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Effects of eight antibacterial agents on cell survival and expression of epithelial-cell- or cell-adhesion-related genes in human gingival epithelial cells

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Objective and background: Our previous studies suggest that little adverse effect on the growth of the periodontal ligament would be expected, if tetracycline, minocycline, ofloxacin, roxithromycin, clarithromycin, and azithromycin were topically administered to the periodontal pocket at their MIC_{90} doses required to inhibit the growth of 90% of periodontopathic bacteria, including *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans*. In the present study, we investigated the cytocidal effects of eight antibacterial agents on the human gingival epithelial cell line NDUSD-1. We also used NDUSD-1 cells to examine the effects of these agents on the mRNA and protein expressions of genes associated with the proliferation, differentiation, or cellular adhesion important to the epithelial regeneration of the periodontal attachment.

Methods: The cytocidal effect of the test agents was measured as a decrease in cell survival. To obtain a quantitative measure of the cytocidal effect, the LD_{50}, i.e. the concentration which results in a 50% decrease in cell survival relative to the controls, was extrapolated from the concentration–response curves. The effects of the agents on the mRNA and protein expressions in NDUSD-1 cells were studied by reverse transcription-polymerase chain reaction (RT-PCR) and western blot analyses, respectively.

Results: The cytocidal effect increased in a concentration-dependent manner as the concentration of each of the eight test agents increased. The order of the agents according to their cytocidal effects (LD_{50}) was minocycline > tetracycline > enoxacin > clarithromycin > roxythromycin ≈ ofloxacin > azithromycin > erythromycin. The cytocidal effects of minocycline, tetracycline, enoxacin, clarithromycin, roxythromycin, ofloxacin, and azithromycin ranged from 1.2 to 23.2 times greater than that of erythromycin. The maximum non-cytocidal concentrations (MNCCs) of these agents for NDUSD-1 cells were: 0.3 μM for minocycline, 1 μM for tetracycline, 3 μM for ofloxacin and erythromycin, 10 μM for enoxacin, clarithromycin, and azithromycin, and 100 μM for roxythromycin. The MNCCs of ofloxacin, azithromycin, clarithromycin, and roxythromycin were greater than

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their MIC_{90} concentrations for periodontopathic bacteria described above. The effects on the mRNA and protein expressions of epithelial-cell- or cell-adhesion-related genes were examined in NDUSD-1 cells exposed to clarithromycin, roxythromycin, ofloxacin, and azithromycin at their MNCCs. None of the agents affected the mRNA expressions of five genes: *keratinocyte growth factor receptor*, *keratin 18*, *integrin* β1, *integrin* β4, and *laminin 5*γ2. Clarithromycin and ofloxacin slightly decreased the protein expression *of integrin* β4. Roxythromycin markedly decreased the protein expressions of *integrin* β4 and *laminin 5*γ2. Azithromycin had little inhibitory effects on the protein expressions of any of the five genes.

Conclusions: These results suggest that little, if any, adverse effects on growth, differentiation, and adhesion of basal epithelial cells would be expected with topical administration of clarithromycin, ofloxacin or azithromycin to the periodontal pocket at a dose equivalent to the MIC_{90}. It is important to note, however, that the extrapolation of these findings to *in vivo* conditions has yet to be undertaken.

Tetracyclines are widely used for the topical treatment of periodontitis. Since prolonged administration of the same class of agents can lead to the development of drug resistance or side-effects (1, 2), attempts have been made to screen other classes of antibacterial agents for efficacy against periodontitis. Fluoroquinolones and macrolide antibiotics are such classes of agents, and their antibacterial activities against periodontopathic bacteria have been thoroughly investigated (3, 4).

