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Inhibition by nifedipine of adherence- and activated macrophage-induced death of human gingival fibroblasts

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Abstract

The effects of nifedipine on the death and proliferation of gingival fibroblasts were investigated to elucidate the mechanism of gingival overgrowth that is associated with chronic administration of Ca^{2+} channel blockers. The number of adhered viable and dead fibroblasts obtained from healthy human gingiva increased after confluence, whereas cell death was inhibited by nifedipine in a concentration-dependent manner. A similar inhibition was also observed in the presence of other calcium channel blockers, such as nicardipine, diltiazem, and verapamil. When gingival fibroblasts were co-cultured with RAW264 (macrophage-like) cells, lipopolysaccharide (LPS) caused the concentration-dependent death of fibroblasts. Nifedipine significantly inhibited the LPS-induced cell death. Although neither LPS nor *N*-ethyl-2-(1-ethyl-2-hydroxy-2-nitroso-hydrazino)-ethanamine, a nitric oxide donor, directly caused fibroblast death, 3-morpholino-sydnonimine (SIN-1), a peroxynitrite donor, induced fibroblast death, regardless of the presence of RAW cells. The cell death induced by SIN-1 was not affected by nifedipine treatment. LPS stimulation caused an increase in the immunoreactivity of inducible nitric oxide synthase (iNOS) and in the nitrite concentration in the incubation medium of RAW cells. The induction of iNOS was completely prevented by the incubation with nifedipine. The inhibition by nifedipine of nitrite production in RAW cells was also observed after treatment with nicardipine, but not with either diltiazem or verapamil. Therefore, the inhibition by nifedipine of both adherence- and LPS-stimulated macrophage-induced death of fibroblasts may be the mechanism of gingival overgrowth seen during chronic treatment with Ca^{2+} channel blockers. © 2001 Published by Elsevier Science B.V.

Keywords: Human gingival fibroblast; Nifedipine; Ca^{2+} channel blocker; Nitric oxide; Peroxynitrite; Gingival overgrowth

1. Introduction

It is well known that chronic medication with various drugs may cause gingival overgrowth. Among them, phenytoin is one of the most representative reagents, and Kimball (1939) first reported gingival overgrowth in association with phenytoin therapy in epileptic patients. Since then, it has been reported that gingival overgrowth is induced by other kinds of drugs such as Ca^{2+} channel blockers and immunosuppressants (Calne et al., 1979; Ramon et al., 1984). Nifedipine is most frequently reported to induce gingival overgrowth during long-term treatment of cardiovascular disorders such as hypertension and angina pectoris (Heupler and Proudfit, 1979; Moskowitz et al., 1979).

Up to the present, however, the mechanism of nifedipine-induced gingival overgrowth has not been determined with certainty, although several hypotheses have been postulated (Brown et al., 1991; Marshall and Bartold, 1998). Gingival hyperplasia refers to a histological enlargement of the tissue caused by an increase in the number of cellular and intercellular elements (Barak et al., 1987; Ellis et al., 1992; Harel et al., 1995). Therefore, its pathogenesis could involve an interaction between the drug and resident gingival fibroblasts, which control extracellular matrix turnover. The proliferation and matrix-producing

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activities of fibroblasts are regulated by environmental agents (Ellis et al., 1993; Seymour et al., 1996). Since nifedipine is known to be concentrated within gingival tissues (Ellis et al., 1992), it has been proposed that nifedipine may directly affect the cell growth of resident fibroblasts.

In addition, previous studies suggest that gingival inflammation may be essential for the onset of nifedipine-induced gingival overgrowth (Harel et al., 1995). Actually, the existence of inflammation was concluded to be responsible for the increased prevalence of gingival hyperplasia during nifedipine therapy, because regression analysis revealed a significant relationship between indices of gingival inflammation and overgrowth (Ellis et al., 1999). Furthermore, extensive control of oral hygiene resulted in no recurrence of gingival overgrowth without substitution or discontinuation of nifedipine (Nishikawa et al., 1991).

Drug-induced gingival overgrowth is not a side effect of nifedipine but is seen after treatment with other Ca^{2+} channel blockers. Although these drugs commonly bind to voltage-operated L-type Ca^{2+} channels to inhibit calcium influx from extracellular fluid, their pharmacological properties and clinical applications are different. Nifedipine and nicardipine are substituted dihydropyridines and are used for the therapy of hypertension, targeting the blood vessels, whereas verapamil and diltiazem are phenylalkylamine and benzothiazepine derivatives, respectively, and are used for cardiac disorders.

Therefore, in the present study, we investigated the effects of nifedipine on the proliferation and survival of gingival fibroblasts to elucidate the mechanism of gingival overgrowth. Moreover, we used RAW264 cells as a representative of inflammatory cells in order to clarify whether nifedipine can indirectly affect the properties of fibroblasts through signals from inflammatory cells.

