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ORIGINAL ARTICLE

Effect of phenytoin on collagen accumulation by human gingival fibroblasts exposed to TNF-α *in vitro*

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OBJECTIVE: Tumor necrosis factor (TNF)-α is associated with chronic gingival inflammation and reported to induce gingival overgrowth (GO), while phenytoin (PHT) is also known to be a causative agent of GO. We examined the synergistic effect of PHT and TNF-α on collagen metabolism in human gingival fibroblasts (HGFs).

MATERIALS AND METHODS: HGFs were cultured with TNF-α and PHT. Quantitative real-time RT-PCR was employed to determine the mRNA levels for collagen, matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and integrin subunits. Cellular collagen endocytosis was determined using a flow-cytometry.

RESULTS: The proliferation of HGFs was not affected by TNF-α or PHT individually, whereas both synergistically increased collagen accumulation in HGFs. Further, collagen mRNA expression was not increased by TNF-α or PHT, although together they markedly prevented cellular collagen endocytosis, associated with the suppression of α2β1-integrin mRNA expression. The mRNA expression of MMP-1 and-2 was suppressed by PHT, while TIMP-1 mRNA expression was enhanced by both TNF-α and PHT.

CONCLUSION: Our results suggest that TNF-α and PHT together cause impaired collagen metabolism by suppression of enzymatic degradation with MMPs/TIMP-1 and integrin-mediated endocytosis. These synergistic effects may also be involved in TNF-α- and PHT-induced collagen accumulation, leading to GO.

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Keywords: gingival overgrowth; phenytoin; tumor necrosis factor-α; collagen; gingival fibroblasts; antiepileptic drug

Introduction

Gingival overgrowth (GO) often causes severe psychological and esthetic problems, and also prevents normal mastication and promotes infection (Hassell and Hefti, 1991). Human gingival fibroblasts (HGFs) are known to not only synthesize extracellular matrix proteins including collagen, but also to degrade them during connective tissue remodeling, thereby maintaining matrix homeostasis (Everts *et al*, 1996). GO is caused by collagen accumulation in those sites, which is related to a lack of collagen breakdown rather than an increase in collagen production by HGFs (Goultschin and Shoshan, 1980; Kato *et al*, 2005). Tumor necrosis factor (TNF)-α, an inflammatory cytokine, induces cellular proliferation (Sugarman *et al*, 1985) and inhibits phagocytosis of collagen by HGFs, resulting in collagen accumulation (Chou *et al*, 1996). In contrast, TNF-α also inhibits collagen synthesis (Solis-Herruzo *et al*, 1988) and increases matrix metalloproteinases (MMP) synthesis by HGFs (Domeij *et al*, 2002), which leads to gingival breakdown. Although TNF-α mediates both breakdown and overgrowth, it remains unclear how this cytokine manages its contrasting functions.

A combination of tissue destruction and fibrosis occurs in chronic inflammatory lesions in gingival tissues. Further, the central regions that contain abundant inflammatory cells exhibit loss of collagen, while more peripheral regions of the connective tissue stroma are replaced with abundant disorganized collagen (Schroeder *et al*, 1973). In a previous study, the concentrations of TNF-α were found to range from 1 to 50 ng ml^{-1} in chronically inflamed sites (Stashenko *et al*, 1991), and it was reported that low levels of TNF-α (<10 ng ml^{-1}) induced collagen accumulation and fibrosis, whereas, higher levels caused loss of collagen in those sites (Chou *et al*, 1996). Thus, the concentration of TNF-α seems to be one of the determining factors for the development of GO.

Phenytoin is a widely used antiepileptic drug and remains the first choice for epilepsy, although it has a marked adverse effect of inducing GO (Marshall and Bartold, 1998). Notably, patients with chronic gingivitis because of poor oral hygiene who take PHT frequently suffer from GO (Stinnett *et al*, 1987; Botha, 1997). It has also been concluded that dental plaque is the most important factor to enhance deterioration caused by PHT-induced GO (Majola *et al*, 2000). It is likely that

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HGFs exposed to TNF-α at low levels show greater susceptibility to PHT, however, the synergistic effect of PHT and TNF-α toward GO has been rarely examined *in vitro*. Therefore, we evaluated the effect of PHT on collagen metabolism by HGFs exposed to TNF-α at a low level (1 ng ml^{-1}).