Antibacterial agents directly administered to the periodontal pocket to treat periodontitis should inflict little or no damage on the host cells, otherwise they might adversely affect recovery from periodontitis. One approach for assessing cellular damage is to assay cytotoxic damage by using cultured human cells derived from periodontal tissues. An estimate of the toxicity of antibacterial agents to be used in dentistry would facilitate maximizing their therapeutic effects while reducing their toxic effects. For this purpose, we previously measured quantitatively the cytocidal effect of four tetracyclines, five fluoroquinolones, and seven macrolides on cultured human periodontal ligament fibroblasts (Pel cells) (5–7). The cytocidal effect of the tetracyclines as determined by LD_{50}, the concentration which results in a 50% decrease in cell survival relative to the control cells, was in the following order: demeclocycline ≈ tetracycline ≈ minocycline ≫ chlortetracycline. The order of the fluoroquinolones according to their cytocidal effects (LD_{50}) was: tosufloxacin > enoxacin > sparfloxacin > lomefloxacin > ofloxacin, and the order for the macrolides was: rokitamycin > roxithromycin > clarithromycin > azithromycin > josamycin > midecamycin ≈ erythromycin. The maximum concentrations of tetracyclines that had little cytocidal effect on Pel cells were: 30 μM for tetracycline, demeclocycline and minocycline, and > 1000 μM for chlortetracycline. The maximum concentrations for the fluoroquinolones were: 10 μM for tosufloxacin, 30 μM for sparfloxacin and enoxacin, 100 μM for lomefloxacin, and 300 μM for ofloxacin. The MIC_{90}, the concentrations required to inhibit the growth of 90% of periodontopathic bacteria, including *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans*, were 0.06–8 μM for tetracycline and minocycline and 0.05–5 μM for tosufloxacin, sparfloxacin, and ofloxacin (3). When extrapolated from the concentration–response curves, the relative survivals of Pel cells exposed to the macrolides at the MIC_{90} concentrations were estimated to be: ≥53.8% for rokitamycin, ≥92.7% for roxithromycin, ≥94.6% for clarithromycin, ≥97.1% for azithromycin, and ≥86.2% for erythromycin (7). Although these findings have yet to be extrapolated to *in vivo* conditions, they suggest that little adverse effect on the growth of the periodontal ligament would be expected, if tetracycline, minocycline, tosufloxacin, sparfloxacin, ofloxacin, roxithromycin, clarithromycin, and azithromycin were topically administered to the periodontal pocket at the MIC_{90} doses.

Because epithelial regeneration of the periodontal attachment is important to healing of periodontal wounds following treatment by surface scaling and curettage, the antibacterial agents topically administered to the periodontal pocket should have little adverse effect on the re-epithelialization that results from the proliferation, migration, and differentiation of the basal epithelial cells. In the present study, we quantitatively investigated the cytocidal effects of enoxacin, ofloxacin, roxithromycin, clarithromycin, azithromycin, and erythromycin on cultured human gingival epithelial cells and compared them to those of tetracycline and minocycline. We also used these cells to examine the effects of these agents on the mRNA and protein expressions of genes for *keratinocyte growth factor receptor* (*KGFR*), *keratin 18* or *8/18*, *integrin* β1, *integrin* β4, and *laminin 5*γ2, which are associated with the proliferation, differentiation, or cellular adhesion important to the epithelial regeneration of the periodontal attachment. Human gingival fibroblasts and periodontal ligament fibroblasts express keratinocyte growth factor (KGF), which plays a major part in re-epithelialization in wound healing via binding to the specific KGF receptor (KGFR) which is expressed in

gingival epithelial cells (8, 9). The expressions of keratins, which are encoded by members of the 10 nm intermediate filament multigene family, varies with proliferation, differentiation, and the states of cell transformation and development. *Keratin 8* and/or *18* are expressed in normal, immortalized, and malignant oral epithelia in organotypic culture (10). Integrins belong to the family of cellular adhesion molecules, which are expressed by numerous cell types. In normal gingival tissues, the basal layer of epithelium is attached to the basal lamina, and integrins on the surface of the epithelial cells act as receptors to bind to laminin in the basal lamina. The integrin subunits α2, α3, α6, β1, and β4 are always expressed by all epithelial cells, including gingival epithelial cells, regardless of whether the tissue involved is healthy, inflamed, or in the process of healing (11). Integrins β1 and β4 are likely to be involved in apical migration of junctional epithelium during the periodontal attachment loss (12). Laminin-5, a laminin isoform specific for epithelial cells, is a major component of the internal basal lamina in both rodent and human tissues (13). Laminin-5 is composed of three truncated polypeptide chains, α3, β3, and γ2 (14). The *laminin 5*γ2 gene is expressed in the cells of the junctional epithelium but not in other parts of the gingival epithelium (13).

