Tenidap, an Anti-Inflammatory Agent, Inhibits DNA and Collagen Syntheses, Depresses Cell Proliferation, and Lowers Intracellular pH in Cultured Human Gingival Fibroblasts

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ABSTRACT
The effect of tenidap ([±]-5-chloro-2,3-dihydro-3-(hydroxy-2-thienylmethylene)-2-oxo-1H-indole-1-carboxamide), a new anti-inflammatory agent, was investigated on DNA synthesis by means of [3H]thymidine incorporation, collagen synthesis by means of [3H]proline incorporation, cell proliferation, and intracellular pH in nicardipine-reactive human gingival fibroblasts. Tenidap significantly inhibited [3H]thymidine incorporation at concentrations greater than 20 μM on the 4th and 8th day of treatment. Tenidap also significantly inhibited [3H]proline incorporation at a concentration greater than 50 μM on the 4th day and at more than 20 μM on the 8th day of treatment. The presence of 1 μM nifedipine or 1 μM nicardipine did not alter the depressing effect of tenidap. Tenidap (20 μM) also lowered intracellular pH. These results suggest that tenidap might be effective for the prevention of gingival overgrowth caused by calcium channel blockers.

Tenidap, ([±]-5-chloro-2,3-dihydro-3-(hydroxy-2-thienylmethylene)-2-oxo-1H-indole-1-carboxamide, is a new anti-inflammatory agent, which has been shown to inhibit IgE-mediated N-acetylglucosaminidase secretion from mast cells (Conklyn et al., 1990), release activated collagenase from neutrophils (Blackburn et al., 1991b), inhibit leukotriene B4 and prostaglandin synthesis in human neutrophils (Morianen et al., 1988), form 5-lipoxygenase products in human subject (Blackburn et al., 1991a), inhibit production of interleukin-1, -6, and tumor necrosis factor from human Hep3B hepatoma cells (Sipe et al., 1992), and inhibit the antigen-induced increase in intracellular Ca2+ and also both antigen- and thapsigargin-induced Ca2+ influx across the plasma membrane in a mast cell line (Cleveland et al., 1993) and in human gingival fibroblasts (Fujii et al., 1995a).

During the course of the study investigating the mechanism of gingival overgrowth by nifedipine, one of the dihydropyridine calcium channel blockers, we demonstrated that the fibroblasts derived from nifedipine responders (reactive patients) gave trends of better cell proliferation rate, DNA synthesis, and collagen synthesis than those from nifedipine nonresponders (nonreactive patients) in the presence of 1 μM nifedipine (Fujii et al., 1994). In our previous report (Fujii et al., 1995b), we also found that gingival fibroblasts derived from nifedipine nonresponder showed a greater cytotoxic calcium response to bradykinin (BK), thrombin, prostaglandins E2 and F2α, and platelet-derived growth factor BB than those derived from nifedipine responder. On the contrary, those derived from nifedipine responder responded more intensively to histamine and bombesin.

The cell proliferation in cultured fibroblasts involves a sequence of biochemical events. Among the earliest of these events are dramatic increases in intracellular free Ca2+ concentration ([Ca2+]i) (Byron et al., 1992; Berridge, 1993). BK is one of the agonists increasing [Ca2+]i response via B2 BK receptor in gingival fibroblasts (Lerner et al., 1992; Fujii et al., 1995b). Since Ca2+ is critical for cell proliferation, it is quite interesting to investigate the depressive effect of tenidap, which might prevent Ca2+ influx through plasma membrane (Cleveland et al., 1993; Fujii et al., 1995a) and decrease [Ca2+]i, of gingival fibroblasts, resulting in the prevention of gingival overgrowth initiated by drug treatment such as phenytoin, cyclosporin A, and calcium channel blockers. Thus, the present investigation was undertaken to clarify the effect of tenidap on gingival fibroblasts in respect to DNA synthesis, collagen synthesis, and cell proliferation.