Materials and methods

Cell culture

Normal HGFs were obtained from three healthy volunteers, after receiving informed consent, and grown in standard explant cultures as described previously (Fujimori *et al*, 2001). Briefly the cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FCS; Invitrogen, Carlsbad, CA, USA), 50 μg ml^{-1} of gentamicin (Sigma Chemicals, St Louis, MO, USA), and 1 μg ml^{-1} of Fungizone (Sigma Chemicals) at 37°C, in a humidified atmosphere of 5% CO_2 and 95% air. All experiments were performed using cells at passages 5–8. PHT (Sigma Chemicals) was dissolved in an aliquot of dimethyl sulfoxide (Wako Pure Chemical Industries Ltd, Osaka, Japan) and added to the culture medium at final concentration of 0, 10, or 20 μg ml^{-1} with or without 1 ng ml^{-1} TNF-α (R & D Systems, Minneapolis, MN, USA).

Assays for cell proliferation and collagen accumulation

Confluent HGFs were cultured for various numbers of days in the presence or absence of PHT in 24-well culture plates. The numbers of viable cells were determined using a trypan blue dye exclusion method. Collagen content was determined by assaying total soluble collagen with a collagen assay kit (Sircol Collagen Assay kit; Biocolor LTD, Belfast, Northern Ireland) according to the manufacturer's instructions. Briefly, HGFs were cultured in 6-well culture plates and homogenized in 250 μl of 0.5 M acetic acid containing 1 mg ml^{-1} pepsin (Wako Pure Chemical Industries), then incubated for 24 h at 4°C with stirring. After centrifugation at $3000 \times g$ for 5 min, 200 μl of each supernatant was mixed with 1 ml of Sircol dye reagent and incubated for 30 min at room temperature. After centrifugation, the pellet was suspended in 1 ml of alkali reagent, included in the kit, and then read at 540 nm using a spectrophotometer. Type I collagen from rat tails was used as the standard. According to the manufacturer's information, the binding of Sircol dye reagent correlates well with the hydroxyproline contents of soluble collagen and the standard collagen solutions in the kit were utilized to construct a standard curve. Collagen contains about 14% hydroxyproline by weight and, according to the manufacture, the collagen contents obtained with this method correlate well with the hydroxyproline content.

Quantification of endocytic collagen

Collagen endocytosis was assayed according to the method of Knowles *et al* (1991). Briefly, 2.5-μm fluorescent latex beads (Molecular Probes Inc., Eugene, OR, USA) coated with soluble bovine dermal type I collagen (3 mg ml^{-1}; Vitrogen Collagen Corp, Palo Alto, CA, USA) were sonicated and dispersed into single-bead suspension. The beads were then added to the cultures (1.0×10^4 cells ml^{-1}) for flow cytometry analysis at a bead:cell ratio of 4:1. Following 3 h of incubation with collagen-coated beads, the cells were detached with 0.01% trypsin, which eliminated membrane-bound beads, but not those internalized. The cells were washed with PBS and resuspended cells were fixed with 70% ethanol at 4°C for 20 min. The cells were then centrifuged at $60 \times g$ for 5 min, resuspended in PBS, and analyzed using a cell sorter (BD Biosciences, San Jose, CA, USA), with 488-nm laser excitation and a 520/539-nm bandpass filter in the emission path to detect the fluorescence of the beads. The percentage of internalized cells was determined by the following formula: (number of cells with internalized beads/total number of cells) × 100.

Real-time reverse transcript (RT)-PCR analysis

For RT-PCR, total RNA was prepared from HGFs using TRIzol reagent (Invitrogen) and 5 μg of total RNA was reverse-transcribed in the presence of oligo(dT) using a reverse transcriptase from the Molony murine leukemia virus (Invitrogen), according to the manufacturer's instructions. Real time RT-PCR was performed to quantify the expression of collagen, MMPs, tissue inhibitor of metalloproteinases (TIMP-1), and integrin mRNA, using a thermal cycler (LightCycler; Roche Molecular Biochemicals, Mannheim, Germany) with SYBRGreen reagent (Qiagen, Hilden, Germany). Samples were subjected to 35 cycles of amplification at 95°C for 15 s followed by 52–57°C for 20 s and 72°C for 25 s using the specific primers (Table 1), as described previously (Kato *et al*, 2005). The results of the assays were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. An amplification plot from a series of dilutions of cDNA from control non-treated HGFs was used to obtain a linear correlation between the threshold cycle and the log amount of template present. The relative ratio of target mRNA for each sample was calculated from its threshold cycle using LightCycler System Software ver. 3.3 (Roche Molecular Biochemicals), according to the manufacturer's instructions.