Materials and methods

Cells and test agents

The human gingival epithelial cell line (NDUSD-1) used in this study and the culture conditions have been described previously (15). NDUSD-1 cells are a cell line immortalized by infection of normal gingival epithelial cells in secondary culture with origin (–)SV40 DNA and human c-*fos*. Tetracycline hydrochloride and minocycline hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Enoxacin (Dai-Nippon Pharmaceutical Co., Tokyo, Japan), ofloxacin (Daiichi Pharmaceutical Co., Tokyo, Japan), azithromycin (Pfizer Pharmaceuticals, Tokyo, Japan), clarithromycin (Taisho Pharmaceutical Co., Tokyo, Japan), and roxithromycin (Eisai Co., Tokyo, Japan) were generously provided by the indicated companies. Erythromycin was purchased from Wako Pure Chemical Industries Ltd, Tokyo, Japan. Tetracycline and minocycline were dissolved at 1 mM in culture medium (Keratinocyte growth medium®: KGM, Cambrex Co., East Rutherford, NJ, USA). Enoxacin and ofloxacin were dissolved at 10 mM in 0.05 N NaOH in KGM. Immediately after dissolving these four agents, they were filter-sterilized, diluted to the desired concentrations with KGM, and used in the experiments. azithromycin, erythromycin, and roxythromycin were dissolved at 100 mM in dimethylsulfoxide. Clarithromycin was dissolved at 60 mM in dimethylsulfoxide. All of these macrolides were diluted to the desired concentrations with KGM.

Cell survival

Cell survival was determined from the colony-forming efficiency of NDUSD-1 cells exposed to test agents. NDUSD-1 cells were plated in triplicate on 60-mm dishes at 1000 cells/dish, incubated overnight, and exposed to different concentrations of test agents for 48 h. The cells were then washed twice with 2 ml KGM and incubated for 12 d to form colonies. The colonies were fixed with absolute methanol and stained with 3% Giemsa solution. The number of surviving colonies with > 50 cells was then counted, and the percentage of cell survival was calculated as the number of colonies in the treated plates divided by the number in control plates and multiplied by 100. In the experiments with test agents dissolved in a NaOH solution or dimethylsulfoxide, cells in the control groups were exposed to KGM containing 0.0015–0.005 N NaOH or 0.5–1% dimethylsulfoxide, respectively. The actual colony-forming efficiency of NDUSD-1 cells exposed to KGM containing 0.005 N NaOH and 1% dimethylsulfoxide was 36.4 ± 2.7 (SD)% and 35.7 ± 1.5%, respectively, and not significantly different from the efficiency of untreated cells (36.1 ± 1.7%). All experiments were carried out under yellow lights because some test agents are photosensitizers (16, 17). Three independent experiments were performed to measure the cytocidal effect of each test agent. The LD_{50} (50% lethal dose) was extrapolated from the concentration-dependent response curves to a value that corresponded to 50% of the survival observed with the control cells. Statistical analysis was performed by Student's *t*-test to assess the significance of the difference between the LD_{50} values, and the order of the test agents according to cytocidal effect was determined. The level of significance in the statistical analysis was determined at $p < 0.05$.

Detection of apoptotic cells

NDUSD-1 cells (1×10^6) were plated into 75-cm^2 flasks. After overnight incubation, cells were exposed for 48 h to each of the test agents at the LD_{50} concentration for NDUSD-1 cells. As a positive control, NDUSD-1 cells were irradiated with an X-ray dose of 2–8 Gy from a linear accelerator (model ML6M; Mitsubishi Iinac, Tokyo, Japan) operated at 6 MV, yielding a dose rate of 2 Gy/min, and then incubated overnight. After harvesting with 0.025% trypsin and 0.01% EDTA, the cells were fixed in 4% neutral buffered formalin for 10 min, and a 50-μl volume of the cell suspension at a density of 2×10^6 cells/ml was dropped on slide glasses and air-dried. To detect apoptotic cells, the cells on the slide glasses were stained by using the TdT-mediated dUTP nick end labeling (TUNEL) method and an apoptosis *in situ* detection kit (Wako Pure Chemical) according to the manufacturer's instructions. More than 500 cells were scored for each group, and the percentage of apoptotic cells was calculated by dividing the number of apoptotic cells by the total number of cells and multiplying by 100.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The effect of the test agents on the mRNA expressions of genes for *KGFR*, *keratin 18*, *integrins* β1 or β4, and *laminin 5*γ2 was assessed by