2. Materials and methods

2.1. Cell culture and treatment with drugs

Under the approval of the Committee for Human Research in Osaka University, Graduate School of Dentistry, the tissues were obtained from healthy human gingiva. The fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco-BRL, Gaithersburg, USA) containing 100 μg/ml streptomycin, 100 IU/ml penicillin, and 1 μg/ml fungizone in a humidified atmosphere of 95% air–5% CO_2 at 37°C. After the fibroblasts were cloned in the same medium, they were used for experiments. Ca^{2+} channel blockers such as nifedipine, nicardipine, verapamil, and diltiazem (Sigma) were added to the same incubation medium after confluence was reached, and cells were cultured for another 1 or 2 weeks.

The cloned murine macrophage-like cell line of RAW264 was obtained from the Riken Gene Bank (Ibaragi, Japan). Cells were grown in DMEM supplemented with 10% FCS. Fibroblasts and RAW cells were plated together at a density of 2×10^5 per ml with a 1-to-1 cell number ratio. For exposure to *N*-ethyl-2-(1-ethyl-2-hydroxy-2-nitroso-hydrazino)-ethanamine (NOC12, Dojindo, Kumamoto, Japan), 3-morpholinosydnonimine (SIN-1, Do-

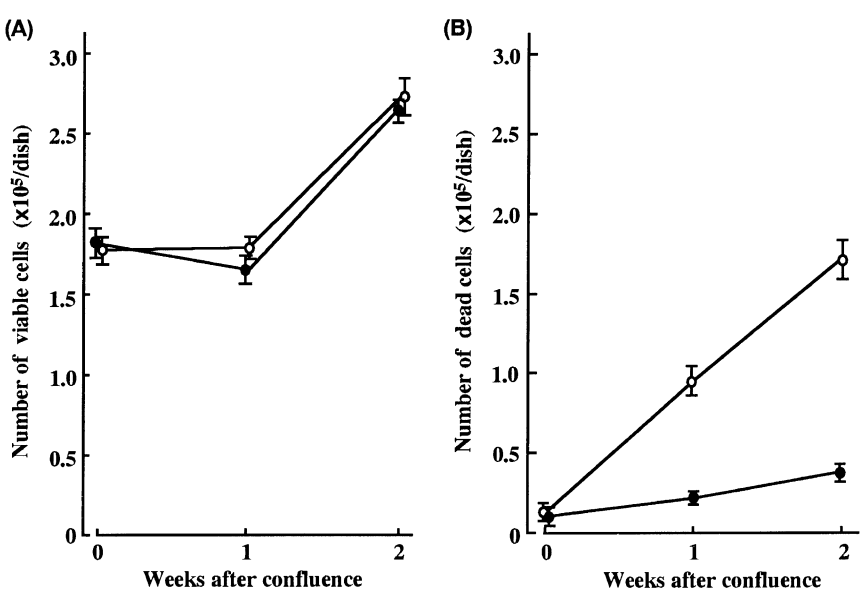

Fig. 1. Effects of nifedipine on number of viable (A) or dead fibroblasts (B). After confluence, gingival fibroblasts were cultured for the designated duration in the absence (control, open circles) or presence of 1 μM nifedipine (solid circles). Viable or dead cells were distinguished by Trypan blue exclusion. $n = 9$.

jindo) or lipopolysaccharide (*E. coli* serotype O111: B4, Sigma), drugs were added in concentrated form to the culture medium. Ca^{2+} channel blockers were also added to the medium at the same time. After a further 24-h incubation, the properties of the cells were investigated.

In order to examine the concentration-dependent effects of nifedipine, fibroblasts and/or RAW cells were incubated with nifedipine (0.1, 0.3, 1, 3, 10 μM) for the designated periods. The obtained concentration–response curves were analyzed by using a personal computer programmed with a non-linear least-square method of Damping Gauss–Newton to fit the equation: $1/Y = (1/\mathrm{MAX}) \times (1 + \mathrm{IC}_{50}/X)$, to calculate IC_{50} values, where Y, MAX and X are response, maximum response and concentration, respectively, of nifedipine (Kamisaki et al., 1991).

2.2. Cell viability and ability to synthesize DNA

To count the number of viable cells, cells were removed from culture dishes by treatment with 0.05% and 0.25% trypsin-EDTA (Gibco-BRL) for fibroblasts and RAW cells, respectively. Since RAW cells were not detached from dishes by the treatment with 0.05% trypsin, we could easily separate these two types of cells. After a brief centrifugation at $1000 \times g$ for 5 min, the cells were re-suspended and washed in phosphate-buffered saline (PBS, 20 mM sodium phosphate [pH 7.4] and 130 mM NaCl). Cell viability was determined by a dye exclusion test, using 0.5% Trypan blue in PBS. The percent cell death was calculated from the ratio of the number of dead cells to the total number of cells examined 10^4. Dead cells were also detected by using a DNA fragmentation assay kit (Boehringer Mannheim, Mannheim, Germany) (Kanesaki et al., 1999).

