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The effects of folic acid application on IL-1β levels of human gingival fibroblasts stimulated by phenytoin and TNFα *in vitro*: a preliminary study

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Abstract: Folic acid (FA), that is required for the integrity of gingival tissues, was found to decrease in patients using phenytoin (PHT). Interleukin-1 beta (IL-1β) was reported to enhance the extracellular matrix synthesis in fibroblasts. The purpose of this study was to assess the role of FA supplementation on PHT-induced overgrowth by investigating its effect on IL-1β production of human gingival fibroblasts induced by tumor necrosis factor alfa (TNFα) in cell culture. PHT (20 μg/ml), FA (20 or 40 ng/ml) + PHT, PHT + TNF (10 ng/ml), FA (20 or 40 ng/ml) + PHT + TNF, or only culture medium (control) was added to 24-well plates containing fibroblasts. After an incubation period of 72 h, culture medium and cells were harvested separately. Then, IL-1β levels in cell lysate were measured using enzyme-linked immunosorbent assay. The cellular IL-1β level in the PHT group was 1 pg/ml. In PHT + 20 or 40 ng/ml FA-added cultures, the results obtained respectively were 0.8 and 0.7 pg/ml, whereas the control group value was 0.7 pg/ml. IL-1β level was 4 pg/ml in the cultures that PHT and TNFα were applied simultaneously ($P < 0.05$). When PHT and either 20 or 40 ng/ml FA were simultaneously added into TNFα-induced cultures, the IL-1β levels were 1.8 and 1.3 pg/ml, respectively. IL-1β level in gingival tissues might play a role in PHT-induced overgrowth by increasing in the gingival tissues, and FA application might play a role in decreasing gingival tissues. However, further studies are needed for a more complete understanding of PHT-induced gingival overgrowth at the cellular level. (J. Oral Sci. 43, 255-260, 2001)

Key words: phenytoin, gingival overgrowth, folic acid, tumor necrosis factor alfa, interleukin-1 beta.

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Introduction

The antiepileptic drug phenytoin (PHT) induces gingival overgrowth as a side effect in approximately 50% of the patients receiving this drug (1). PHT-induced gingival overgrowth is characterized with increased amount of extracellular matrix production (2), but the mechanism behind this increase is still unclear. Overgrowth of the gingival tissues can result in the accumulation of oral bacteria on the blunted margins; therefore, the amount of the overgrowth was further increased when associated with inflammation induced by microbial dental plaque (3). Elevated levels of particular mediators, such as interleukin-1 beta (IL-1β) and tumor necrosis factor alfa (TNFα), were detected in the crevicular fluid in inflamed gingival tissues (4-7). These cytokines that were accepted to derive from gingival fibroblasts, monocytes and lymphocytes, had regulatory effects on fibroblasts (8,9). The cytokine IL-1β was also reported to enhance the extracellular matrix synthesis in human gingival fibroblasts (10-12). TNFα has been known for its ability to induce hemorragic necrosis of tumours (13).

Recently, TNFα was shown to induce IL-1β production in human gingival fibroblasts (14). It was reported that when

TNFα and PHT were applied simultaneously, they further enhanced IL-1β production of the fibroblasts *in vitro* (15). It was known that PHT interferes with folic acid (FA) metabolism (16) and inhibited the use of FA at the cellular level (17). Supplementation with FA was found to inhibit the amount and incidence of PHT-induced gingival overgrowth in cats (18) and in man (19). The purpose of this study was to assess the role of FA supplementation on PHT-induced overgrowth by investigating its effect on IL-1β production of fibroblasts induced by TNFα in cell culture.

Materials and Methods

Human gingival fibroblasts were isolated from gingival papilla internally during orthodontic tooth extraction procedures in four patients. The gingival explants were minced with sterile blades, placed in a Petri dish and supplemented with Dulbeco's Modified Eagle Medium (DMEM) and 10% foetal calf serum (FCS) at 37 °C in an atmosphere of 95% air and 5% CO_2. After overgrowth of the cells from the explants, cells were released by trypsinization and were placed into culture flasks. The cells reaching confluency were washed three times with phosphate buffer solution then incubated with 2 ml of 0.05% trypsin for 3 minutes. The eighth and ninth passage cells were re-suspended in medium then placed in 24-well plates in a concentration of 1×10^5cell/well in a triplicate manner. PHT (20 μg/ml), FA (20 or 40 ng/ml) + PHT, PHT + TNF (10 ng/ml), and FA (20 or 40 ng/ml) + PHT + TNF were added into the culture wells in a final concentration. The normal range of serum folate is 6-20 ng/ml (16), so 20 ng/ml, or the double concentration of the highest serum folate level (40 ng/ml), was used for the experiment. Control wells containing only culture medium were also included. After an incubation period of 72 h (20), culture medium and cells were harvested separately and were collected in the tubes. However, culture mediums were discarded and not used in this study. Then the cells were lysed by freeze-thawing and ultrasonication. After centrifugation, the amount of IL-1β in the samples was determined by enzyme-linked immunosorbent assay (ELISA) (Peninsula Lab., Europe Ltd., England). Statistical differences between the groups were determined with the Mann Whitney-U test using a computer program (SPSS 9.0 for Windows 98).

