Comparative study of calcium-channel blockers on cell proliferation, DNA and collagen syntheses, and EGF receptors of cultured gingival fibroblasts derived from human nifedipine, nicardipine and nisoldipine responders

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Abstract: Our previous study indicated that fibroblasts derived from patients reactive to nifedipine might be susceptible to the other calcium-channel blockers (nicardipine, verapamil and diltiazem) in terms of cell proliferation, DNA synthesis, and collagen synthesis. Thus, the present investigation was designed to clarify the cross-reactivity among dihydropyridine calcium-channel blockers (nifedipine, nicardipine, and nisoldipine). Human gingival fibroblasts derived from seven, two, and one patients who developed gingival overgrowth as a result of nifedipine, nicardipine, and nisoldipine medications, respectively, were examined in terms of the effect of calcium-channel blockers (nifedipine, diltiazem, verapamil, and nicardipine) on cell proliferation, DNA synthesis, collagen synthesis, and the number of epidermal growth factor (EGF) receptors. Phenytoin was used as a positive control. With most of the calcium-channel blockers and phenytoin, fibroblasts from patients reactive to nifedipine and nicardipine medications gave a better cell proliferation rate, DNA synthesis, and an increased number of EGF receptors, compared to non-drug-treated control. However, this was not the case for calcium-channel blockers tested in fibroblasts from patients reactive to nisoldipine medication. (J. Oral Sci. 43, 261-268, 2001)

Key words: calcium-channel blockers; gingival overgrowth; human gingival fibroblasts (HGF).

Introduction

Many case reports have implicated nifedipine, one of the dihydropyridine calcium-channel blockers, as a cause of gingival overgrowth (first reported by Ramon et al. (1) and Lederman et al. (2)). The incidence of gingival overgrowth due to nifedipine has been reported to be 6.5% (3), more than 10% (4), 13.7% (5), 15% (6), and 20% (7). This unwanted side effect has also been reported in patients taking three other calcium-channel blockers; diltiazem (8-10), verapamil (11-13), and nicardipine (5,14). We have recently found a patient who developed gingival overgrowth as a result of nisoldipine medication. Excellent reviews on drug-induced gingival overgrowth are available (15-17). Nifedipine is increasingly used in the treatment of angina pectoris and hypertension and is the most frequently cited causal agent in calcium-channel blocker-induced gingival overgrowth (4).

We have previously demonstrated that the fibroblasts from patients reactive to nifedipine gave trends toward better cell proliferation rates, DNA synthesis, and collagen synthesis than those from non-reactive patients in the presence of 1 μM of calcium-channel blockers (nifedipine, diltiazem, nicardipine, and verapamil) or phenytoin (17). Therefore, it is possible that fibroblasts from reactive patients may be also susceptible to the other calcium-channel blockers, which indicates that those patients who developed gingival overgrowth because of nifedipine
medication may also develop it in response to other calcium-channel blockers. In order to clarify this cross-susceptibility, we have isolated gingival fibroblasts from patients reactive to nifedipine (seven strains), nicardipine (two strains), and nisoldipine (one strain), and tested the effect of calcium-channel blockers (nifedipine, diltiazem, nicardipine, and verapamil) and phenytoin on cell proliferation rate, DNA synthesis, collagen synthesis, and the number and Kd value of epidermal growth factor (EGF) receptors in those gingival fibroblasts.

**Materials and Methods**

**Cells**

Cultures of fibroblast-like cells were established from gingival specimens of seven, two, and one patients who develop gingival overgrowth as a result of nifedipine, nicardipine, and nisoldipine medications, respectively (Table 1). All the specimens were obtained during gingivectomy, clearance of remaining teeth, or orthopedic surgery of the alveolar ridge from the patient who gave informed consent, under a protocol approved by the Committee on Studies involving Human Beings of Nihon University School of Dentistry at Matsudo. Disappearance or decreased severity of the overgrowth after discontinuance of medication was regarded as indicating a responsive state. Isolation and culture of gingival fibroblasts were performed by the methods described previously (17,18). Homogeneity of fibroblasts was determined by flow cytometry (FACS Vantage, Nippon Becton Dickinson Co. Ltd., Japan). The fibroblasts used for experiments proliferated in the logarithmic phase between the 5th and the 8th passage.

