth potential of HGF cells

The effects of progesterone and 17β-estradiol on proliferation of HGF cells were tested (Fig. 5). The results indicated that 24 hours treatment of HGF cells with various concentrations of progesterone and 17β-estradiol (10^{-9}, 10^{-7}, 10^{-5}M) had no significant effect on proliferation of these cells. Treatments of cells with combination of progesterone and 17β-estradiol (10^{-9}, 10^{-7}, 10^{-5}M of each) had a very small inhibitory, but not significant, effect on HGF cell growth (Fig.5). The experiment was performed on cells obtained from either sex and no significant differences was observed (data not shown).

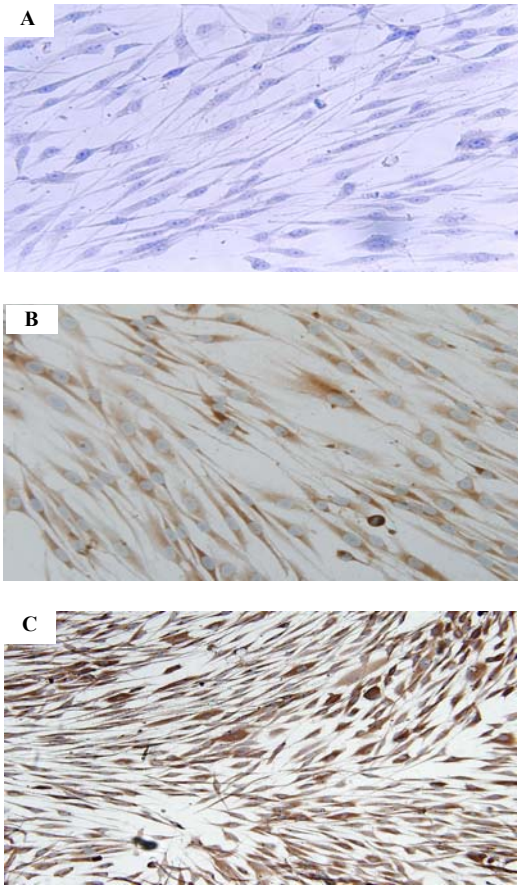

Figure 2. Immunocytochemically analysis of COX-2 expression in HGF cells. HGF cells with no hormonal treatment (*A*, negative control), stimulated by 17β-estradiol (*B*) or progesterone (*C*) were immunocytochemically stained for COX-2 expression.

DISCUSSION

Using immunocytochemical staining of COX-2 protein, it was demonstrated that COX-2 is upregulated in HGFs treated by 17β-estradiol and progesterone (Fig.2, 3). It is well documented that COX-2 plays a pivotal role in generating high levels of proinflammatory prostaglandins locally resulting in gingival inflammation (11, 17-20). Thus, it is reasonable to suggest estradiol- and progesterone-mediated upregulation of COX-2 expression in HGFs. The HGF cells are the most abundant cells in the gingival and could contribute to the phenomenon of female sex hormone-associated gingivitis.

In recent years, in vitro cell culture assessment has been widely used to address the cellular responses to specific factors in an attempt to predict the in vivo effects of these bioactive molecules. Although fibroblasts have classically been described as engineers of the extracellular connective tissue matrix, recent investigations have markedly expanded their functions such as synthesis of various important mediators of inflammatory processes including prostaglandins (5, 11, 20-24). It is noteworthy that the presence of both estrogen (25, 26) and progesterone receptors (22) in HGFs have been demonstrated previously, indicating that these cells possess the cellular elements necessary for response to these hormones.