The proliferation of cells was determined by measuring the incorporation of radioactive thymidine. After cells were incubated for 4 h with [^{3}H]thymidine (final concentration, 3.7×10^4 Bq/ml, NEN, Boston, USA), PBS-washed cells were treated with 1% trichloroacetic acid.

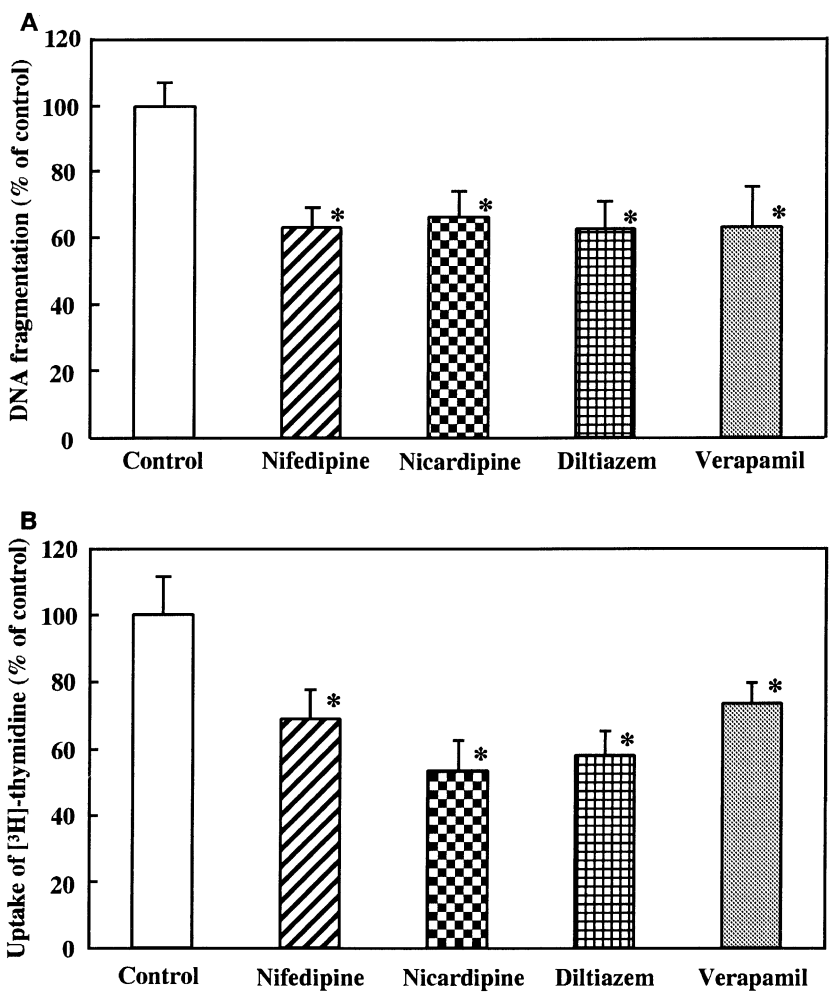

Fig. 2. Effects of Ca^{2+} channel blockers on DNA fragmentation (A) and uptake of [^{3}H]-thymidine (B) in fibroblasts. Cells were cultured for 1 week after the indicated Ca^{2+} channel blockers (1 μM, each) were added under confluent conditions, and then DNA fragmentation and proliferation ability were examined as described in Materials and methods. Results were obtained from nine separate experiments and are expressed as percentages of those for untreated controls. $^*P < 0.01$ vs. control.

Insoluble materials were solubilized with 0.25 M NaOH and used for the measurement of radioactivity.

2.3. Measurement of nitrite concentrations

After RAW cells were incubated with LPS (1 or 10 μg/ml) for various times, the accumulation of NO_2^-, a stable end-product extensively used as an indicator of nitric oxide (NO) production in cultured cells, was assayed by a method using the Griess reaction. Briefly, cell-free supernatants were mixed with equal amounts of Griess reagent (*p*-aminobenzene sulfonilamide 1% and naphthylethylenediamide 0.1% in phosphoric acid 2.5%). Samples were incubated at room temperature for 10 min and their absorbance was recorded at 540 nm.