Results

The mean cellular IL-1β level in the PHT group was 1 pg/ml. There were decreases observed in IL-1β level when 20 or 40 ng/ml FA were added to the PHT-added cultures. The results respectively were 0.8 and 0.7 pg/ml. IL-1β level in the control group was 0.7 pg/ml. There were no statistically significant differences found between the PHT or PHT + FA and the control groups (Fig. 1). The maximum production of cellular IL-1β level was 4 pg/ml in the cultures that PHT and TNFα were applied simultaneously. When compared with the groups that had PHT + FA added, the differences between the IL-1β levels were found to be significantly different from the levels of the PHT + TNFα applied cultures ($P < 0.05$). There were

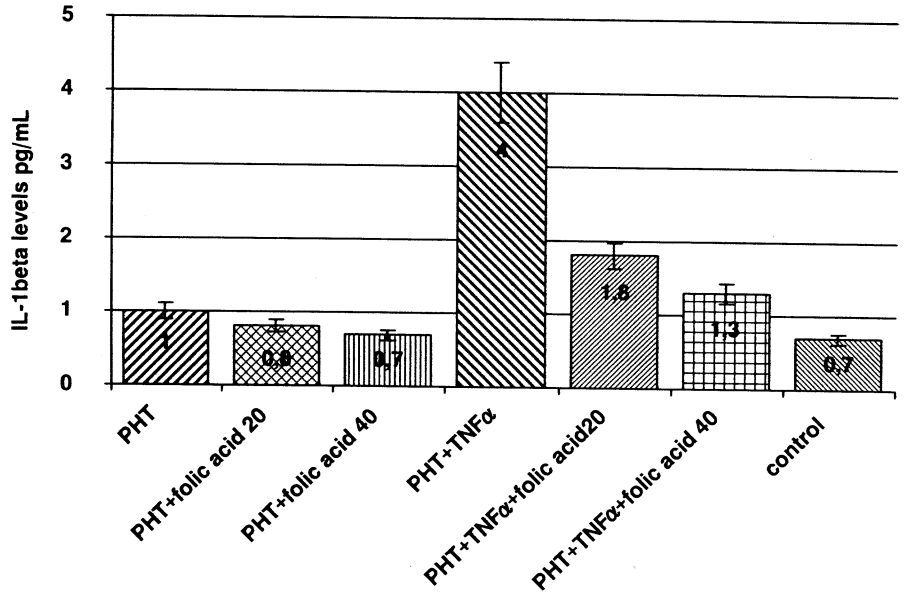


Fig. 1 Cell-associated IL-1β level (pg/ml) of HGF stimulated by Phenytoin (20 μg/ml) and TNFα (10 ng/ml), in the presence or absence of folic acid (20 or 40 ng/ml). Human gingival fibroblasts were seeded at 1×10^5cell per well. Supernatants obtained from cell lysates (n = 3) were measured for IL-1β production by ELISA after incubation for 72h.

decreases observed in the TNFα-induced cultures when PHT and 20 or 40 ng/ml FA were simultaneously added. The results obtained respectively were 1.8 and 1.3 pg/ml. Reductions were found in the FA-treated cultures when compared to the TNFα- and PHT-treated cultures. However, the differences were not statistically significant in either group.

Discussion

PHT is known to induce gingival overgrowth in half of PHT-treated individuals and development of this overgrowth is associated with gingival inflammation (2,3). The pathogenesis of PHT-induced gingival overgrowth was explained with a multifactorial etiology including adrenal gland function (21), changes in epidermal growth factor density (22), non-specific immuno-suppression (23) and drug effects on a selective cell population of fibroblasts (24,25). Recently, monocytes were reported to play a key role in regulating connective tissue formation by secreting anabolic growth factors (26). However, irrespective of the possible etiologies, the gingival overgrowth was related to the increased synthetic/mitotic activity of fibroblasts and their secretion of extracellular matrix components (2,27,28). Besides the other theories, the effects of PHT on FA metabolism were cited as possible mechanisms and the application of folate was also reported to inhibit gingival hyperplasia (18,19,29-31). However, the mechanism of this interference is still not well- understood.

It has been reported that reversible cytological changes due to folate deficiency occur in epithelial cells from a variety of organs where there is a rapid turnover rate (32,33), preventing the completion of cell maturation (34). Degenerative changes in the gingival epithelium have also been observed in experimentally-produced FA-deficient marmosets (35). Previously, authors postulated that an end organ deficiency of FA at the cellular level could exist in spite of the fact that blood levels were within normal limits (33,36). Such an end organ deficiency for FA had been previously demonstrated in human cervical epithelium (33). FA, that is required for the integrity of gingival tissues, has been found to decrease in PHT-using patients and render the gingiva more susceptible to inflammatory changes induced by microbial dental plaque, and thereby contribute to the phenytoin-induced hyperplasia (29).