**Cell-proliferation assay**

The cell-proliferation assay was carried out by a previously reported method (17). All ten strains of fibroblasts were subjected to the experiment in the following manner: Fibroblasts (approximately 3×10⁵) in 500 µl of Dulbecco’s modified Eagle media (DMEM) supplemented with 10% fetal calf serum (FCS) and antimicrobial agents (streptomycin 100 µg/ml, penicillin G 100 U/ml, and amphotericin B 0.2 µg/ml; DMEM-10) were allowed to settle in a 24-well plate (Falcon tissue culture plate 3047) for 24 h. Cells were washed and the medium was replaced with DMEM containing 1% FCS and the same antimicrobial agents as above (DMEM-1) for 24 h. Cells were again washed, a fresh 500 µl of DMEM-1 poured and 20 µl of 25 µM calcium-channel blockers (nifedipine, diltiazem, verapamil, nicardipine; Sigma Chemical Co., St. Louis, MO, U.S.A.) and phenytoin (Sigma Chemical Co., St. Louis, MO, U.S.A.) were added to make the final concentration of 1 µM in 4 wells each and kept for 48 h. Our previous dose-response experiment revealed that 1 µM of nifedipine gave the best growth in fibroblasts from nifedipine-reactive patients (17). Cells were then washed again and treated in the same manner as above except that DMEM-10 was used. Cells were harvested using 0.25% trypsin and 0.02% EDTA in Dulbecco’s phosphate buffered

<p>| Table 1 Source of cell strains |
|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>age</th>
<th>sex</th>
<th>nifedipine dose mg/day</th>
<th>duration years</th>
<th>site of gingival hyperplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nifedipine-reactive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>F</td>
<td>20</td>
<td>3.0</td>
<td>43,42: labial; gingival</td>
</tr>
<tr>
<td>39</td>
<td>M</td>
<td>20-40</td>
<td>6.0</td>
<td>36,37: buccal; gingival</td>
</tr>
<tr>
<td>50</td>
<td>M</td>
<td>20</td>
<td>1.5</td>
<td>34,35: buccal; alveolar ridge</td>
</tr>
<tr>
<td>74</td>
<td>M</td>
<td>30</td>
<td>2.0</td>
<td>26: buccal; gingival</td>
</tr>
<tr>
<td>66</td>
<td>F</td>
<td>20</td>
<td>1.0</td>
<td>36: buccal; gingival</td>
</tr>
<tr>
<td>65</td>
<td>F</td>
<td>20</td>
<td>4.0</td>
<td>33: labial; gingival</td>
</tr>
<tr>
<td>55</td>
<td>M</td>
<td>30</td>
<td>3.0</td>
<td>11,21: labial; gingival</td>
</tr>
<tr>
<td>Nicardipine-reactive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>F</td>
<td>30</td>
<td>1.5</td>
<td>43: labial; gingival</td>
</tr>
<tr>
<td>75</td>
<td>M</td>
<td>40</td>
<td>1.5</td>
<td>27: buccal; gingival</td>
</tr>
<tr>
<td>Nisoldipine-reactive</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>F</td>
<td>4</td>
<td>1.0</td>
<td>46,44: buccal; gingival</td>
</tr>
</tbody>
</table>

*Degree of gingival overgrowth: 2, moderate; 3, severe; none, clinically healthy with a Gingival Index value of 0, and a Bleeding Index value of 0.
saline (DPBS) and the population count was performed using a Coulter Counter ZM (Coulter Electronics, Ltd., Luton, England). The number of cells in the absence of drugs served as the control.

DNA synthesis

DNA synthesis was determined using the method previously reported (17) with a slight modification. Specifically, fibroblasts (approximately 6×10⁵ cells) in 100 µl of DMEM-10 were allowed to settle in a 96-well plate (Falcon tissue culture plate 3072) for 24 h. The adherent cells were washed and the medium was replaced with DMEM-1 and then 4 µl of 25 µM calcium-channel blockers and phenytoin were added in the same manner as above except in 6 wells each and kept for 48 h. The medium was changed again with DMEM-1 and calcium-channel blockers and phenytoin were added. ³H-thymidine (22.2 kBq, Amersham Life Science, Tokyo, Japan) was added to each well 24 h after the last medium change and then incorporated into the cells for 24 h. During this pulse-label period, cells were in the S-phase stage and proliferation was very low because the medium (DMEM-1) contained only 1% of FCS. After the treatment using 0.25% trypsin and 0.02% EDTA in DPBS, cells were harvested with a cell collector (Micromate 196, Packard Japan, Tokyo, Japan), and the resulting radioactivities on the cells were measured by liquid scintillation counting using Ultima Gold (Packard Japan, Tokyo, Japan) as a scintillator. The radioactivities of the wells with non-drug treatment served as the control.

