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Effects of nifedipine and interleukin-1α on the expression of collagen, matrix metalloproteinase-1, and tissue inhibitor of metalloproteinase-1 in human gingival fibroblasts

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Background and Objective: Nifedipine-induced gingival overgrowth is known to be characterized by fibrosis and some degree of inflammation. However, the molecular mechanism of the fibrosis is not fully understood. The purpose of this study was to investigate *in vitro* the effects of nifedipine and interleukin-1α on the molecules involved in fibrosis, namely type I collagen, matrix metalloproteinase-1 (MMP-1), and tissue inhibitor of metalloproteinase-1 (TIMP-1).

Material and Methods: Four human gingival fibroblast strains, derived from four healthy volunteers, were cultured in media containing nifedipine (1 μg/ml), with or without interleukin-1α (0.05 ng/ml). The mRNAs of type I collagen, MMP-1, and TIMP-1 were measured by reverse transcription–polymerase chain reaction (RT–PCR). The proteins of MMP-1 and TIMP-1 were examined by enzyme-linked immunosorbent assay (ELISA), and the ratios of MMP-1 to TIMP-1 proteins were calculated.

Results: The mRNA expression of type I collagen showed no significant change. Both mRNA expression and protein production of MMP-1 were up-regulated by interleukin-1α, either alone or in combination with nifedipine, whereas those of TIMP-1 were up-regulated by nifedipine alone or in combination with interleukin-1α. The ratio of MMP-1 to TIMP-1 was not changed by nifedipine alone, but it was increased by interleukin-1α alone or in combination with nifedipine. However, in two of the four cell strains tested, nifedipine reduced the ratio of MMP-1 to TIMP-1 compared with that for interleukin-1α alone.

Conclusion: These results suggest that nifedipine may predispose to fibrosis in some individuals in situations where interleukin-1 levels are raised.

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Nifedipine, a calcium-channel blocking drug, is used widely to treat ischemic cardiovascular diseases, such as angina pectoris and hypertension (1). However, gingival overgrowth may occur as a side-effect in patients receiving nifedipine medication (2–4). Gingival overgrowth causes esthetic problems and aggravates oral hygiene, possibly resulting in an increased susceptibility to dental caries and periodontitis. Nifedipine-induced gingival overgrowth is clinically and histopathologically similar to phenytoin- and cyclosporin A-induced gingival overgrowth. These drug-induced gingival overgrowths are known to be characterized by fibrosis with some degree of inflammation (5–7). However, the molecular mechanism of the drug-induced fibrosis is not fully understood.

Generally, fibrosis is known to be the result of an imbalance between the synthesis and degradation of extracellular matrix, mainly collagen. As to the effect of nifedipine on the collagen synthesis, there have been conflicting *in vitro* studies that nifedipine increased or decreased or did not affect the collagen synthesis in normal gingival fibroblasts or those isolated from gingival overgrowth (8–15). Regarding the effect of nifedipine on collagen degradation, it has been reported that nifedipine affected both intracellular and extracellular pathways of the collagen degradation (11,16–18). In the intracellular pathway, nifedipine has been reported to decrease the phagocytosis of collagen by gingival fibroblasts (16,17). In the extracellular pathway, two studies have reported that the collagenase activity of fibroblasts from nifedipine-induced overgrown gingiva was lower than that of healthy gingival fibroblasts (11), and that the protein production of matrix metalloproteinase-1 (MMP-1) in normal gingival fibroblasts was decreased by exogenous nifedipine (18).

The activity of MMPs in the extracellular degradation of collagen is controlled mainly by tissue inhibitor of metalloproteinases (TIMPs) (19). Because the balance between MMPs and TIMPs is important in physiological and pathological conditions (20), the investigation of both MMPs and TIMPs is needed to elucidate whether the connective tissue tends to be fibrosis or fibrolysis. However, to date there have been no reports of the effects of nifedipine on both MMPs and TIMPs in gingival fibroblasts, although the effects of cyclosporin A and phenytoin have been studied (21–24).

Many studies have suggested that the plaque-induced inflammation is associated with the onset or the severity of a drug-induced gingival overgrowth (5–7,25–27), and histological studies have shown the presence of some degree of inflammatory infiltrate in the overgrown gingiva (2–4). Interleukin-1α is a pro-inflammatory cytokine that is expressed by the epithelium and the infiltrating leukocytes in the gingival tissue of patients with periodontitis (28,29), and may modify the cellular response to drugs such as nifedipine. Therefore, we used interleukin-1α in this study.