The levels of inflammatory mediators TNFα and IL-1β were reported to increase in the crevicular fluid of sites with gingival inflammation (6,7). In lung, fibroblasts recombinant IL-1 stimulates fibroblast IL-1β mRNA accumulation and that rIL-1 and rTNF synergize to further increase the levels of IL-1β mRNA in these cells (37). Furthermore, environments rich in related mediators favor bone loss and connective tissue breakdown that occurs in periodontitis. However, IL-1β is a potent regulator of osteoprogenitor cells and fibroblasts and its secretion has been found to stimulate the glycosaminoglycan formation by fibroblasts that might contribute to the gingival overgrowth (8,9). When compared with healthy gingival tissues, increased amounts of glycosaminoglycans and decreased amounts of collagen content were the characteristics of the gingival overgrowth induced by PHT (2,27,38). It was reported that PHT induces IL-1 activity and potentiates LPS-induced IL-1 production *in vitro*. Thus, PHT-induced IL-1 activity was suggested to have importance in the development of gingival overgrowth (39). In addition, PHT alone, as well as in combination with IL-1, was suggested to have the ability to up-regulate the production of IL-6 and IL-8 in human gingival fibroblasts and lead to gingival overgrowth (40). In a previous study, PHT was also shown to potentiate TNFα-induced IL-1β production of fibroblasts *in vitro* (14). Thus, the aim of this study was to investigate the possible mechanism and the treatment of the overgrowth by assessing the effect of FA supplementation on TNFα- and PHT-induced IL-1β production of gingival fibroblasts at the cellular level.

The effect of PHT on IL-1β production was investigated using 20 μg/ml of this drug, which is the mean recommended serum concentration in epilepsy treatment. It was found in our study that the antiepileptic drug, PHT, by itself did not induce cell-associated IL-1β production. The levels of cell-associated IL-1β in PHT-added cultures were found to be similar to the controls. However, when PHT and TNFα were added to cultures simultaneously, PHT was found to potentiate the stimulatory effect of TNFα on IL-1β production, and this result is in agreement with a previous finding (14). The complex interaction between several cytokines, the levels of which are dependent on the degree of inflammation, is likely to contribute to the clinical symptoms (41). In a clinical study, PHT-treated patients exhibiting overgrowth demonstrate no significant increase in IL-1β mRNA. However, in the severely inflamed gingival samples, IL-1β mRNA levels exhibit significant increases (41). As mentioned above, overgrowth was generally accompanied by inflammation, which accounts for the severe gingival overgrowth. The levels of TNFα and IL-1β have been reported to be enhanced in the crevicular fluid of sites with gingival inflammation (6,7), and TNFα was found to induce IL-1β production in fibroblasts from various tissues (13,42).

Previously, maximum stimulation of IL-1β production was found at the 10 ng/ml concentration of TNFα (15), thus the 10 ng/ml concentration of TNFα was chosen to

induce IL-1β production in this study. IL-1β level was 4 pg/ml when PHT and TNFα were applied together. The result showed the interaction of inflammatory mediators in gingival overgrowth.

The involvement of the PHT on TNFα-induced cell-associated IL-1β production was studied in the presence or absence of FA (20 or 40 ng/ml) in this study. However, it was not possible to compare our results with the related studies; as to our knowledge, this was the only report describing the *in vitro* effect of folic acid on IL-1β level in PHT-treated cell cultures. It was found in this study that the treatment of cells with FA resulted in a decrease of IL-1β level compared with those cells treated with PHT and TNFα. As compared to the PHT + FA (20 or 40 ng/ml)-treated groups, IL-1β level was found to be significantly higher in the PHT + TNFα group ($P < 0.05$). Previously, Drew et al. (1987) suggested that the topical application of FA increases the concentration of FA in gingival tissues in a dose-dependent manner (19). The assumption is that FA might reduce TNFα-induced IL-1β production in gingival fibroblast in a dose-dependent manner. Thus, 20 ng/ml, the normal serum concentration, or double the concentration (40 ng/ml) of FA, was added to PHT + TNFα-treated cultures. However, there was no statistical difference found between the effects of either concentration with the respective results being 1.8 and 1.3 pg/ml. This result suggests the possible effect of folic acid that predisposes a reduction of cell-associated IL-1β in fibroblasts. However, the small sample was insufficient to demonstrate statistical significance. Besides the present findings, supplementation of cultures with FA was reported to compensate the cellular need for FA caused by PHT application, leading to the gingival tissues becoming susceptible to inflammation from local factors. However, other possible interactions need to be investigated.

In conclusion, hyperplasia associated with phenytoin administration can be reduced or prevented by the effect of FA on reducing cell-associated IL-1β, especially reducing the inflammatory response by such a mechanism in inflamed hyperplastic tissues. However, further *in vitro* studies should be conducted to determine whether FA inhibits IL-1β mRNA transcription, decreases IL-1β mRNA stability, inhibits IL-1β mRNA into protein, or inhibits post-translation cleavage of the translated product that yields the active form of IL-1β.

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