Collagen synthesis

Collagen synthesis was determined by the method described previously (17) with a slight modification. Specifically, fibroblasts (approximately 3×10⁴ cells) in 500 µl of DMEM-10 were allowed to settle in a 24-well plate (Falcon tissue culture plate 3047) for 24 h. The adherent cells were washed and the medium was replaced with DMEM-1 and then 20 µl of 25 µM calcium-channel blockers and phenytoin were added in the same manner as above in 4 wells each and kept for 48 h. The medium was changed again with DMEM-1 and calcium-channel blockers and phenytoin were added in the same manner as above. ³H-proline (92.5 kBq, Amersham Life Science, Tokyo, Japan) was added to each well 24 h after the last medium change and then incorporated into the cell for 24 h. During this pulse-label period, cells were in the S-phase stage and proliferation was very low because the medium (DMEM-1) contained only 1% FCS. After the treatment using 0.4% trypsin and 0.0576% L-proline was added. The radioactivities on the cells were measured using a direct beta counter (Matrix 96, Packard Japan, Tokyo, Japan). Radioactivity of the cell in the absence of drug served as a control.

EGF receptor assay

EGF receptor assay was performed by the method reported by King and Cuatrecasas (19) and Modéer et al. (20,21) with a slight modification. Specifically, fibroblasts (approximately 3×10⁴ cells) in 1.5 ml of DMEM-10 were allowed to settle in a 6-well plate (Falcon tissue culture plate 3046) for 24 h. The adherent cells were washed and the medium was replaced with DMEM-1 and then 60 µl of 25 µM calcium-channel blockers and phenytoin were added in the same manner as above in 3 wells each and kept for 24 h. The medium was discarded and the monolayer fibroblasts in a 6-well culture plate were washed 3 times with 500 µl each of cold DPBS, and then replaced with cold Hank’s solution (400 µl) substituted with 0.1% bovine serum albumin. Calcium-channel blockers and phenytoin were added to the solution to make the final concentration of 1 µM, and then different concentration of unlabeled EGF (0-1250 ng/ml in cold DPBS) were added into the well and incubated for 10 min at 4°C. Next, radioiodinated EGF (125I-EGF; 1.8 kBq) was added into the well, and incubated for a further 3h at 4°C. Following the incubation, the well was washed 3 times with cold DPBS and then dissolved into 500 µl of 0.5 M NaOH. The radioactivity was determined by gamma counting (COBRA, Packard Japan, Tokyo, Japan). The experiment was performed three times and the data are the mean of three trials. Bmax (Bmax₁ and Bmax₂) and Kd (Kd₁ and Kd₂) values were obtained in the Scatchard
analysis. Data are expressed as relative $B_{\text{max1}}$ and $B_{\text{max2}}$ and $K_{d1}$ and $K_{d2}$ for high affinity and low affinity receptors, respectively, compared to non-drug-treated control.

Statistical methodology
Statistical analyses on the data for fibroblasts obtained from nifedipine (seven strains) reactive patients on the means of 3-5 multiple experiments were carried out by the multiple analysis of variance (multiple ANOVA) and the significance was established on the basis of the Newman-Keuls test at $P < 0.05$.

Results
Homogeneity of fibroblasts
Each group of cell-strains used in the present experiment were subjected to flow cytometry and were each found to give homogeneous samples in terms of the appearance of SC-F (difference of size and characteristics of cell surface) and SC-S (difference of inner substance).

Cell proliferation
The effect of calcium-channel blockers and phenytoin on relative growth rates (number of cells in experiment /number of cells in non-drug-treated control) in nifedipine, nicardipine, and nisoldipine responders are summarized in Fig. 1. With all calcium-channel blockers and phenytoin, fibroblasts from subjects reactive to nifedipine showed better growth than ones reactive to nicardipine and nisoldipine. Fibroblasts from subjects reactive to nicardipine showed better growth by nifedipine and those reactive to nisoldipine by nicardipine and phenytoin. All fibroblasts from subjects reactive to calcium-channel blockers showed better growth with all calcium-channel blockers and phenytoin than non-drug-treated controls (the relative growth rate was greater than 1). No statistical difference was found among the calcium-channel blockers in the subjects reactive to nifedipine.