Statistical analyses

All data are expressed as the mean ± standard deviation (s.d.). Multiple comparisons were performed with Sheffe's *F*-test (STAT View software; SAS Institute, Cary, NC, USA).

Results

Effects of TNF-α and PHT on cellular proliferation and collagen accumulation in HGFs

We first determined whether TNF-α and PHT separately had effects on cellular proliferation and/or collagen accumulation in HGFs. TNF-α (1 ng ml^{-1}) and PHT at various concentrations showed no effect on proliferation (Figure 1). In contrast, PHT alone induced collagen

Table 1 PCR primers

Target gene	*Sense primer (5′-3′)* *Antisense primer (5′-3′)*	*Template size (bp)*	*Annealing temperature (°C)*
GAPDH	GTCTTCACCACCATGGAGAAG GTTGTCATGGATGACCTTGGC	210	55
Type I collagen	CTCAAGAGAAGGCTCACGATG GTCTCACCAGTCTCCATGTTG	280	55
MMP-1	CACAGCTTTCCTCCACTGCTGCTGC GGCATGGTCCACATCTGCTCTTGGC	396	57
MMP-2	GAGACAGTGGATGATGCCTTTG TGGAAGCGGAATGGAAACTTGC	481	57
TIMP-1	CCTGGCTTCTGGCATCCTGTT GGGACCTGTGGAAGTATCCGC	280	57
α2-integrin	GCCTATTCTGAGACTGCCAAG GGCAACAGACTTCTGAGATGC	309	55
β1-integrin	ACTGTGATGCCTTACATTAGC CTGCAACTTGCATGATGGCA	210	55

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinase.

accumulation, and TNF-α alone also significantly increased accumulation (Figure 2). Further, TNF-α-induced collagen accumulation was increased by combination with PHT in a dose-dependent manner. Next, the effects of TNF-α and PHT on collagen mRNA expression were examined, and the mRNA expression of collagen type I was significantly down-regulated by both (Figure 3), showing that the collagen accumulation induced by TNF-α and PHT was not due to an increased production of collagen.

Effects of TNF-α and PHT on endocytosis of collagen by HGFs

Next, we examined whether TNF-α and PHT can inhibit the endocytosis of collagen in HGFs. Flow cytometric analysis revealed that both TNF-α and PHT significantly inhibited the uptake of collagen by HGFs, while PHT enhanced the effect of TNF-α on cellular endocytosis in a dose-dependent manner (Figure 4). It is known that α2β1-integrin is a cellular receptor for collagen that mediates collagen endocytosis (Lee *et al*, 1996). Therefore, we also determined the mRNA expression levels of the α2- and β1-integrin subunit molecules. The mRNA expression of α2-integrin was significantly inhibited by PHT up to 4 h, but not by TNF-α, and the expression was enhanced at 8 h after TNF-α was added (Figure 5a). On the contrary, the mRNA expression of β1-integrin was significantly suppressed by PHT in both the presence and absence of TNF-α, and TNF-α alone also showed a marked inhibitory effect at 4 h (Figure 5b).