2.4. Western blot analysis

Twelve hours after RAW cells were treated with LPS (6 μg/ml) and/or nifedipine (1 or 10 μM), cells were washed twice with PBS before the addition of 10 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 1 mM sodium ortho vanadate. After sonication twice for 30 s and centrifugation at 70,000 × *g* for 30 min at 4°C, adjusted amounts of protein (10 μg) from whole-cell lysates were subjected to 7.5% polyacrylamide gel electrophoresis. The resolved proteins were transferred to nitrocellulose membranes, which were then incubated with anti-inducible NO synthase (iNOS) antibody (Transduction Lab., Kentucky, USA) overnight at 4°C. Immunoreactive proteins were visualized, using an alkaline phosphatase conjugated anti-rabbit Immunoglobulin G antibody (Wako, Osaka, Japan).

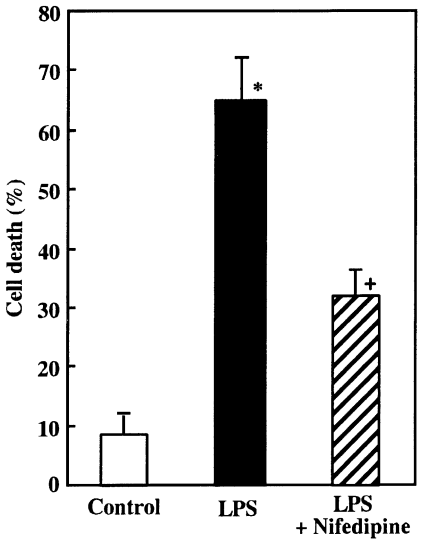

Fig. 4. Effect of nifedipine on death of fibroblasts induced by LPS-stimulated RAW cells. Nifedipine (1 μM) was added to the media for both fibroblasts and RAW cells at the same time with LPS (10 μg/ml) treatment. Results are expressed as percentages of the total number of cultured fibroblasts and compared with those for unstimulated control and LPS-treated cells. $^{*}P < 0.01$ and $^{+}P < 0.01$ are compared to control and LPS treatment, respectively. $n = 9$.

2.5. Statistical evaluation

The results are expressed as means ± S.E.M. from three to nine experiments. Statistical differences between groups were determined using Student's *t*-test or Kruskal–Wallis

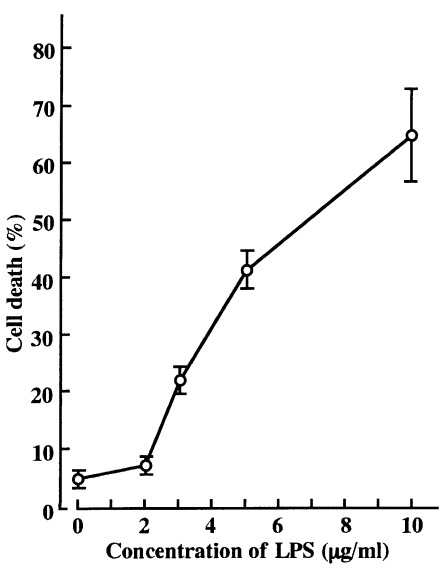

Fig. 3. Death of fibroblasts induced by LPS-stimulated RAW cells. Fibroblasts and RAW cells were co-cultured and stimulated by the indicated amounts of LPS for 24 h. The number of dead fibroblasts was determined in a Trypan blue exclusion test and expressed as a percentage of the total number of fibroblasts. $n = 9$.

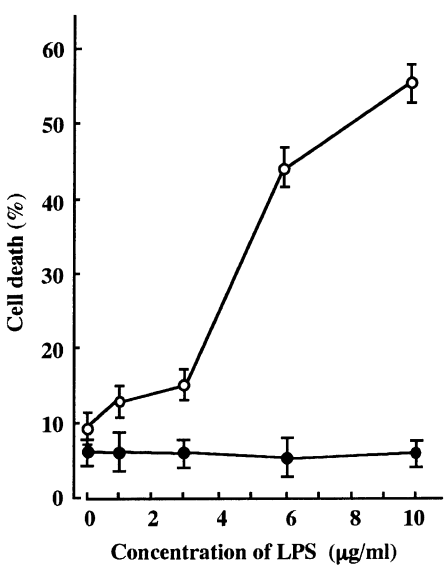

Fig. 5. Concentration-dependent effect of LPS on death of RAW cells or fibroblasts. RAW cells (open circles) or fibroblasts (solid circles) were stimulated by various concentrations of LPS (1–10 μg/ml) for 24 h. The number of dead cells is expressed as a percentage of the total number of cultured cells. $n = 9$.