RT-PCR analysis. Cells (3×10^5) were plated into 25-cm^2 flasks, incubated overnight, and exposed to the desired concentrations of the test agents for 48 h. After harvesting, total cellular RNA was isolated and reverse-transcribed as described previously (18). PCR was performed in a reaction mixture (25 µl) containing 1 µl of the RT reaction products, 0.025 units of *Taq* polymerase (Amplitaq Gold, Applied Biosystems, Foster City, CA, USA), 200 µM dNTP, 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, and 20 pmol of sense and antisense primers for *KGFR* (8), *keratin 18* (19), *integrins* β1 (20) or β4 (21), *laminin 5*γ2 (22), and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) (23). The reaction mixtures were subjected to 35 cycles of amplification under the conditions described previously (19–23). The PCR products were resolved on a 2% agarose gel, stained with SYBR Green (Biowhittaker Molecular Applications, Rockland, ME), and analyzed with a fluorescence imaging analyzer.

Western blot analysis

Cells (1×10^6) were plated into 75-cm^2 flasks, incubated overnight, and exposed to the desired concentrations of test agents for 48 h. Western blots were performed as described previously (24). Cells were lysed in buffer [0.1% Nonidet P-40/250 mM KCl/50 mM HEPES, pH 7.9/10% (v/v) glycerol/4 mM NaF/4 mM sodium orthovanadate/0.2 mM EDTA/0.2 mM EGTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/aprotinin, pepstatin, and leupeptin, each at 1 µg/ml]. A 50-µl sample of total protein per lane was then run on a 6% SDS-PAGE gel for KGFR, integrin β1, integrin β4, and laminin 5γ2, and a 12% SDS-PAGE gel for keratin 8/18 analyses, and then transferred to nitrocellulose (Millipore, Tokyo, Japan). KGFR and keratin 8/18 were observed with the KGFR and keratin 8/18 monoclonal antibodies, respectively (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a horseradish-peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology), and detection by enhanced chemiluminescence (Amersham Pharmacia Biotech, Tokyo, Japan). X-ray films (RX-U, Fuji Film, Tokyo, Japan) were exposed to the blot. Integrin β1 or integrin β4, and laminin 5γ2 were observed with the integrin β1, integrin β4, and laminin 5γ2 polyclonal antibodies, respectively (Santa Cruz Biotechnology). A horseradish-peroxidase-conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnology) was used for the integrin β1 and integrin β4 blottings and a horseradish-peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) for the laminin 5γ2 blotting, and followed by detection with enhanced chemiluminescence. The probe for extracellular-signal-related kinase 1 (ERK1) was used as an internal control.

Results

The concentration–response curves for the survival of NDUSD-1 cells exposed to test agents for 48 h are shown in Fig. 1. No statistically significant decreases in cell survival were observed when NDUSD-1 cells were exposed to minocycline at ≤ 0.3 µM or tetracycline at ≤ 1 µM. Exposure to ofloxacin or erythromycin at ≤ 3 µM did not reduce cell survival, and exposure to enoxacin, azithromycin, or clarithromycin at ≤ 10 µM, or roxythromycin at ≤ 100 µM had little effect on cell survival. However, cell survival decreased in a concentration-dependent manner as the concentration of each of the test agents increased. To quantitatively compare the cytocidal effect of the test agents, the concentration of each agent that resulted in a 50% decrease in cell survival was estimated by extrapolating from the concentration–response curves (LD_{50}) (Table 1). The LD_{50} value of erythromycin was > 1000 µM, and the LD_{50} values of the other seven agents ranged from 43.0 ± 6.1 µM to 791.0 ± 135.5 µM. There were statistically significant differences among the LD_{50} values of all the test agents, except between roxythromycin and ofloxacin. Thus, the order of the test agents according to their cytocidal effects based on the LD_{50} values was: minocycline > tetracycline > enoxacin > clarithromycin > roxythromycin ≈ ofloxacin > azithromycin > erythromycin. The cytocidal effects of minocycline, tetracycline, enoxacin, clarithromycin, roxythromycin, ofloxacin, and azithromycin ranged from 1.2 to 23.2 times greater than that of erythromycin (Table 1).