ABBREVIATIONS: BK, bradykinin; [Ca2+]i, intracellular free Ca2+ concentration; DMEM-10, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; DPBS, Dulbecco’s phosphate-buffered saline; pH, intracellular pH; BCECF-AM, 3’-O-acetyl-2’7’-bis(carboxyethyl)-4’,5’-carboxyfluorescein, diacetoxymethyl ester; BCECF, 2’7’-Bis(carboxyethyl)-4’ or 5-carboxyfluorescein, diacetoxymethyl ester.
Materials and Methods

Chemicals and Reagents. Tenidap was kindly provided by Pfizer Pharmaceutical Co. Ltd., Tokyo, Japan. [3H]Thymidine and [3H]proline were purchased from Amersham Life Science, Tokyo, Japan. Scintillator (Ultima Gold) was purchased from Packard Japan K. K. (Tokyo, Japan). All the chemicals and reagents for tissue culture were purchased from Invitrogen Laboratories, Carlsbad, CA. BCECF-AM was purchased from Dojindo Laboratories, Kumamoto, Japan.

Cells. Cultures of fibroblast-like cells were established from the noninflamed gingival specimen of a male patient, 75 years old, who had been receiving nicardipine and developed gingival overgrowth during clearance of his remaining teeth. The plan to use gingival samples was accepted by the Committee of Studies involving Human Beings of Nihon University School of Dentistry at Matsudo. Fibroblasts were obtained by trypsinization of the primary outgrowth of the cells by the procedure described by Fujii et al. (1994, 1995a).

Briefly, the cells were incubated in an atmosphere of 5% CO₂/95% air at 37°C in the medium, DMEM-10 supplemented with 10% fetal calf serum (FCS), 100 μg/ml streptomycin, 100 μg/ml penicillin G, and 0.2 μg/ml amphotericin B, and routinely passaged using 0.25% trypsin and 0.02% EDTA in Dulbecco’s phosphate-buffered saline (DPBS). Homogeneity of fibroblasts was determined by flow cytometry (FACS Vantage; Nippon Becton Dickinson Co. Ltd., Tokyo, Japan). The fibroblasts used for experiments proliferated in the logarithmic phase between the fifth and the eighth passage.

DNA, Collagen Syntheses, and Cell Proliferation. Measurement of DNA, collagen syntheses, and cell proliferation was performed in the same manner as reported previously (Fujii et al., 1994) with a slight modification. Briefly, for DNA synthesis assay, fibroblasts (approximately 6 × 10⁴ cells) in 100 μl of DMEM-10 were allowed to settle in a 96-well multiwell plate for 24 h. The adherent cells were washed, the medium was replaced with DMEM-1 (the same as DMEM-10, except DMEM-1 contains 1% FCS), and then 4 μl of different concentrations of tenidap was added to make the final concentration of 0, 1, 5, 10, 20, 50, and 100 μM and kept for 48 h. The medium was changed again with DMEM-1, and tenidap was added in the same manner as above. The medium was also supplemented with nifedipine (for a final concentration of 1 μM) and nicardipine (for a final concentration of 1 μM). [3H]Thymidine (22.2 kBq) was added to each well on the 4th and 8th day 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 essentially uniform throughout the experimental period.

The incorporated radioactivity of 0 μM tenidap was used as control. The data are expressed as relative [3H]thymidine incorporation rate (1–100 μM tenidap/0 μM tenidap).

For collagen synthesis assay, fibroblasts (approximately 2 × 10⁵ cells) in 500 μl of DMEM-10 were allowed to settle in a 24-well multiwell plate for 24 h. The adherent cells were washed, and the medium was replaced with DMEM-1 for 24 h. The medium was changed again with DMEM-1, and tenidap was added. The medium was also supplemented with nifedipine (for a final concentration of 1 μM) and nicardipine (for a final concentration of 1 μM). [3H]Proline (92.5 kBq) was added to each well on the 4th and 8th day 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. Cells were harvested after the treatment with 0.25% trypsin and 0.02% EDTA in DPBS, and the total collagen was collected. The radioactivity of incorporated [3H]proline was measured by liquid scintillation counting (TRI-CARB 900CA Liquid Scintillation Analyzer; Packard Japan K. K.). A group without the addition of calcium channel blockers served as the control. The data are expressed as relative [3H]proline incorporation rate (1–100 μM tenidap/0 μM tenidap).