In this study, we investigated the effects of nifedipine, with or without interleukin-1α, on the mRNA expression of type I collagen, MMP-1 (interstitial collagenase produced by fibroblast and degrades types I and III collagen), and TIMP-1 (endogenous inhibitor of MMP-1), in the normal human gingival fibroblasts *in vitro*. In addition, we examined the proteins of MMP-1 and TIMP-1, and then calculated the ratio of MMP-1 to TIMP-1.

Material and methods

Cells

Four human gingival fibroblast strains (GF-1, -2, -3, -4) were obtained from the healthy gingivae located above the impacted teeth of four volunteers (18–23 years old; one man and three women) at the time of extraction. Prior to surgical removal, informed consent was obtained from the donors. The gingiva was washed with Dulbecco's phosphate-buffered saline (PBS; Nissui Pharmaceutical Co. Ltd, Tokyo, Japan), soaked in 0.1% (v/v) chlorhexidine solution (Dainippon Sumitomo Pharma Co. Ltd, Osaka, Japan) for 30 s for disinfection, and immediately washed thoroughly three times with PBS. The gingival connective tissue was separated from the epithelium under a stereoscopic microscope. After mincing, the tissue pieces were placed in a 35-mm culture dish (Falcon, Franklin Lakes, NJ, USA), allowed to adhere, and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 20% (v/v) heat-inactivated fetal bovine serum (FBS; Medical Biological Laboratories Co. Ltd, Nagoya, Japan), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Meiji Seika Kaisha Ltd, Tokyo, Japan). The dish was incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. When fibroblasts (spindle-shaped cells), which were identified by a phase-contrast microscope, began to migrate from the explant, the medium was replaced with DMEM supplemented with 10% (v/v) FBS containing the above antibiotics (growth medium). On reaching confluence, the fibroblasts were harvested with 0.1% (w/v) trypsin and PBS containing 0.02% (v/v) EDTA, and stored in liquid N_2 until use. The fibroblasts used in this study were between the fifth and the seventh passage.

Exposure to nifedipine and interleukin-1α

Human gingival fibroblasts were seeded into six-well plates (Falcon) at 2×10^5 cells/well and incubated in the growth medium. After reaching subconfluence, the growth medium was replaced with DMEM supplemented with 2% (v/v) FBS containing 100 U/ml penicillin G and 100 μg/ml streptomycin (basal medium). After 24 h, the basal medium was replaced with fresh basal medium containing 1 μg/ml nifedipine (Sigma, St Louis, MO, USA), with or without 0.05 ng/ml interleukin-1α (Sigma). The control medium contained neither nifedipine nor interleukin-1α. All culture media contained 0.1% (v/v) ethanol, a solvent for nifedipine. The cells were incubated in the above-mentioned media for 24 h and 48 h.

Extraction of total RNA and reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was isolated from the cells after 24 h of culture using an ISOGEN reagent (Nippon Gene Co. Ltd, Toyama, Japan) and then dissolved in RNAse-free H_2O at a concentration of 1 μg/ml. RT–PCR was carried out using an RNA PCR kit (AMV), version 2.1 (Takara Bio Inc., Otsu, Japan), according to the manufacturer's instructions. Briefly, the synthesis of cDNAs from total RNAs was performed for 30 min at 42°C in a reaction buffer containing AMV reverse transcriptase XL, Oligo dT-adaptor primer, and dNTP mixture. The resulting cDNA products were amplified in a solution containing *TaKaRa Taq*™ and the specific primers (Sigma-Aldrich Japan, K.K. Genosys Division, Ishikari, Japan), at various cycles, using a thermal cycler (Perkin Elmer Co., Norwalk, NJ, USA) (Table 1). Each cycle consisted of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min. The resulting PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and photographed under ultraviolet (UV) light with a CCD camera (Kodak digital science ID; Kodak, New York, NY, USA). The intensities of the stained bands were determined using NIH image software, version 1.62 (http://rsb.info.nih.gov/nih-image/). The expression levels of each mRNA were presented as the ratio to β-actin mRNA, a housekeeping gene. When the PCR products reached a plateau, cDNAs were diluted 1 : 2 or 1 : 4, amplified under the same experimental conditions described above, and the expression levels determined.