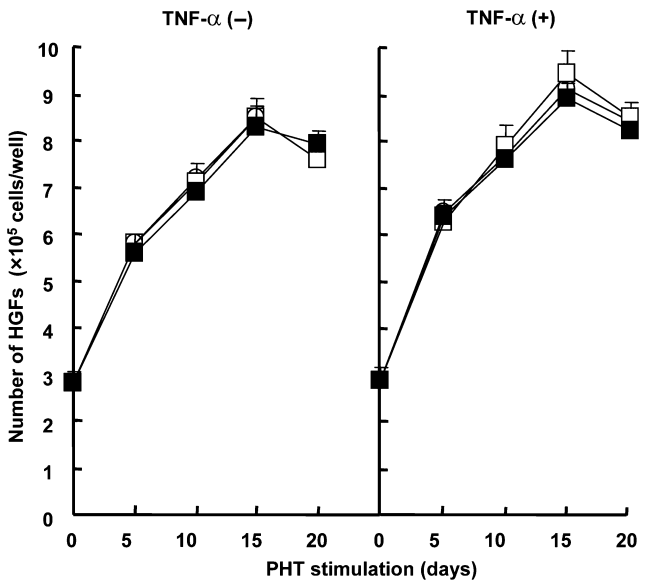

Figure 1 Effects of phenytoin (PHT) and tumor necrosis factor (TNF)-α on cellular proliferation of human gingival fibroblasts (HGFs). HGFs were incubated in the presence or absence of TNF-α with various concentrations of PHT (0 μg ml^{-1}, ○; 10 μg ml^{-1}, □; 20 μg ml^{-1}, ■). Data shown represent the mean ± s.d. of three individual experiments performed in triplicate

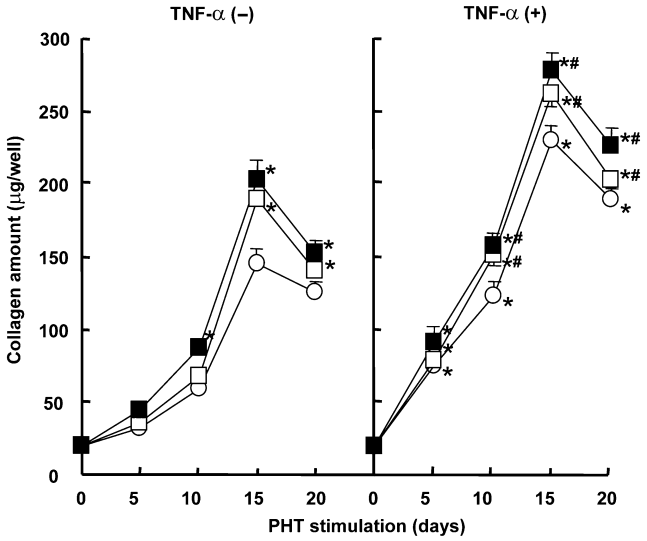

Figure 2 Effects of phenytoin (PHT) and tumor necrosis factor (TNF)-α on collagen accumulation in human gingival fibroblasts (HGFs). HGFs were incubated in the presence or absence of TNF-α with various concentrations of PHT (0 μg ml^{-1}, ○; 10 μg ml^{-1}, □; 20 μg ml^{-1}, ■). Data shown represent the mean ± s.d. of three individual experiments in triplicate. *$^{\#}$Significant difference ($P < 0.05$) in comparison with the control value (PHT = 0 μg ml^{-1}) in the *absence or $^{\#}$presence of TNF-α