For cell proliferation assay, fibroblasts (approximately 3 × 10⁴ cells) in 500 μl of DMEM-10 were allowed to settle in a 24-well multiwell plate for 24 h. The adherent cells were washed, and the medium was replaced with DMEM-1 for 24 h. Cells were again washed, 500 μl of fresh DMEM-1 was poured, and 20 μl of different concentrations of tenidap were added as above and kept for 48 h. Cells were then washed again and treated in the same manner as above except DMEM-10 was used. Cells were harvested using 0.25% trypsin and 0.02% EDTA in DPBS, and the population count was measured using Coulter Counter ZM (Coulter Electronics, Ltd., Luton, UK). A group without the addition of calcium channel blockers served as the control. The data are expressed as relative cell proliferation rate (1–100 μM tenidap/0 μM tenidap).

Each assay was performed with six wells, and the mean was obtained. This was repeated three times, and the mean was shown as the result. Statistical analysis was made by using the multiple analysis of variance (the Newman-Keuls test) to determine the significance level of the difference between mean values. P values of <0.05 were considered significant.

Intracellular pH was assayed in the following manner: fibroblasts in HEPES buffer (153 mM NaCl, 5 mM KCl, 5 mM glucose, and 20 mM HEPES, pH 7.4) were loaded onto 3 μM BCECF-AM with gentle shaking for 30 min at 37°C. BCECF-loaded cells were washed three times with the same buffer and suspended to make 5 × 10⁴ cells/ml. Intracellular pH was measured for 5 min using CAF-110 intracellular ion analyzer (JASCO, Tokyo, Japan).

Results

Homogeneity of Fibroblasts. The cell strain used in the present experiment was subjected to flow cytometry to find its homogeneity in the appearance on SC-F (difference of size and characteristics of cell surface) and SC-S (difference of inner substance).

Effect of Tenidap on DNA Synthesis. Relative [3H]thymidine incorporation rate is summarized in Fig. 1. Relative [3H]thymidine incorporation rate was calculated by dividing the incorporated radioactivity of experimental by the incorporated radioactivity of 0 μM tenidap. Tenidap significantly inhibited [3H]thymidine incorporation at concentrations greater than 20 μM in the absence of calcium channel blockers (control, tenidap only) and the presence of nicardipine on both the 4th and 8th day of treatment (Fig. 1, A and B).

Effect of Tenidap on Collagen Synthesis. Relative [3H]proline incorporation rate is summarized in Fig. 2. Relative [3H]proline incorporation rate was calculated by dividing the incorporated radioactivity of experimental by the incorporated radioactivity of 0 μM tenidap. Tenidap significantly inhibited [3H]proline incorporation at concentrations of greater than 50 μM in the absence of calcium channel blockers (control, tenidap only) and the presence of nifedipine and greater than 100 μM in the presence of nicardipine on the 4th day (Fig. 2A), and also inhibited it at concentrations greater than 10 μM in the presence of nifedipine and greater than 20 μM in the absence of calcium channel blockers (control, tenidap only) and the presence of nifedipine on the 8th day (Fig. 2B).

Effect of Tenidap on Cell Proliferation. Relative cell proliferation rate is summarized in Fig. 3. Relative cell proliferation rate was calculated by dividing the cell count of experimental by the cell count of 0 μM tenidap. Tenidap did...
not show a significant difference between 0 μM tenidap and 1 to 100 μM tenidap; however, 100 μM tenidap significantly inhibited cell proliferation compared with 5, 10, and 20 μM tenidap in the absence of calcium channel blockers (control, tenidap only) on the 8th day of treatment (Fig. 3B).

**Effect of Tenidap on Intracellular pH.** The time course of pHₐ after the tenidap treatment is shown in Fig. 4. Tenidap (20 μM) lowered pHₐ by 0.12 unit during the measurement, 5 min. The presence of nifedipine or nicardipine did not alter the pHₐ change by tenidap (data not shown).

**Discussion**

We have previously reported that the connective tissue showed larger bundles of dense collagenous fibers with a moderate increase of fibroblasts in gingival overgrowth.
caused by nifedipine medication (Akimoto et al., 1991). There are also many reports describing the enhancement of collagenous fibers and number of fibroblasts in the loci of gingival overgrowth (Ramon et al., 1984; Lucas et al., 1985; Nagata et al., 1985; Barak et al., 1987; Tagawa et al., 1989; Seymour, 1991; Fujii et al., 1994). Therefore, the reductions of collagen formation and fibroblast cell proliferation might play an important role in preventing gingival overgrowth.