Enzyme-linked immunosorbent assay (ELISA)

The conditioned media were collected after 48 h of culture, centrifuged at 750 *g* for 5 min in order to remove cell debris, and then stored at −30°C until use. The protein contents of MMP-1 and TIMP-1 in the conditioned media were measured by using a human MMP-1 ELISA kit (Amersham Bioscience, Piscataway, NJ, USA) and a human TIMP-1 ELISA kit (Amersham Bioscience), respectively, according to the manufacturer's instructions. The conditioned media were diluted 2–10 times for MMP-1 assay, and 20–30 times for TIMP-1 assay, according to the mRNA expression levels. Because FBS has been reported to contain varying amounts of TIMP-1 (30), the TIMP-1 content in the basal medium was also measured, as described above. The net TIMP-1 content of the conditioned medium was obtained by subtracting the TIMP-1 content in the basal medium from the TIMP-1 content in the conditioned medium.

Statistical analysis

The data were expressed as mean ± standard deviation and were assessed using Tukey–Kramer's multiple comparison test. Significant differences were noted at a p-value of < 0.05.

Table 1. Primer sequences used in this study

Primer	Sequence		Cycles	Product size (bp)	GenBank accession number
Collagen I[1)]	Forward	5′-GTGGAAATGATGGTGCTACT-3′	23	379	XM_012651
	Reverse	5′-TTAGCACCAGTGTCTCCTTT-3′			
MMP-1	Forward	5′-AAAGGGAATAAGTACTGGGC-3′	24	237	NM_002421
	Reverse	5′-AATTCCAGGAAAGTCATGTG-3′			
TIMP-1	Forward	5′-ACACCAGAAGTCAACCAGAC-3′	22	330	XM_033878
	Reverse	5′-GATGGATAAACAGGGAAACA-3′			
β-actin	Forward	5′-ACCCAGATCATGTTTGAGAC-3′	23	211	NM_001101 >
	Reverse	5′-TGAGGTAGTCAGTCAGGTCC-3′			

1) Type I collagen α1 chain.

Results

Type I collagen

The mRNA expression of type I collagen was unaffected by all combinations of nifedipine and interleukin-1α tested, except for an apparent reduction in expression in strain GF-2 by nifedipine plus interleukin-1α (Fig. 1).

MMP-1

Both mRNA expression and protein production of MMP-1 showed a similar pattern in the four cell strains (Fig. 2). Nifedipine alone caused a nonsignificant increase in mean levels of both mRNA expression and protein production of MMP-1 in most strains compared with the control. By contrast, interleukin-1α alone increased significantly both the mRNA expression and protein production of MMP-1 in three cell strains, but had no effect on strain GF-4. Similarly, the combination of nifedipine and interleukin-1α significantly increased both mRNA expression and protein production of MMP-1 compared with the control in all cell strains, and compared with interleukin-1α alone, in strains GF-1 and -4. The addition of nifedipine to interleukin-1α had little effect on MMP-1 expression in the other two strains (GF-2 and -3).

TIMP-1

The effect of nifedipine and interleukin-1α on both mRNA expression and protein production of TIMP-1 was similar in all cell strains and differed from that found on MMP-1 (Fig. 3). Nifedipine alone increased significantly both mRNA expression and protein production of TIMP-1 compared with the control. By contrast, interleukin-1α alone generally caused small, nonsignificant increases in both mRNA expression and protein production of TIMP-1 compared with the control. The combination of nifedipine + interleukin-1α increased significantly or markedly both mRNA expression and protein production of TIMP-1