In the inflammation phenomena, the role of prostaglandins (PGs) has been well characterized. Although the production of PGs is being managed by the COX-1 (constitutive) and COX-2 (inducible) forms of the COX enzyme, the role of COX-2 in inflammation has been explicit (17, 18, 27). It is known that gingival fibroblast contributes to inflammation process of gingiva by production of proinflammatory mediator including PG (11, 28, 29). It has been shown that lipopolysaccharide from periodontal pathogens, IL-1β, EGF, nicotin, serum and sex hormones can increase PG synthesis (5, 10, 12, 20, 23, 28, 29). Previous study on gingival homogenate has reported that progesterone and 17β-estradiol can induce PG formation at low concentrations (10^{-9} and 10^{-7} M) but can inhibit that in higher concentrations (10^{-5} and 10^{-3} M) (12), suggesting a regulatory role for female sex hormones on PG production. Furthermore, it has been demonstrated that the structure of COX-2 gene is consistent with that of an "immediate early-response" gene product which can be rapidly upregulated in response to appropriate bioactive molecules (19), such as hormones. It has been reported that COX-2 expression is increased during inflammation in gingival fibroblasts (11, 20, 21, 28-30). Moreover, progesterone and estrogen can stimulate COX-2 expression in human uterine myometrial cells (10), human uterine microvascular endothelial cells (31) and human endothelial cells (7, 8). These findings are in agreement with our in vitro findings. Which indicate an upregulation of COX-2 expression at 10^{-9} and 10^{-7} M of 17β-estradiol and progesterone (Fig. 3). Furthermore, the unstimulated HGFs is expressed at very low and statistically not significant levels of COX-2 protein, whereas stimulated HGFs are expressed at high levels of this enzyme. These data taken together with the results from tissue homogenate (12) indicates an increase in PGs and the enzyme responsible for PG production, i.e. COX-2 protein. Since HGF cells express progesterone and estrogen receptors (22, 25), the stimulation of COX-2 expression in HGF cells suggests a regulatory role for sex hormone receptors in this finding. Although we have utilized cells from either sexes, the growth potential (data not shown) as well as effect of 17β-estradiol and progesterone on COX-2 expression was not affected (Fig. 4). This is interesting since it could indicate the elimination of the effect of sex hormone or lack of

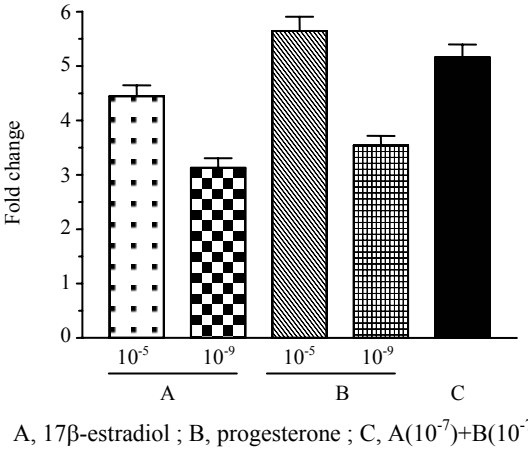

Figure 3. The COX-2 expression level in HGF cells. The cells obtained from six different individuals were treated with 17β-estradiol and progesterone with indicated concentrations. The intensity of the expression was measured as described in methods and materials and the change in expression was calculated as fold change compared to control cells (no treatment). The results are indicated as Mean ± SE of six independent cell clones (n=6) treated in triplicate.

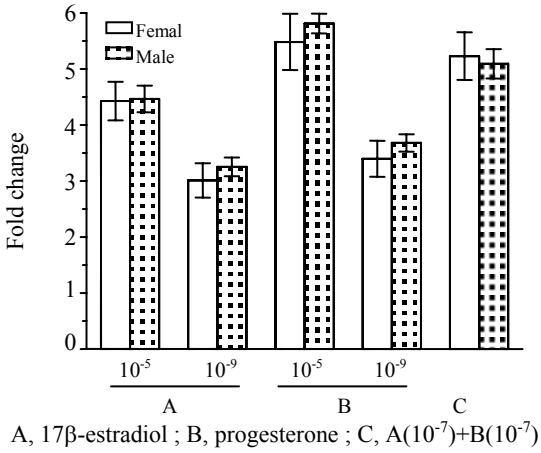

Figure 4. The effect of sex on the COX-2 expression level in HGF cells. The cells obtained from three male and three female individuals were treated with 17β-estradiol and progesterone with indicated concentrations. The intensity of the expression was measured as described in materials and method and the change in expression was calculated as fold change compare to control cells (no treatment). The results are indicated as Mean ± SE of three independent cell clones for either sex (n=3) treated in triplicate.