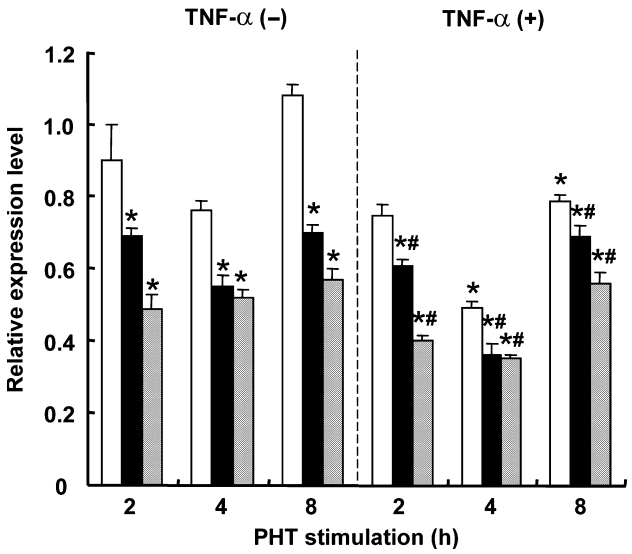

Figure 3 Effects of phenytoin (PHT) and tumor necrosis factor (TNF)-α on collagen gene expression by human gingival fibroblasts (HGFs). HGFs were incubated with various concentrations of PHT (0 μg ml^{-1}, □; 10 μg ml^{-1}, ■; 20 μg ml^{-1}, ▩) for 2, 4 and 8 h in the presence or absence of TNF-α. Type I collagen mRNA expression was quantified with real-time RT-PCR analysis. The amount of glyceraldehyde-3-phosphate dehydrogenase mRNA expressed at 0 h was designated as 1.0, and relative expression amounts are shown with the mean ± s.d. of three individual experiments in triplicate. *#Significant difference ($P < 0.05$) in comparison with the control value (PHT = 0 μg ml^{-1}) in the *absence or #presence of TNF-α

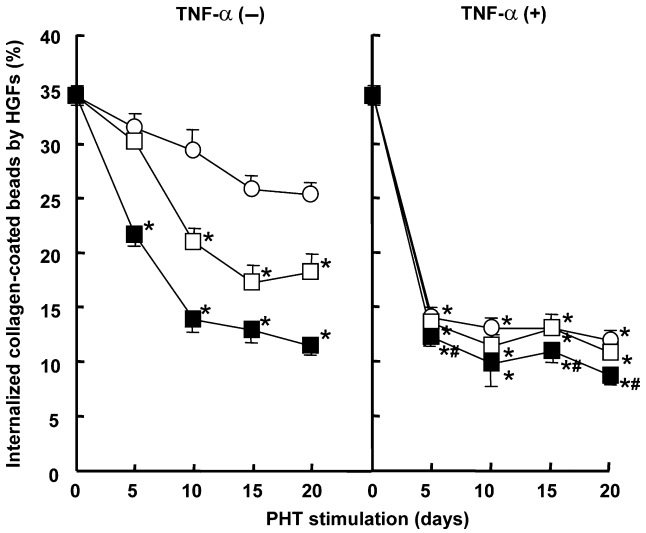

Figure 4 Effects of phenytoin (PHT) and tumor necrosis factor (TNF)-α on collagen endocytosis in human gingival fibroblasts (HGFs). HGFs were incubated in the presence or absence of TNF-α with various concentrations of PHT(0 μg ml^{-1}, ○; 10 μg ml^{-1}, □; 20 μg ml^{-1}, ■). Type I collagen coated beads added to the cultures (1.0×10^4 cells ml^{-1}) at a beads:cell ratio of 4:1 for flow cytometry analysis. After 3 h of incubation, the number of internalized beads was counted. The percentage of internalized cells was determined as described in the text. Data shown represent the mean ± s.d. of three individual experiments in triplicate. *#Significant difference ($P < 0.05$) in comparison with the control value (PHT = 0 μg ml^{-1}) in the *absence or #presence of TNF-α

Effects of TNF-α and PHT on MMP and TIMP mRNA expression by HGFs

The MMP family and its inhibitor, TIMP, are metabolic enzymes involved in collagen degradation (Brew *et al*, 2000). Our RT-PCR assay results showed that HGFs express mRNAs for MMP-1, MMP-2 and TIMP-1, and the basal level of TIMP-1 mRNA was markedly higher than that of MMP-1 and MMP-2 (Figure 6a). Thus, the