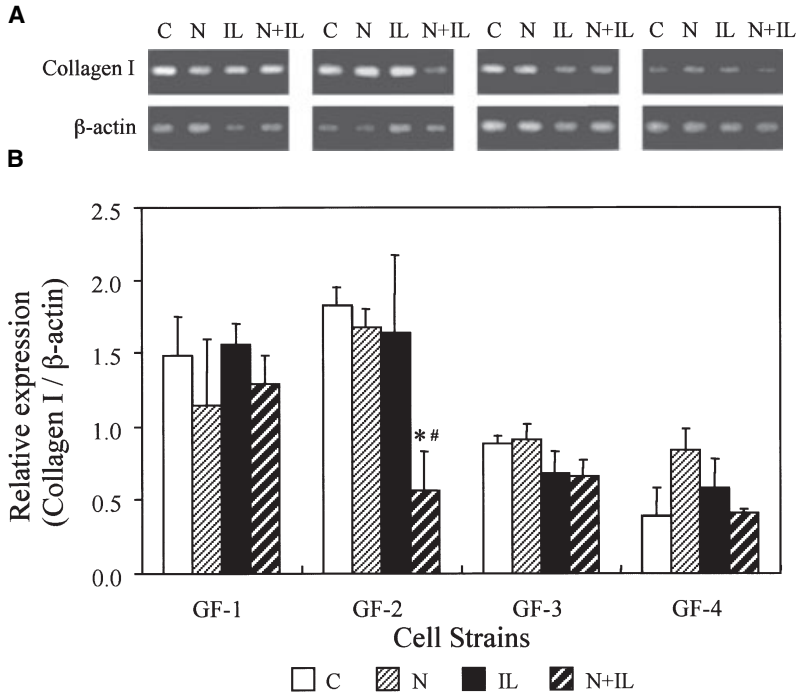

Fig. 1. The effect of nifedipine, with or without interleukin-1α, on the mRNA expression of type I collagen. (A) Gel electrophoregrams of the reverse transcription–polymerase chain reaction (RT–PCR) products of type I collagen and β-actin mRNAs. (B) Relative expression levels are presented as the ratio of type I collagen to β-actin mRNAs, as determined by RT–PCR. Each column and its vertical bar indicate the mean value and standard deviation of three wells. $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. interleukin-α alone. C, control; N, nifedipine alone; IL, interleukin-1α alone; N + IL, nifedipine plus interleukin-1α.

compared with the control or interleukin-1α alone in all cell strains. However, the expression levels detected with nifedipine + interleukin-1α were essentially similar to those obtained with nifedipine alone.

The ratio of MMP-1 and TIMP-1

The ratio of MMP-1 to TIMP-1 was calculated using the protein contents determined by ELISA (Fig. 4). Nifedipine alone did not change the ratio of MMP-1 to TIMP-1, compared with the control, in the four cell strains. Interleukin-1α alone increased the ratio in all cell strains. Similarly, the combination of nifedipine + interleukin-1α increased significantly the ratio compared with the control. However, in two of the four cell strains (GF-2 and -3), nifedipine reduced significantly the ratio to ≈65% compared with interleukin-1α alone. In strain GF-3, all ratios were higher than those in the other cell strains.

Discussion

Nifedipine-induced gingival overgrowth is known to be characterized by the excess accumulation of extracellular matrix, particularly collagen, and the presence of some degree of inflammation (5–7). The excess accumulation of collagen may be accelerated by collagen production and/or may be reduced by collagen degradation as a result of the imbalance between MMPs and TIMPs. However, the molecular mechanism of the nifedipine-induced fibrosis is not fully understood. Therefore, we investigated the effects of nifedipine and interleukin-1α on the expression of collagen, MMP-1, and TIMP-1 in normal human gingival fibroblasts.

We showed that nifedipine, with or without interleukin-1α, had no significant or consistent effect on the mRNA expression of type I collagen. Previous *in vitro* studies have shown similar effects of nifedipine on the synthesis of type I collagen in human normal fibroblasts and those derived from overgrown gingival tissue (8–15). Interestingly, McKevit & Irwin (12) demonstrated that the fibroblasts of patients with nifedipine-induced gingival overgrowth produced increased levels of protein and collagen as compared with those of patients without overgrowth, but the addition of exogenous nifedipine to cultures induced an inhibitory response in the fibroblasts from overgrown gingiva. Thus, our results and those of others suggest that nifedipine may not directly affect the collagen synthesis by gingival fibroblasts.