DNA synthesis
The effect of calcium-channel blockers and phenytoin on relative [$^3$H]-thymidine incorporation rates (radioactivity in experiment/radioactivity in non-drug-treated control) in nifedipine, nicardipine, and nisoldipine responders are summarized in Fig. 2. With all calcium-channel blockers and phenytoin, except for nicardipine in fibroblasts from nifedipine-reactive patients, all fibroblasts from subjects reactive to calcium-channel blockers showed greater [$^3$H]-thymidine incorporation than non-drug-treated controls (the relative [$^3$H]-thymidine incorporation rate was greater than 1). No statistical difference was found among the calcium-channel blockers in the subjects reactive to nifedipine.
Collagen synthesis

The effects of calcium-channel blockers and phenytoin on the relative $[^{3}H]$-proline incorporation rate (radioactivity in experiment/radioactivity in non-drug-treated control) in the collagenase digestible collagen are shown in Fig. 3.

With most calcium-channel blockers and phenytoin, fibroblasts from subjects reactive to nifedipine showed less collagen synthesis than non-drug-treated controls (the relative $[^{3}H]$-proline incorporation rate was smaller than 1). Only phenytoin treatment on fibroblasts reactive to nifedipine and nisoldipine gave greater collagen synthesis. No statistical difference was found among the calcium-channel blockers in the subjects reactive to nifedipine.

Assay for EGF

The effects of calcium-channel blockers and phenytoin on relative number of the high affinity EGF receptors (Bmax$_1$) and the low affinity EGF receptors (Bmax$_2$) (number in experiment/number in non-drug-treated control) are summarized in Fig. 4. With all calcium-channel blockers and phenytoin, fibroblasts from subjects reactive to nifedipine and nicardipine had greater relative numbers of Bmax$_1$. Only with verapamil and phenytoin, fibroblasts from subjects reactive to nifedipine and nisoldipine showed greater relative numbers of Bmax$_1$. In case of Bmax$_2$, fibroblasts from subjects reactive to nifedipine and nicardipine showed greater relative numbers of Bmax$_2$. Thus, both fibroblasts from subjects reactive to nifedipine and nicardipine were affected to increase the number of Bmax$_1$ and Bmax$_2$ by calcium-channel blockers and phenytoin.

The effects of calcium-channel blockers and phenytoin on relative affinities of high and low affinity EGF receptors (Kd$_1$ and Kd$_2$, respectively) (Kd of experimental/Kd of non-

Fig. 3 The effect of calcium-channel blockers and phenytoin on collagenase digestible collagen synthesis in monolayer cultures of 7 strains of fibroblasts from nifedipine-reactive patients, 2 strains from nicardipine-reactive patients, and 1 strain from a nisoldipine-reactive patient. Cells were kept in DMEM-1 and pulse-labeled with $[^{3}H]$-proline for 24 h in the presence of 1 $\mu$M of calcium-channel blockers or phenytoin. Data presented as the mean ratio to non-drug-treated control. NIF, Nifedipine; DIL, diltiazem; VER, verapamil; NIC, nicardipine; PHT, phenytoin. ■, Fibroblasts derived from nifedipine-reactive patients; ◇, Fibroblasts derived from nicardipine-reactive patients; □, Fibroblasts derived from a nisoldipine-reactive patient.

Fig. 4 The effect of calcium-channel blockers and phenytoin on the number of high affinity EGF receptors (A) and low affinity EGF receptors (B) in the monolayer cultures of 7 strains of fibroblasts from nifedipine-reactive patients, 2 strains from nicardipine-reactive patients, and 1 strain from a nisoldipine-reactive patient. Cells were kept in cold Hank’s solution substituted with 0.1% bovine serum albumin. Calcium-channel blockers and phenytoin were added to the solution to make the final concentration of 1 $\mu$M, and then different concentrations of unlabeled EGF (0-1250 ng/ml in cold 20 $\mu$l of DPBS) were added and incubated for 10 min at 4°C. Next, $^{125}$I-EGF was added and incubated for a further 3 h at 4°C. Data presented as the mean ratio to non-drug-treated control. NIF, Nifedipine; DIL, diltiazem; VER, verapamil; NIC, nicardipine; PHT, phenytoin. ■, Fibroblasts derived from nifedipine-reactive patients; ◇, Fibroblasts derived from nicardipine-reactive patients; □, Fibroblasts derived from a nisoldipine-reactive patient.
drug-treated control) are summarized in Fig. 5. Most of relative $K_{d1}$s in fibroblasts from subjects reactive to nifedipine, nicardipine, and nisoldipine showed smaller relative $K_{d1}$, except in the one from subjects reactive to nisoldipine, for nifedipine and nicardipine showed greater relative $K_{d1}$ (Fig. 5A). All $K_{d2}$s in fibroblasts from subjects reactive to nifedipine were greater than those of non-drug-treated controls (Fig. 5B). No statistical differences were found among the calcium-channel blockers in the subjects reactive to nifedipine in $B_{max}$ and $K_d$ values.