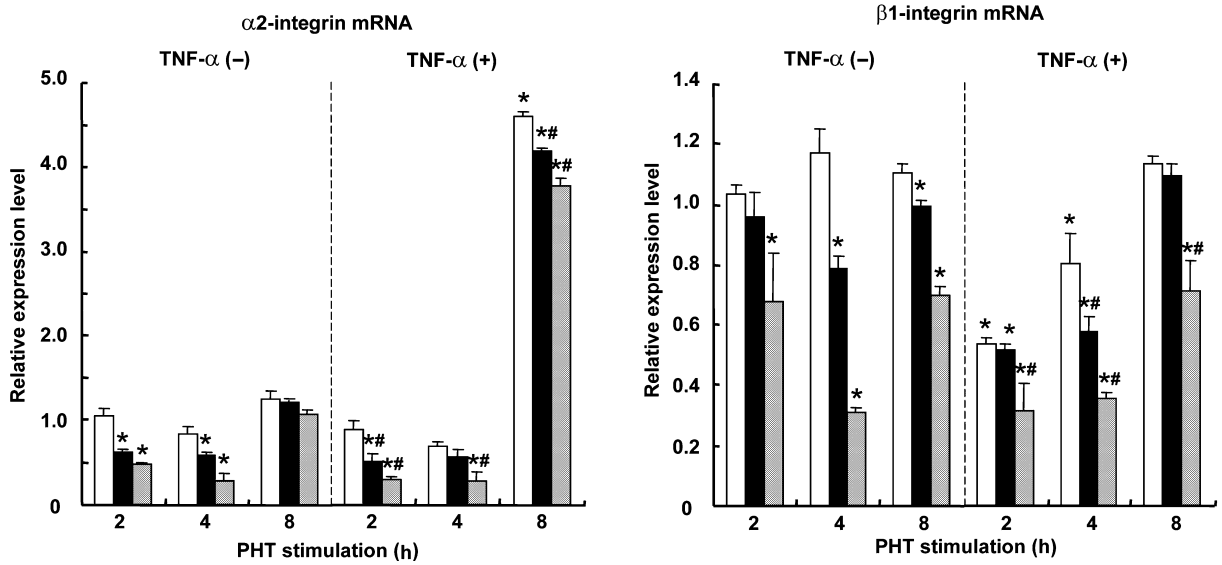

Figure 5 Effects of phenytoin (PHT) and tumor necrosis factor (TNF)-α on α2- and β1-integrin gene expression by human gingival fibroblasts (HGFs). HGFs were incubated with various concentrations of PHT (0 μg ml^{-1}, □; 10 μg ml^{-1}, ■; 20 μg ml^{-1}, ▩) for 2, 4 and 8 h in the presence or absence of TNF-α. α2- and β1-integrin subunit mRNA expressions were quantified using real-time RT-PCR analysis. The amount of glyceraldehyde-3-phosphate dehydrogenase mRNA expressed at 0 h was designated as 1.0, and relative expression amounts are shown with the mean ± s.d. of three individual experiments in triplicate. *#Significant difference ($P < 0.05$) in comparison with the control value (PHT = 0 μg ml^{-1}) in the *absence or #presence of TNF-α