Our previous study indicated that the fibroblasts from patients reactive to nifedipine and nicardipine medications gave better cell proliferation rates, DNA synthesis and collagen synthesis due to nifedipine or nicardipine treatment were treated with tenidap and compared with the cells without tenidap treatment (0 μM tenidap). Consequently, the present in vitro data suggest that tenidap significantly inhibits DNA and collagen syntheses at a concentration of greater than 20 μM (6.85 μg/ml). In the first phase of the clinical trial, although plasma C_{max} was 8.305, 17.006, and 21.009 μg/ml after a single oral dose of 40, 80, and 120 mg tenidap, respectively (Pfizer Pharmaceutical Co. Ltd., unpublished data), more than 99% of tenidap bound to plasma protein. Cleveland et al. (1993) also indicated that the plasma drug level at therapeutic doses in arthritis patients reaches 60 μM (20.6 μg/ml), but tenidap is substantially bound by serum albumin. The distribution of tenidap to oral tissue is hardly available. In the case of rats, the distribution to salivary gland is 17.4 to 19.7% (unpublished data, Pfizer Pharmaceutical Co. Ltd.). Thus, it could be estimated that enough tenidap concentration might not be able to reach to the oral tissue, which is enough to reduce DNA and collagen syntheses in gingiva by a systemic tenidap administration.

In our preliminary experiment using rats (Matsumoto et al., 1995), the local application of a high dose of tenidap might be effective to prevent gingival overgrowth caused by calcium channel blockers, especially nifedipine. Therefore, tenidap may be one of the drugs that prevent gingival overgrowth.

Since the cells were established from the gingiva of nicardipine-reactive patients, it was thought that nicardipine and nifedipine might alter the depressive effect of tenidap. However, the presence of 1 μM nifedipine or nicardipine did not alter the depressive effect of tenidap. This fact indicates that tenidap can be locally applied during the administration of nicardipine or nifedipine to depress gingival overgrowth. Thus, one can continue systemic calcium channel blocker...
medication along with local application of tenidap, without gingival overgrowth.

Ives and Daniel (1987) demonstrated that bradykinin and ionomycin caused rapid acidification along with rapid increase of intracellular Ca$^{2+}$. Tenidap has been reported to reduce pH in mouse L cells (McNiff et al., 1994, 1995). We have also demonstrated here that 20 μM tenidap decreased pH, by 0.12 pH unit. Boyle et al. (1997) reported the clamping reduce pHi in mouse L cells (McNiff et al., 1994, 1995). We tracellular Ca$^{2+}$ influx in gingival fibroblasts to reduce its growth through apoptosis.

We have previously reported that tenidap discharges intracellular Ca$^{2+}$ store, resulting in a depletion of intracellular Ca$^{2+}$ store and that tenidap functions on inhibition of Ca$^{2+}$ influx in gingival fibroblasts (Fujii et al., 1995a). Thap-sigargin, one of the compounds that accelerates cell prolifer-pHi by 0.12 pH unit. Boyle et al. (1997) reported the clamping reduce pHi in mouse L cells (McNiff et al., 1994, 1995). We tracellular Ca$^{2+}$ influx in gingival fibroblasts to reduce its growth through apoptosis.

References


Ives HE and Daniel TO (1987) Interrelationship between growth factor-induced pH changes and intracellular Ca$^{2+}$.


Takemura H, Hughes AR, Thastrup O, and Putney JW Jr (1989) Activation of inflammatory agent, discharges intracellular Ca$^{2+}$ store, however, it does not increase intracellular Ca$^{2+}$ store and that tenidap functions on inhibition of Ca$^{2+}$ influx through plasma membrane (Take-mura et al., 1989; Putney, 1990; Putney and Bird, 1993). This indicates that the nature of tenidap, which inhibits Ca$^{2+}$ influx through plasma membrane, plays a quite important role for depression of gingival overgrowth.

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