To investigate the possible involvement of apoptosis in the cellular lethality of the eight test agents, NDUSD-1 cells exposed to each of the

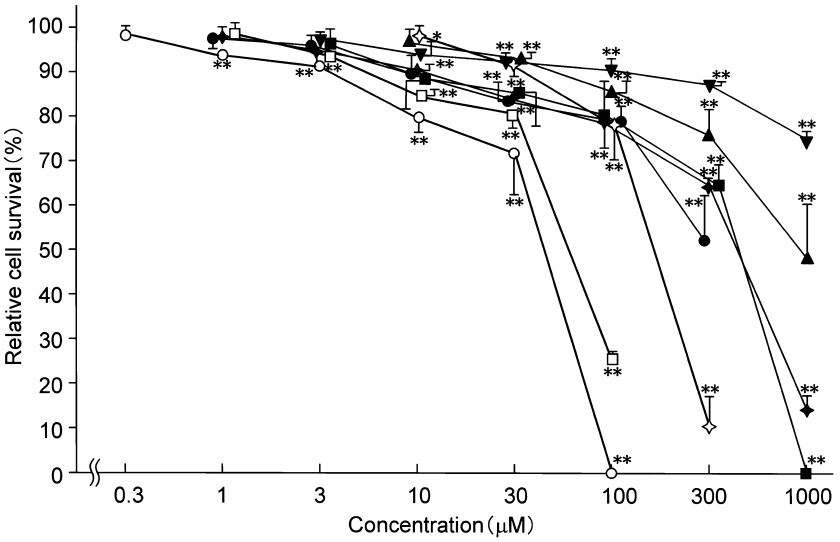

Fig. 1. Relative cell survival of NDUSD-1 cells exposed to each test agent for 48 h. ○, minocycline; □, tetracycline; ◇ , enoxacin; ◆ , ofloxacin; ▲, azithromycin; ▼, erythromycin; ●, clarithromycin; ■, roxythromycin. Each data point represents the average of three independent experiments. The actual colony-forming efficiency in control cultures was 36.1 ± 1.7% (SD). Bars denote SD. When not indicated, SD are within symbols. Significantly different from the control: $*p < 0.05$; $**p < 0.01$, *t*-test.

Table 1. Comparison of the cytocidal effect of various antibacterial agents on NDUSD-1 cells

Antibacterial agents	LD_{50} (μM)	Ratio of cytocidal effect
MINO	43.0 ± 6.1	> 23.3
TC	59.3 ± 2.3*	> 16.9
ENX	159.0 ± 17.5**	> 6.3
CAM	283.0 ± 37.0**	> 3.5
RXM	399.0 ± 33.3*	> 2.5
OFLX	424.3 ± 17.9	> 2.4
AZM	791.0 ± 135.5**	> 1.3
EM	> 1000.0**	1.0

LD_{50}, concentrations of antibacterial agents that resulted in a 50% decrease in cell survival when compared with control cells. The values are presented as the means ± SD of the data obtained from three independent experiments. Ratio of cytocidal effects, the inverse ratio of the LD_{50} of minocycline (MINO), tetracycline (TC), enoxacin (ENX), clarithromycin (CAM), roxythromycin (RXM), ofloxacin (OFLX), or azithromycin (AZM) to that of erythromycin (EM).
Significantly different from the antibacterial agent listed on the line above: *$p < 0.05$; **$p < 0.01$, *t*-test.