the effects of male sex hormone on COX-2 expression. Since the cells were used after their 3rd passage, it is reasonable to consider that the sex hormone dependent gene transcription of protein has been eliminated. Thus, sex dependency of COX-2 expression has been abolished in this method. In addition, our results indicate that hormone treatment of HGF cells has no significant effect on the proliferation of these cells and proliferation has been reduced when treated with either hormones (Fig.5). These results are in agreement with Christoffers et.al findings (32) where they have found no significant effect on gingival fibroblast proliferation by sex hormones at low concentrations. However, in concentrations higher that 50 μM, progesterone inhibits the growth. Our result indicates that the effect of sex hormones on induction of COX-2 is not dependent on the proliferation of these cells.

Even though the half-life of COX-2 protein has been reported to be relatively short (33), our results show that expression of this enzyme by HGFs in response to 17β-estradiol and progesterone was elevated even after 24 hours of stimulation. These results are consistent with data in smooth muscle cells (34) and suggest that COX-2 protein may be more stable in these cell types. One advantage of immunocytochemical analysis of protein expression is to study the localization of the protein in the cell. There has been suggestions of localization of COX-2 protein to membrane upon induction or even physical interaction with p53 protein (35) in cancer cells. Our results indicate that the expression COX-2 has

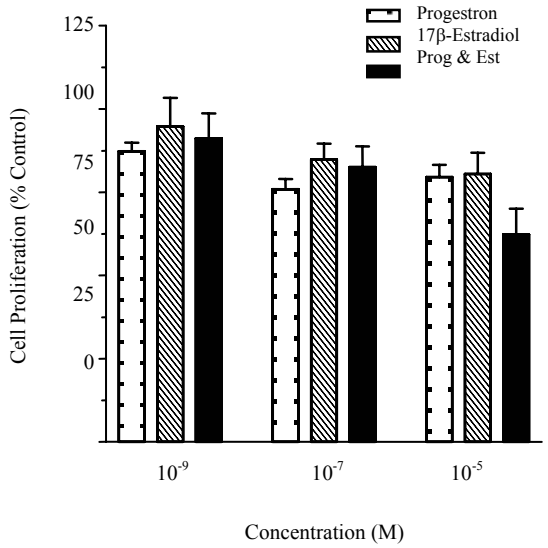

Figure 5. The effect of Progesterone and 17β-estradiol on the growth of HGF cells. The cells were treated with either progesterone, 17β-estradiol or both at indicated concentrations for 24 hours. The growth was measured using MTT assay. The data obtained from drug treatments were normalized to control non treated cells and plotted. The results indicate Mean ± SE of eight replica (n=8). The experiment was repeated twice independently.

been disperse and mainly cytoplasmic (Fig. 3) and we could not detect any nuclear or membrane associated expression similar to our findings in BAE-1 endothelial cell (14).

CONCLUSION

Data obtained from this in vitro experiment suggest that one possible pathogenetic mechanism of the female sex hormone-associated gingivitis *in vivo* may be the synthesis of proinflammatory prostaglandins, such as prostaglandin E2, via upregulation of COX-2 expression by present in gingiva in response to elevated levels of circulating estrogens and progesterone. However, further *in vitro* and *in vivo* studies with selective COX-2 inhibitors are needed to elucidate the potential usefulness of these agents as therapeutic adjuncts in the management of female sex hormone-associated gingivitis.

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