We showed that MMP-1 production was increased significantly (compared with the control) by interleukin-1α, alone or in combination with 1 μg/ml nifedipine, but not by 1 μg/ml nifedipine alone. Our data show that nifedipine has little effect on MMP-1 production and do not agree with the results of Maita *et al.* (18), who reported that 10 μM (3.46 μg/ml) nifedipine alone decreased significantly the protein production of MMP-1 in normal gingival fibroblasts. Ellis *et al.* (31) reported that the concentration of nifedipine in gingival crevicular fluid (GCF) in patients receiving nifedipine medication was 0.92–9.30 μg/ml, 15–90-fold higher than that in plasma. Considering the high concentration of nifedipine in GCF, our results, and those of Maita *et al.*, suggest that nifedipine may not affect or might decrease MMP-1 production in the tissue of nifedipine-induced gingival overgrowth without inflammation.

The effect of nifedipine on TIMP-1 production in human gingival fibroblast has not been reported. We showed that TIMP-1 production was increased significantly by nifedipine alone or in combination with interleukin-1α, but not by interleukin-1α alone. Our data indicate that the effects of nifedipine and interleukin-1α on TIMP-1 production differ from those on MMP-1 production and support the results of Domeij *et al.* (32), who reported that MMP-1 and TIMP-1 production was regulated differently by interleukin-1β and calcium in human gingival fibroblasts. However, our

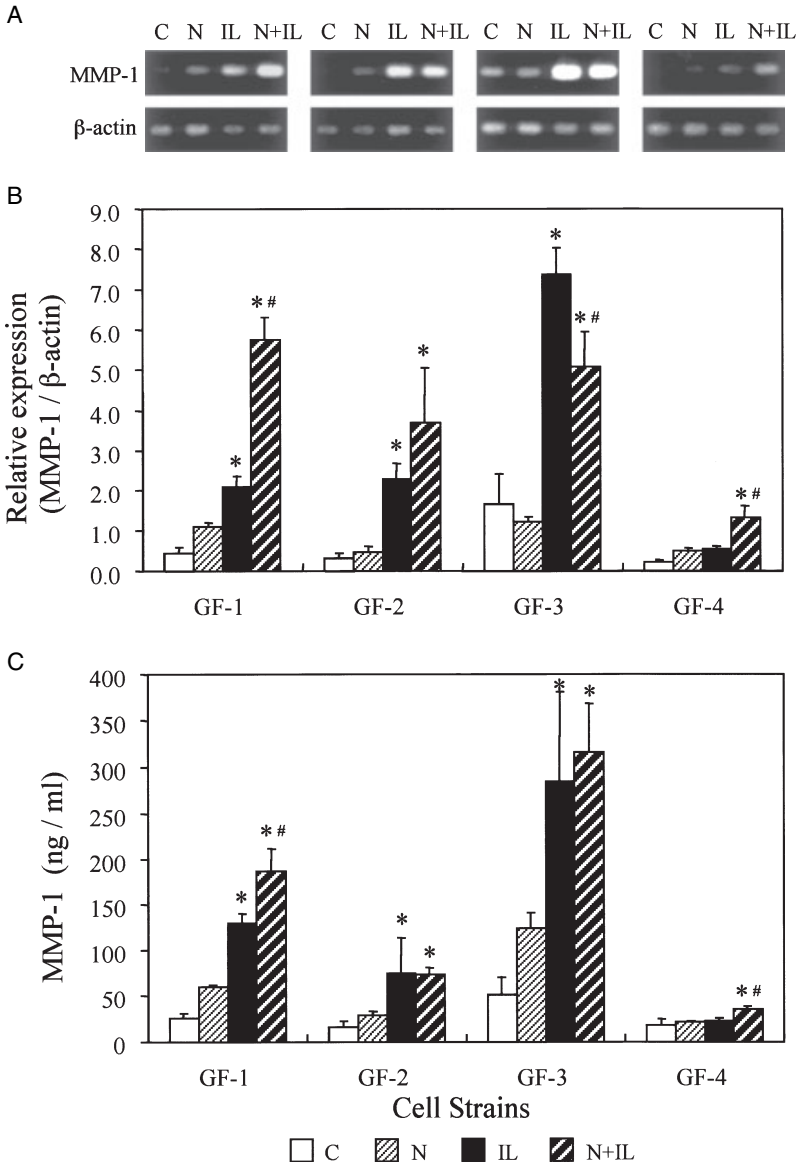

Fig. 2. The effect of nifedipine, with or without interleukin-1α, on the mRNA expression and protein production of matrix metalloproteinase-1 (MMP-1). (A) Gel electrophoregrams of the reverse transcription–polymerase chain reaction (RT–PCR) products of MMP-1 and β-actin mRNAs. (B) Relative expression levels are presented as the ratio of MMP-1 to β-actin mRNAs, as determined by RT–PCR. (C) The protein contents of MMP-1 shown are the concentrations determined by enzyme-linked immunosorbent assay (ELISA). Each column and its vertical bar indicate the mean value and standard deviation of three wells. $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. interleukin-α alone. C, control; N, nifedipine alone; IL, interleukin-1α alone; N + IL, nifedipine plus interleukin-1α.