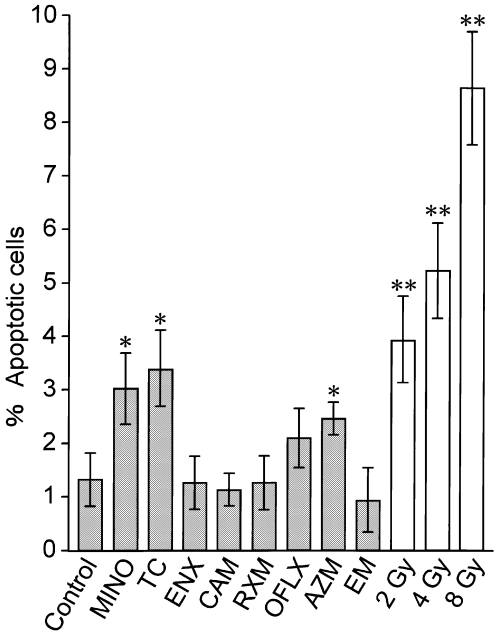

Fig. 2. Percentage of apoptotic cells following exposure of NDUSD-1 cells to minocycline (MINO), tetracycline (TC), enoxacin (ENX), clarithromycin (CAM), roxythromycin (RXM), ofloxacin (OFLX), azithromycin (AZM) and erythromycin (EM) at the LD_{50} for 48 h. 2 Gy, 4 Gy, and 8 Gy refer to NDUSD-1 cells irradiated at 2 Gy, 4 Gy, or 8 Gy (2 Gy/min) and then cultured for 24 h. Significantly different from the control: *$p < 0.05$; **$p < 0.01$, *t*-test.

test agents at the LD_{50} concentration were assayed by the TUNEL method, and the frequency of apoptotic cells was investigated. X-ray irradiations of NDUSD-1 cells at 2–8 Gy used as the positive controls resulted in apoptosis in a dose-dependent manner (Fig. 2). NDUSD-1 cells exposed to minocycline, tetracycline, and azithromycin contained significantly more apoptotic cells than the control cells, but the percentages of apoptotic cells after exposure to enoxacin, clarithromycin, roxythromycin, ofloxacin, or erthromycin were not significantly increased compared with the control cultures.

Next we examined the levels of mRNA and protein expressions of genes for *KGFR*, *keratin 8* and *8/18*, *integrins* β1 and β4, and *laminin 5*γ2 in NDUSD-1 cells exposed to clarithromycin, roxythromycin, ofloxacin, or azithromycin. The other test agents tetracycline, minocycline, enoxacin, and erythromycin were excluded from these experiments because tetracycline, minocycline, and enoxacin exhibited a greater cytocidal effect on NDUSD-1 cells than the other test agents, and the MIC_{90} values of tetracycline, minocycline, and erythromycin against *P. gingivalis*, *P. intermedia*, and *A. actinomycetemcomitans* exceed the maximum concentrations that had little cytocidal effect on NDUSD-1 cells (maximum non-cytocidal concentration: MNCC). In this experiment, NDUSD-1 cells were exposed for 48 h to each of these test agents at two different concentrations, i.e. the MNCC and the LD_{50}. Reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that none of the four test agents had much effect on the mRNA expressions of the five genes in NDUSD-1 cells (Fig. 3). RT-PCR analysis conducted with 25 cycles of amplification yielded the same results (data not shown).

Cells exposed to clarithromycin at the MNCC (10.0 μM) or the LD_{50} (283.0 μM) retained their constitutive levels of protein expression for *KGFR*, *keratin 8/18*, and *integrin* β1. However, cells treated with clarithromycin at the MNCC exhibited a 20% decrease in the protein expression of *integrin* β4 compared with the control cells. The decrease in integrin β4 expression increased to 60% with clarithromycin at the LD_{50} (Fig. 4). Exposure of cells to clarithromycin at the MNCC had little inhibitory effect on the protein expression of *laminin 5*γ2, but exposure at the LD_{50} resulted in a 30% decrease in protein expression relative to the control cells. Cells treated with roxythromycin at the MNCC (100.0 μM) or the LD_{50} (399.0 μM) retained their constitutive levels of protein expression for *keratin 8/18* and *integrin* β1. When exposed to roxythromycin at the MNCC, cells exhibited a level of KGFR protein expression similar to the control cells, whereas cells exposed to roxythromycin at the LD_{50} exhibited a 40% decrease in KGFR protein expression relative to the control cells.