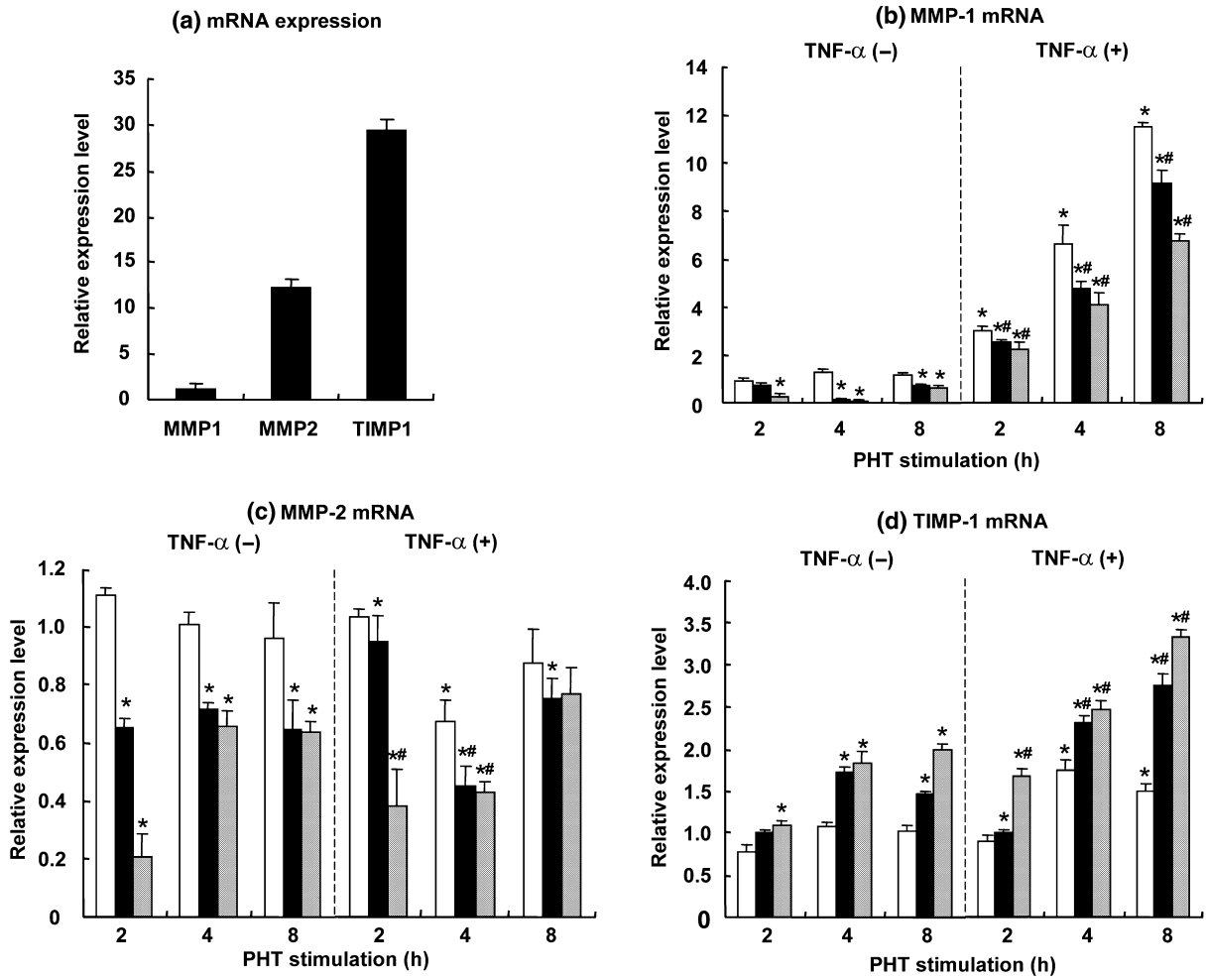

Figure 6 Effects of phenytoin (PHT) and tumor necrosis factor (TNF)-α on matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMP)-1 gene expression by human gingival fibroblasts (HGFs). HGFs were incubated with various concentrations of PHT (0 μg ml^{-1}, □; 10 μg ml^{-1}, ■; 20 μg ml^{-1}, ▨) for 0, 2, 4 and 8 h in the presence or absence of TNF-α. MMP-1, MMP-2, and TIMP-1 mRNA expressions were quantified using real-time RT-PCR analysis. (**a**) Expression levels of MMP-1, MMP-2 and TIMP-1 in HGFs at 0 h. The expression amount of glyceraldehyde-3-phosphate dehydrogenase mRNA was designated as 1.0, and relative expression amounts are shown. (**b, c, d**) Relative expression levels of MMP-1, MMP-2 and TIMP-1 in HGFs. The expressed amount of mRNA for each gene expressed at 0 h was designated as 1.0, and relative expression amounts are shown. *$^{\#}$Significant difference ($P < 0.05$) in comparison with the control value (PHT = 0 μg ml^{-1}) in the *absence or $^{\#}$presence of TNF-α

effects of TNF-α and PHT on the mRNA expression of MMP-1, and MMP-2 and TIMP-1 by HGFs were determined. Exogenous PHT at various concentrations significantly suppressed the mRNA expression of both MMP-1 and MMP-2 after 8 h in the presence and absence of TNF-α, in a dose-dependent manner (Figure 6b,c). TNF-α suppressed the MMP-2 mRNA expression, however, increased that of MMP-1. In contrast, TIMP-1 mRNA expression was upregulated by TNF-α, and the degree of the mRNA expression markedly enhanced by PHT with a dose-dependent manner (Figure 6d). Although the effects of PHT and TNF-α on protein expression and enzymatic activity were not examined, these results indicates that an impaired enzymatic degradation by PHT and TNF-α may be also involved in collagen accumulation in HGFs.