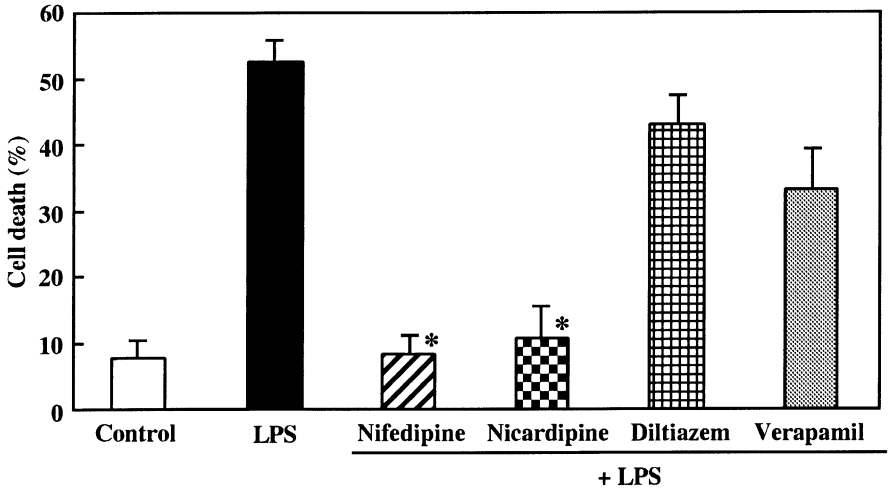

Fig. 6. Effects of Ca^{2+} channel blockers on LPS-induced death of RAW cells. Twenty-four hours after the addition of Ca^{2+} channel blockers (1 μM, each) with LPS (10 μg/ml), the viability of RAW cells was determined in a Trypan blue exclusion test. $^{*}P < 0.01$ vs. LPS-treatment. $n = 9$.

test. When P is less than 0.05, the difference was considered significant.

3. Results

3.1. Effects of nifedipine on proliferation and death of gingival fibroblasts after confluence

Culture of human gingival fibroblasts for 1 week after confluence was reached resulted in the death of about 30% of the fibroblasts. Even after confluence, the fibroblasts continued to increase in number. Nifedipine (1 μM) inhibited the increase in the number of dead cells, although it did not affect the number of viable cells (Fig. 1). The inhibition by nifedipine of cell death was concentration dependent, with an IC_{50} of 1 μM (data not shown). The death of fibroblasts after confluence was accompanied by DNA fragmentation, which was attenuated by Ca^{2+} channel blockers such as nifedipine, nicardipine, diltiazem, and verapamil (Fig. 2A).

Under these conditions, the fibroblasts still possessed the ability to synthesize DNA, which was evaluated by a method of [^{3}H]-thymidine incorporation (Fig. 2B). However, the ability of fibroblasts to proliferate was also inhibited in part by any Ca^{2+} channel blockers (1 μM). The inhibition by nifedipine was also concentration dependent, with an IC_{50} of 1 μM (data not shown). These

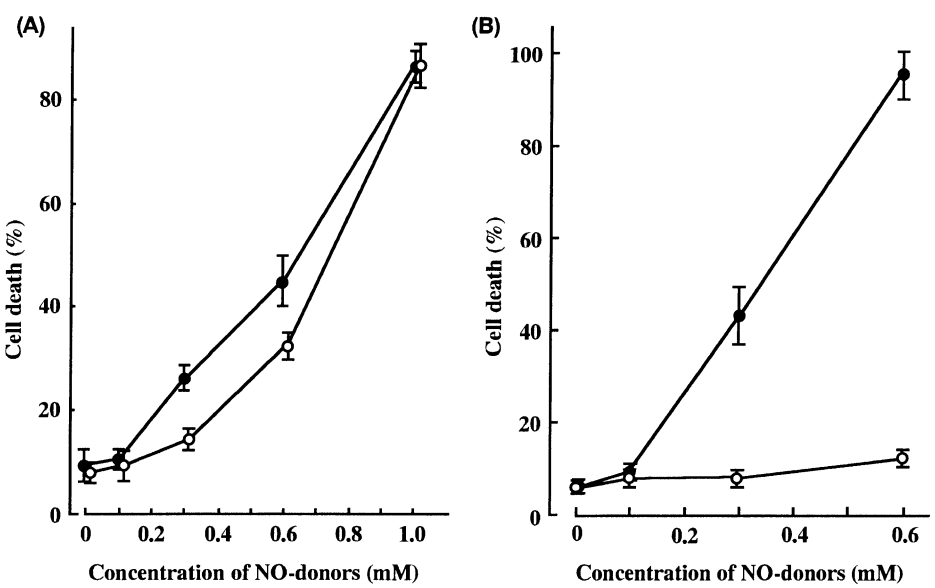

Fig. 7. Cell death by NO-donors in RAW cells or gingival fibroblasts. RAW cells (A) or fibroblasts (B) were treated with the designated concentration of NOC12 (open circles) or SIN-1 (solid circles) for 24 h and cell viability was determined. Each group consists of nine experiments.