results differ from those of cyclosporin A and phenytoin: several studies reported that cyclosporin A and phenytoin decreased both MMP-1 and TIMP-1 production (21–24). Trackman and colleagues (7,33) showed that the level of connective tissue growth factor (CTGF) and the degree of inflammation varied among nifedipine-, cyclosporin A- and phenytoin-induced gingival overgrowth tissues, and they proposed that the cellular and molecular mechanisms in these three lesions were different. Our results on the effects of nifedipine on MMP-1 and TIMP-1 expression support the proposal that the molecular mechanisms underlying drug-induced gingival overgrowth are drug dependent.

Many studies have demonstrated that pathological conditions, such as fibrosis or tissue destruction, are caused by an imbalance between MMPs and TIMPs in various tissues (20,34–39). Moreover, it has been reported that the changes of the ratio of MMPs to TIMPs in GCF (37), in human synovial fibroblasts (38), and in the serum of patients with chronic hepatitis (39), are associated with fibrosis or tissue destruction. We showed that the ratio of MMP-1 to TIMP-1 was increased significantly by interleukin-1α alone or in combination with nifedipine, but not by nifedipine alone. However, in two of the four cell strains tested, nifedipine reduced significantly the ratio of MMP-1 to TIMP-1 compared with that for interleukin-1α alone, indicating that nifedipine may perhaps predispose to fibrosis in some individuals in situations where interleukin-1 levels are raised, such as inflammation. Our data also showed that all ratios in strain GF-3 were higher than those in the other cell strains, indicating that this cell strain may be bioactive. The reason for this is unknown, as all fibroblasts used in this study were obtained in a similar manner from the gingival connective tissues of healthy adolescent individuals. Tipton *et al.* (21) reported variation among individuals, as well as intrastrain heterogeneity, of human gingival fibroblasts with regard to both collagenase activity and also to the production of collagenase and TIMP. Therefore, these high ratio values may be caused by individual heterogeneity.

In general, the extracellular degradation of collagen has been known to be regulated in a number of ways, including the production of pro-MMPs as a latent form, the activation from pro-MMPs to active MMPs by proteinases such as plasmin and other MMPs, and the inhibition of active MMPs by TIMPs (20). It has been reported that the majority of synthetic MMP-1s stimulated by interleukin-1α or interleukin-1β are latent forms (33,40). Therefore, additional studies will be required to elucidate the precise