**Discussion**

This is our first and a rare case of gingival overgrowth found in a patient receiving nisoldipine (22). At present, only one case was found out of 76 nisoldipine-receiving patients. Since nifedipine, nicardipine, and nisoldipine are all dihydropyridine calcium-channel blockers (Fig. 6), it was of interest to investigate the influence of calcium-channel blockers (nifedipine, diltiazem, verapamil, and nicardipine) and phenytoin on fibroblasts from patients reactive to nifedipine, nicardipine, and nisoldipine in order to find cross reactivity. Fibroblasts from subjects reactive to nifedipine and nicardipine showed almost the same trend to calcium-channel blockers and phenytoin in terms of increased proliferation rate and DNA synthesis. However, fibroblasts from the subject reactive to nisoldipine showed a different pattern. As suggested previously, with calcium-channel blockers and phenytoin, fibroblasts from patients reactive to nifedipine medication gave better cell proliferation rates and DNA synthesis than ones from non-reactive patients (17). The same trend was found in fibroblasts from patients reactive to nicardipine medication, but not in fibroblasts from the patient reactive to nisoldipine. Thus, nifedipine and nicardipine might act in quite a similar manner to gingival fibroblasts obtained from patients reactive to nifedipine or nicardipine medication. The statistical analysis of the data for fibroblasts obtained from the nisoldipine-reactive patient was not performed in the present study because of the limitation of number of strains available.

Nishikawa et al. (23) reported that both responder and non-responder cells to nifedipine were not significantly sensitive to EGF on DNA synthesis as compared to the control cells. We demonstrated in this investigation that responder cells to nifedipine and nicardipine gave an increasing number of cell-surface EGF receptors when they were treated with calcium-channel blockers and phenytoin than those of non-drug-treated controls. Although we did not compare fibroblasts from reactive and non-reactive patients, the treatment with all calcium-channel blockers or phenytoin enhanced reactivity to EGF in fibroblasts from reactive patients.

Modéer and Andersson (20) reported that phenytoin regulated EGF receptor metabolism in human gingival fibroblasts by increasing the number of cell-surface EGF receptors which might contribute to the alteration of gingival connective tissue observed in patients undergoing phenytoin medication. Modéer et al. (21) also reported that in fibroblasts derived from a patient who developed gingival overgrowth during phenytoin medication (responder) as

![Fig. 5](image-url)
well as in the fibroblasts derived from a patient where gingival overgrowth did not develop (non-responder), the affinity of the EGF receptor for EGF was not significantly changed. However, phenytoin medication resulted in a down-regulation of EGF receptor metabolism in fibroblasts derived from a responder patient, whereas in the non-responder patient EGF receptor metabolism was up-regulated. In case of nifedipine and nicardipine, the number of EGF receptors was increased in fibroblasts from patients reactive to nifedipine and nicardipine with calcium-channel blockers and phenytoin. The present data support the former explanation that suggests an increase in EGF receptors. Since the relative Bmax was generally greater than 1 (the ratio of drug treated/non-drug-treated was greater than 1) in the present study, it was not likely that calcium-channel blockers cause down-regulation in fibroblasts obtained from reactive patients. The relative Kd\textsubscript{s} in fibroblasts from subjects reactive to nifedipine, nicardipine and nisoldipine were generally smaller than those of non-drug-treated controls. On the contrary, Kd\textsubscript{s} in fibroblasts from subjects reactive to nifedipine were greater than those of non-drug-treated controls. This indicates the presence of two different kinds of EGF receptors on negative cooperativity. Although the nature of EGF receptor was not clarified in the present study, the further study might be necessary to clarify it. These findings suggest that fibroblasts obtained from subjects reactive to a specific calcium-channel blocker showed cross-reactivity to the other calcium-channel blockers. Thus, care should be taken when one changes from one calcium-channel blocker to another.

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**References**


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Fig. 6 Structure of nifedipine and related compounds.