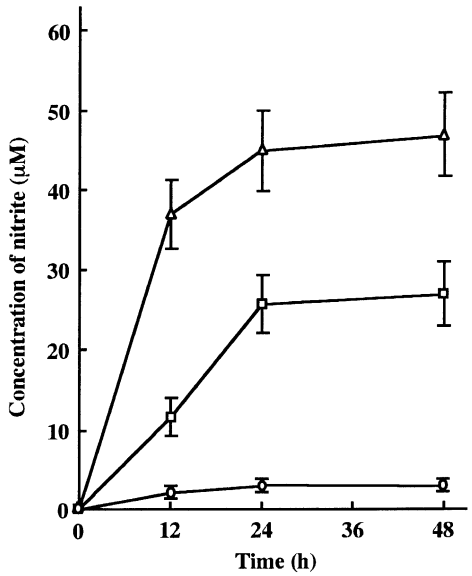

Fig. 8. Production of nitrite from LPS-stimulated RAW cells. RAW cells were incubated with LPS (1 or 10 μg/ml, squares or triangles, respectively) for the indicated times. Nitrite concentrations in media were determined by the Griess method and compared with those of untreated control (circles). $n = 3$.

results suggest that the nifedipine-induced protection against cell death after confluence may be closely related to the inhibition of cell proliferation.

3.2. Effects of nifedipine on death of fibroblasts co-cultured with RAW cells

In order to investigate the effects of nifedipine on the death of fibroblasts during inflammation, gingival fibroblasts were co-cultured with RAW264 cells (macrophage-like cell line). Stimulation with *E. coli* lipopolysaccharide (LPS) induced the death of fibroblasts in a concentration-dependent manner (Fig. 3). However, all concentrations of LPS (1–10 μg/ml) failed to cause the death of fibroblasts cultured alone (Fig. 5). In addition, the death of fibroblasts induced by LPS (10 μg/ml) was inhibited by the addition of 1 μM nifedipine (Fig. 4). However, the LPS-induced death of fibroblasts was not affected by 1 μM diltiazem (data not shown). These results indicate that the nifedipine-induced prevention of fibroblast death may be important to explain the mechanism of gingival overgrowth caused by the long-term use of nifedipine.

3.3. Effects of nifedipine on LPS-induced death of RAW cells

We estimated the direct damage to RAW cells induced by LPS, using a dye exclusion test. LPS-induced cell death was concentration dependent (Fig. 5). Treatment with LPS (10 μg/ml) for 24 h induced the death of approximately 60% of RAW cells. However, the simultaneous addition of nifedipine with LPS (10 μg/ml) caused the inhibition of cell death in a concentration-dependent manner, with an IC_{50} of 0.6 μM (data not shown). Cell death was suppressed by 80% by nifedipine (1 μM) treatment. This significant inhibition was also observed with nicardipine (1 μM), which belongs to the same class (dihydropyridine derivatives) of Ca^{2+} channel blocker as nifedipine, but not with either diltiazem or verapamil (benzothiazepine or phenylalkylamine, respectively) (Fig. 6).

In addition, the death of RAW cells was induced by the NO donors NOC12 and SIN-1 without stimulation by LPS (Fig. 7A), indicating that NO may play an important role in the death of RAW cells. The effects of NOC12 (0.8 mM) or SIN-1 (0.8 mM) were not attenuated by treatment with any concentration of nifedipine (0.1–10 μM) (data not shown).

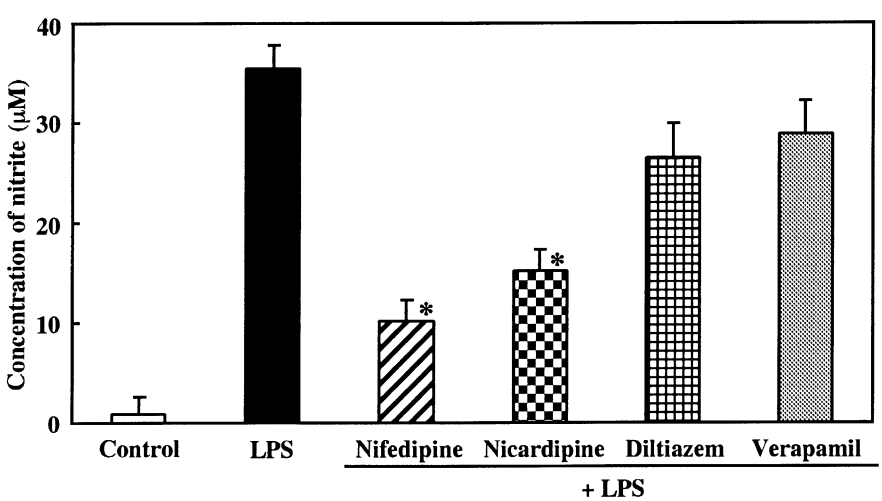

Fig. 9. Effects of Ca^{2+} channel blockers on nitrite production from LPS-stimulated RAW cells. Ca^{2+} channel blockers (1 μM, each) were added to the culture media of RAW cells, simultaneously with LPS (10 μg/ml). Released nitrite concentrations were measured after a further 24-h incubation. $^{*}P < 0.05$ vs. LPS treatment. $n = 9$.