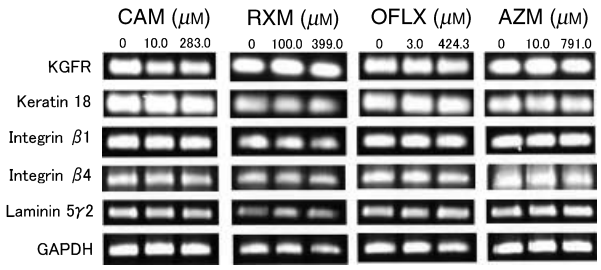

Fig. 3. RT-PCR analysis to detect the mRNA expression of *KGFR*, *keratin 18*, *integrins* β1 or β4, or *laminin 5*γ2 in NDUSD-1 cells exposed to the indicated concentrations of test agents clarithromycin (CAM), roxythromycin (RXM), ofloxacin (OFLX) and azithromycin (AZM) for 48 h. After exposure to the agents, total RNA was extracted, reverse-transcribed into cDNA, and amplified by using the specific primers for each gene (see Materials and methods). KGFR (141 bp); keratin 18 (86 bp); integrin β1 (265 bp); integrin β4 (369 bp); laminin 5γ2 (452 bp); GAPDH, glyceraldehyde-3-phosphate dehydrogenase. GAPDH expressions are shown as the internal controls.

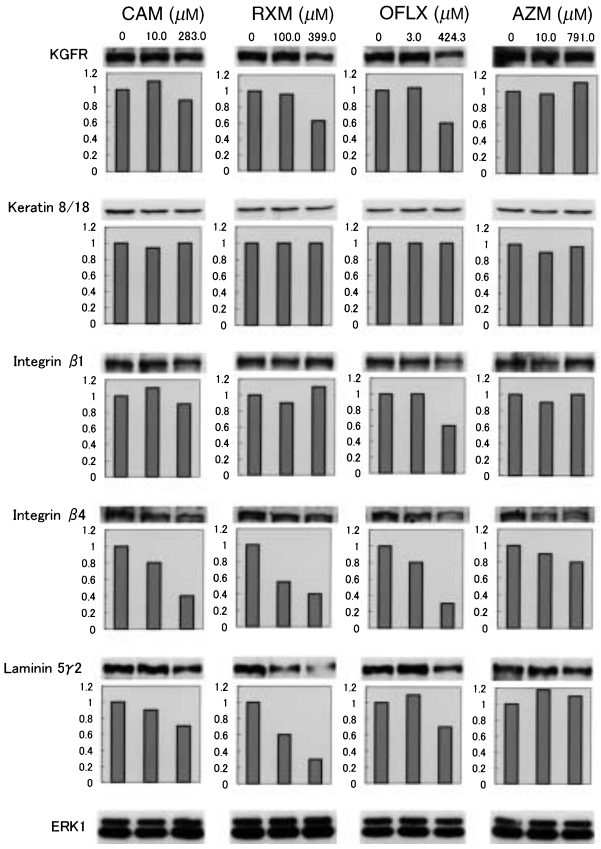

Fig. 4. Western blot analysis to detect the protein expression of *KGFR*, *keratin 8/18*, *integrin* β1, *integrin* β4, and *laminin 5*γ2 in NDUSD-1 cells exposed to the indicated concentrations of test agents clarithromycin (CAM), roxythromycin (RXM), ofloxacin (OFLX) and azithromycin (AZM) for 48 h. KGFR (120 kDa); keratin 8/18 (45 kDa); integrin β1 (120 kDa); integrin β4 (207 kDa); laminin 5γ2 (140 kDa). The protein expression of *extracellular-signal related kinase 1* (*ERK1*) was used as the internal control. Protein levels in cells exposed to the test agents were quantified by densitometry, expressed relative to the protein levels in the control cells, and normalized to the ERK1 by using a Software LabWorks® (version 4.0) (UVP, Inc., Upland, CA, USA).