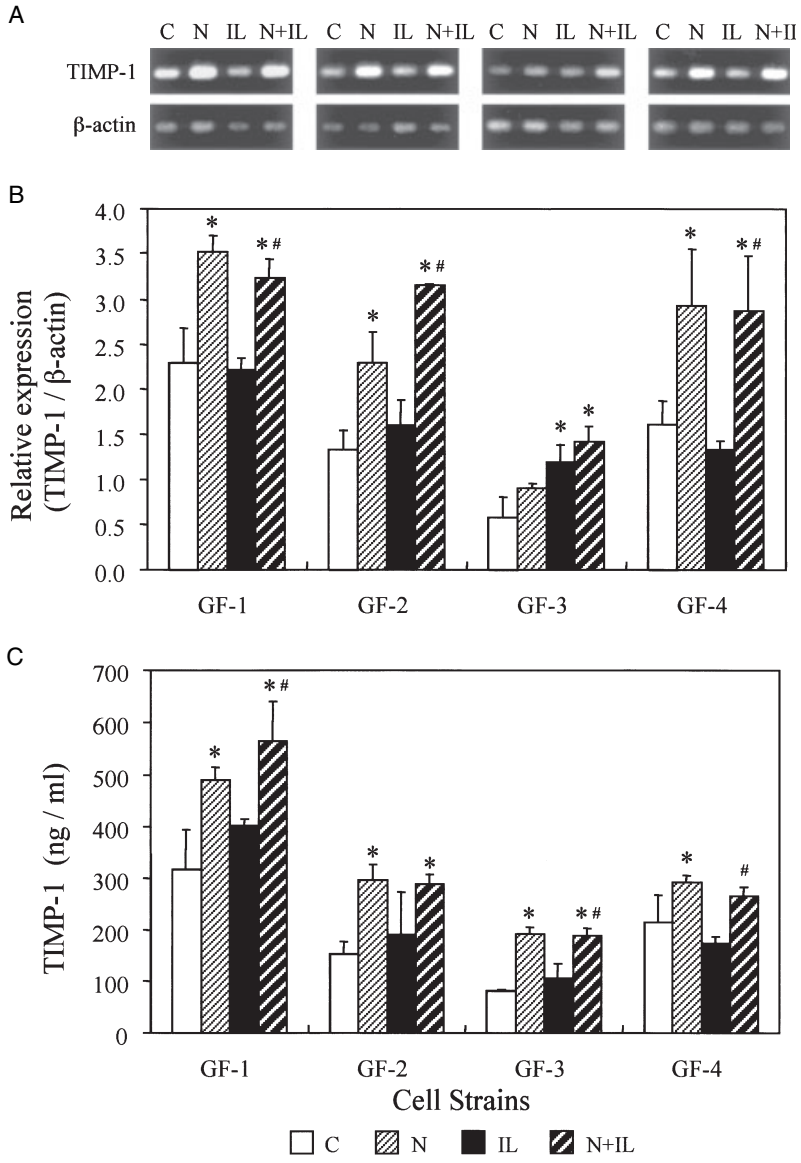

Fig. 3. The effect of nifedipine, with or without interleukin-1α, on the mRNA expression and protein production of tissue inhibitor of metalloproteinase-1 (TIMP-1). (A) Gel electrophoregrams of the reverse transcription–polymerase chain reaction (RT–PCR) products of TIMP-1 and β-actin mRNAs. (B) Relative expression levels are presented as the ratio of TIMP-1 to β-actin mRNAs, as determined by RT–PCR. (C) The protein contents of TIMP-1 shown are the concentrations determined by enzyme-linked immunosorbent assay (ELISA). Each column and its vertical bar indicate the mean value and standard deviation of three wells. $*p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. interleukin-α alone. C, control; N, nifedipine alone; IL, interleukin-1α alone; N + IL, nifedipine plus interleukin-1α.

molecular mechanism of the extracellular degradation of collagen in nifedipine-induced gingival overgrowth, whether MMP-1 stimulated by interleukin-1α is a latent or an active form, and also whether other proteinases and those inhibitors are expressed.

In summary, MMP-1 is up-regulated by interleukin-1α alone or in combination with nifedipine, whereas TIMP-1 is up-regulated by nifedipine alone or in combination with interleukin-1α. It appears that the ratio of MMP-1 to TIMP-1 is increased by interleukin-1α alone, or by interleukin-1α in combination with nifedipine, but not by nifedipine alone. However, in two of the four cell strains tested, nifedipine

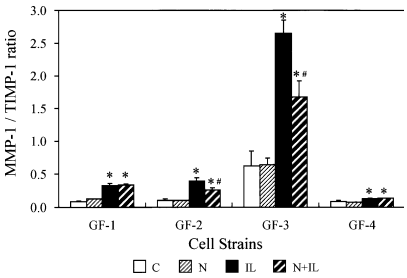

Fig. 4. The effect of nifedipine, with or without interleukin-1α, on the ratio of matrix metalloproteinase-1 (MMP-1) to tissue inhibitor of metalloproteinase-1 (TIMP-1). The ratio of MMP-1 to TIMP-1 was calculated by using the protein contents determined by enzyme-linked immunosorbent assay (ELISA). Each column and its vertical bar indicate the mean value and standard deviation of three wells. $*p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. interleukin-α alone. C, control; N, nifedipine alone; IL, interleukin-1α alone; N + IL, nifedipine plus interleukin-1α.

reduced the ratio of MMP-1 to TIMP-1 compared with that for interleukin-1α alone, indicating perhaps that nifedipine may predispose to fibrosis in some individuals in situations where interleukin-1 levels are raised, such as inflammation.

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