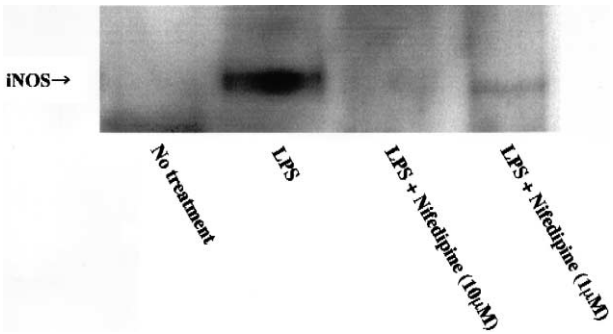

Fig. 10. Induction of inducible NOS by LPS stimulation of RAW cells and effect of nifedipine. RAW cells were treated with LPS (6 μg/ml) and/or nifedipine (1 or 10 μM) for 12 h. The anti-iNOS immunoreactive substances were detected by a Western blotting procedure. The presented photograph is one of representative results. Lanes 1–4 contain cell lysates treated with vehicle (control), LPS, LPS with nifedipine (10 μM), and LPS with nifedipine (1 μM), respectively.

3.4. Effects of nifedipine on NO production by LPS-stimulated RAW cells

We investigated the production of NO by RAW cells stimulated with LPS. Measurement of the nitrite (a final product of NO) concentration in the medium revealed that LPS stimulation caused an increase in NO production, related to the stimulation time and concentration of LPS (Fig. 8). However, LPS stimulation (1–10 μg/ml) failed to release NO from fibroblasts. The NO production by RAW cells was reduced by nifedipine in a concentration-dependent manner with an IC_{50} of 0.8 μM (data not shown), corresponding to the inhibition of cell death. The same inhibition of NO production was observed with nicradipine (1 μM), but not with either diltiazem or verapamil (1 μM, each) (Fig. 9).

We investigated whether nifedipine affected the synthesis of iNOS protein, using anti-iNOS antibody in a Western blotting procedure. Treatment with 6 μg/ml LPS for 12 h clearly increased the immunoreactivity for iNOS in RAW cells (Fig. 10). The iNOS induction was inhibited partly by 1 μM nifedipine and completely by 10 μM.

3.5. Effects of NO-donors on death of gingival fibroblasts

Although the number of RAW cells was deceased by both NO donors, NOC12 and SIN-1 (Fig. 7A), the death of fibroblasts was not stimulated by NOC12, but was by SIN-1 in a concentration-dependent manner (Fig. 7B). The effect of SIN-1 (0.8 mM) was not inhibited by treatment with any concentration of nifedipine (0.1–10 μM) (data not shown).

4. Discussion

In the present experiments, human gingival fibroblasts possessed the ability to synthesize DNA and to proliferate after confluence was reached, probably due to the space provided by dead cells. However, the increase in the number of viable cells was slow and was probably balanced by the number of dead cells, which may be induced by cell–cell contact (adherence). Although the mechanism remains unknown, any kind of Ca^{2+} channel blocker, such as nifedipine, nicardipine, verapamil and diltiazem, significantly inhibited the death of fibroblasts after confluence was reached, without changing the number of viable cells. The reduction in the number of dead cells indicates that Ca^{2+} channel blockers may substantially enhance the proliferation of fibroblasts. These drugs were used at 1 μM, a concentration reached in the gingiva of patients receiving the drug as treatment for cardiovascular diseases (Ellis et al., 1992). In addition, there is a report about the existence of voltage-sensitive L-type Ca^{2+} channels in cultured fibroblasts (Chen et al., 1988; Baumgarten et al., 1992), although their roles in fibroblasts were not clarified. Moreover, the other gingival hyperplasia-inducing drugs, such as phenytoin and cyclosporin, are also reported to interfere with intracellular Ca^{2+} concentrations (Messing et al., 1985; Gelfand et al., 1986, 1987). Therefore, these results suggest that nifedipine may inhibit the adherence-induced death of fibroblasts, probably through the blockade of L-type Ca^{2+} channels, resulting in gingival overgrowth due to the disrupted balance between the proliferation and death of fibroblasts.