Discussion

Several drugs such as PHT, nifedipine (calcium channel blocker) and cyclosporine (immunosuppressive agent) are known to induce GO as a side effect, with dental plaque considered to be the most critical factor influencing GO induced by medication (Stinnett *et al*, 1987; Hassell and Hefti, 1991; Steele *et al*, 1994; Botha, 1997; Marshall and Bartold, 1998; Majola *et al*, 2000). In addition, chronic gingival inflammation was reported to sporadically cause GO in a normal population with poor oral hygiene, likely due to the effect of TNF-α (Steele *et al*, 1994; Miranda *et al*, 2001). TNF-α is a major proinflammatory cytokine found in chronically inflamed gingival tissue (Roberts *et al*, 1997; Tervahartiala *et al*, 2001; Wang *et al*, 2003), and has been reported to mediate GO and participate in the destruction of

periodontal tissue (Brunius *et al*, 1996; Chou *et al*, 1996; Dogan *et al*, 2001). A previous investigation showed that PHT induced TNF-α, interleukin (IL)-1 and prostaglandin E2 (PGE2) production by HGFs, and suggested that IL-1 and PGE2 play important roles in the pathogenesis of GO induction by PHT (Brunius *et al*, 1996). However, more recently it was demonstrated that collagen accumulation is the most important process in GO induced by drugs and TNF-α (McCulloch and Knowles, 1993; Chou *et al*, 1996; Kato *et al*, 2005). Therefore, we examined the synergistic effect of PHT and TNF-α on collagen metabolism in HGFs.

Our results showed that TNF-α and PHT induced collagen accumulation in HGFs, but not cellular proliferation. The addition of PHT enhanced TNF-α-induced collagen accumulation, while collagen mRNA expression was suppressed by TNF-α and PHT, thus, the accumulation observed was likely due to impaired collagen metabolism. Collagen turnover and remodeling are considered to be predominantly regulated by two pathways, cellular endocytosis and enzymatic digestion of MMPs/TIMPs (Everts *et al*, 1985, 1996; Meikle *et al*, 1989). We found that both TNF-α and PHT significantly inhibited the cellular endocytosis of collagen, while PHT synergistically enhanced the effect of TNF-α, as shown in Figure 4. Similarly, the mRNA expressions of α2- and β1-integrin subunits were inhibited by TNF-α and PHT (Figure 5). Interaction between integrins and extracellular matrix proteins such as type I collagen are known to mediate migration and endocytosis of HGFs (Lee *et al*, 1996; Cox and Huttenlocher, 1998; Defilippi *et al*, 1999; Paine and Ward, 1999). Further, TNF-α was shown to induce integrin inactivation associated with impaired phosphorylation of focal adhesion proteins, resulting in decreased affinity of integrins for their receptors (Chou *et al*, 1996), and we have previously reported that PHT inhibited cellular endocytosis of collagen, which was associated with decreased integrin expression (Kato *et al*, 2005). Therefore, the decreased affinity and decreased expression of α2β1-integrin caused by the addition of TNF-α and PHT likely inhibits endocytosis of collagen.

It is considered that collagen degradation is also controlled by MMPs and their antagonist TIMPs (Meikle *et al*, 1989; Sternlicht and Werb, 2001). Our results showed that PHT decreased MMP-1 and -2 mRNA expressions, and although longer stimulation with TNF-α enhanced MMP-1 mRNA expression by HGFs, PHT clearly suppressed the mRNA expression of MMP-1, whereas, TIMP-1 mRNA expression was markedly enhanced by PHT and TNF-α. TIMP-1 was shown to have a marked inhibitory effect on the enzymatic degradation of extracellular matrix proteins (Meikle *et al*, 1989; Brew *et al*, 2000), thus, an upregulated mRNA expression of TIMP-1 would considerably contribute to the suppression of collagen degradation.

It is unclear if PHT and/or its reactive metabolites induced collagen accumulation in HGFs. It was reported that gingival tissues form the patients contained cytochrome P450 2C which plays an important role in PHT metabolism, and also showed significant PHT hydroxylase activity (Zhou *et al*, 1996). In the present assay, PHT might have been metabolized by HGFs. Further study is necessary to analyze the effects of the metabolites on GO.

Taken together, these results suggest that TNF-α plays an important role to decrease collagen endocytosis by HGFs, while PHT induces dysregulation of MMPs/TIMPs, resulting in suppressed degradation of collagen. In addition, their synergistic actions further enhanced impaired collagen metabolism. Thus, PHT is suggested to enhance collagen accumulation in HGFs exposed to TNF-α at low levels in inflammatory sites and poor oral hygiene leading to chronic gingival inflammation is likely a determining factor for development of GO in patients taking PHT.

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