Since it has been reported that inflammation may play an important role in the onset of drug-induced gingival overgrowth (Nishikawa et al., 1991; Harel et al., 1995; Ellis et al., 1999), we investigated the effects of nifedipine on the interaction between inflammatory cells and fibroblasts. Inflammatory stimulators such as LPS are reported to cause the induction of iNOS and to increase NO production in macrophages (Nathan, 1992; Yamashita et al., 1997). The released NO participates in the regulation of diverse physiological processes including cytotoxicity (Moncada et al., 1991; Murad, 1996, 1998). In the present studies, nifedipine attenuated the production of NO from LPS-stimulated RAW264 cells, a murine macrophage-like cell line, with a concomitant reduction in LPS-induced death of RAW cells. These data suggest the possibility that nifedipine may inhibit the induction of iNOS by LPS, or that the activity of iNOS may be inhibited by the Ca^{2+} channel blocker. Because of the Ca^{2+} independence of iNOS, the latter possibility is unlikely. Actually, nifedipine inhibited the induction of iNOS by LPS in RAW cells in a concentration-dependent manner. However, the effects of nifedipine on the induction of iNOS are controversial. It is reported that Ca^{2+} channel blockers, such as nifedipine, diltiazem and verapamil, do not affect the iNOS induction by interferon-γ or LPS in RAW264.7 cells (Schmidt et al., 1992). However, dihydropyridine Ca^{2+} channel modulators, such as nifedipine, nitrendipine, nimodipine, and BAY K8644, are reported to inhibit the induction of iNOS by bacterial LPS in J774.2 cells, a similar macrophage-like

clone (Szabo et al., 1993a,b), suggesting that the inhibition of iNOS induction may not be due to a reduction in the intracellular Ca^{2+} level. Similarly, we observed that the death of LPS-stimulated RAW cells was inhibited by dihydropyridines (nifedipine and nicardipine), but not by another types of Ca^{2+} channel blockers such as verapamil and diltiazem. These data may indicate the mechanism of the inhibition by nifedipine of RAW cell death stimulated by LPS.

Moreover, a simple NO-donor, NOC12, failed to induce the death of fibroblasts, but it was effective in causing the death of RAW cells. In contrast, SIN-1, a co-donor of NO and superoxide, could induce the death of both fibroblasts and RAW cells. Since NO interacts with superoxide in a rapid reaction to form a strong oxidative agent, peroxynitrite, the cytotoxic effects of NO may be the result of the formation of peroxynitrite (Beckman et al., 1990; Stamler et al., 1992). Therefore, the discrepancy between the effects of NO-donors on fibroblasts and RAW cells may be explained by the ability of RAW cells to produce superoxide radicals. In addition, this also explains the mechanism of death of fibroblasts co-cultured with RAW cells. The LPS stimulation caused the production of both NO and superoxide to form peroxynitrite, which then induced the death of fibroblasts. The reaction of NO and superoxide is reported to be 2×10^{10} M^{-1} s^{-1} (Koppenol, 1998). It might be too rapid for treatment with superoxide dismutase (10 IU/ml) to attenuate the death of fibroblasts induced by LPS-stimulated RAW cells. Therefore, the protective effects of nifedipine against the cytotoxicity may be considered due to the inhibition by dihydropyridine of LPS-stimulated iNOS induction.

Gingival hyperplasia was induced not only by nifedipine, but also by the other Ca^{2+} channel blockers. Although they inhibit the same L-type Ca^{2+} channels, their clinical properties are somewhat different. Dihydropyridines mainly target blood vessels, but verapamil and diltiazem are used for cardiac disorders. Together with the reported data, the present results suggest that the gingival overgrowth induced by Ca^{2+} channel blockers may be the result of a disruption of the balance between the proliferation and death of fibroblasts, and that the inhibition of cell death by Ca^{2+} blockers may be separated into two types: one is adherence-induced cell death, which is inhibited by all Ca^{2+} channel blockers, probably through a mechanism related to reduced intracellular Ca^{2+} concentrations as a result of the blockade of L-type Ca^{2+} channels. The other type of cell death is induced during the inflammatory process through the induction of iNOS and the production of NO (peroxynitrite), and is inhibited by dihydropyridines (nifedipine and nicardipine), probably through a Ca^{2+}-independent mechanism. Therefore, these Ca^{2+} channel blockers may cause gingival hyperplasia by different mechanisms.

The results of the present studies suggest that LPS-stimulated RAW cells produce peroxynitrite, which may induce the death of cultured cells, and that the protection provided by nifedipine against cell death may be due to the reduction in iNOS synthesis and NO production in RAW cells. Therefore, together with the inhibition of cell growth under confluent conditions, these protective effects against cell death may explain the gingival overgrowth seen during clinical treatment with Ca^{2+} channel blockers with or without stimulatory conditions such as inflammation.

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