Exposure of the cells to roxythromycin at the MNCC resulted in a 40% or more decrease in the protein expression of *integrin* β4 and *laminin 5*γ2 relative to the control cells. Exposure to roxythromycin at the LD_{50} induced a further decrease in the protein expression for both genes. Cells exposed to ofloxacin at the MNCC (3.0 μM) retained their constitutive levels of protein expression of *KGFR*, *keratin 8/18*, *integrin* β1, and *laminin* 5γ2, but exhibited a 20% decrease in protein expression of *integrin* β4. Exposure of cells to ofloxacin at the LD_{50} (424.3 μM) had little inhibitory effect on the protein expression of *keratin 8/18* but resulted in 30–70% decreases in the protein expression of the other genes. The levels of protein expression of *KGFR*, *keratin 8/18*, *integrin* β1, and *laminin 5*γ2 were unaffected by exposure to azithromycin at either the MNCC or the LD_{50}. Cells exposed to azithromycin at the MNCC exhibited little decrease in the protein expression of *integrin* β4, whereas cells exposed at the LD_{50} showed a 20% decrease in protein expression compared with the control cells.

Discussion

The cytocidal effects of eight antibacterial agents were quantitatively measured in the human gingival epithelial cell line, NDUSD-1 cells. The cytocidal effects varied with the agents, and the order of the agents according to their cytocidal effects was minocycline > tetracycline > enoxacin > clarithromycin > roxythromycin ≈ ofloxacin > azithromycin > erythromycin. The cytocidal effects of minocycline and tetracycline on NDUSD-1 cells were similar to those observed in normal human gingival epithelial cells in secondary culture that we reported previously (25), indicating that the sensitivity of NDUSD-1 cells to the cytocidal effects of test agents may be similar to that of normal human gingival epithelial cells. Most of the tetracyclines are adequately, but incompletely, absorbed from the gastrointestinal tract (26). When the stomach is empty, the percentage of an oral dose that is absorbed is 100% for minocycline and 60–80% for tetracycline (26). Sato and

Tsutsui (25) demonstrated that the cytocidal effect of minocycline on normal human gingival epithelial cells in secondary culture is greater than that of tetracycline, and the effect depends both on the intracellular concentration of the tetracyclines and on the persistence of the tetracyclines in the cells (26). The difference between the cytocidal effects of minocycline and tetracycline on NDUSD-1 cells may be due to mechanisms similar to those observed in normal gingival epithelial cells.

When normal human gingival epithelial cells are exposed to the fluoroquinolones enoxacin or ofloxacin, the cytocidal effect is enoxacin > ofloxacin (27), but the mechanism of the cytocidal effects of the fluoroquinolones is not yet known. Fluoroquinolones are well absorbed after oral administration and are widely distributed in body tissues. The peak serum concentration (C_{max}) of enoxacin occurs in 1 h after an oral dose of 200 mg and 3 h after the same dose of ofloxacin, with peak levels of 1.70 μg/ml and 1.65 μg/ml, respectively (28). The plasma elimination half-time ($t_{1/2}$) is 5.9 h for enoxacin and 4.5 h for ofloxacin (28). The difference in the cytocidal effects of enoxacin and ofloxacin on NDUSD-1 cells may be related to the differences in their pharmacokinetic properties.

The mechanism responsible for the difference in the cytocidal effects of various macrolides on NDUSD-1 cells is unknown. After oral administration of clarithromycin, azithromycin or erythromycin, their C_{max} is reached in approximately 2 h (25), and the order of the macrolides according to their C_{max} values after a single dose of 500 mg, which range from 0.3 to 3 μg/ml, is: clarithromycin > erythromycin ≥ azithromycin (25). The $t_{1/2}$-values of the macrolides range from 1.6 to 68 h, and the order is: azithromycin ≫ clarithromycin > erythromycin (25). These pharmacokinetic properties are unrelated to the order of the macrolides according to their cytocidal effects, meaning that the differences in their pharmacokinetic properties may not be responsible for the difference in their cytocidal effects on NDUSD-1 cells.

When NDUSD-1 cells were exposed to the LD_{50} concentrations, apoptosis was induced by minocycline, tetracycline, and azithromycin, but not by the other five agents. Although we cannot exclude the possibility that exposure to the five agents at concentrations higher than the LD_{50} would induce apoptosis in NDUSD-1 cells, the percentages of apoptotic cells following exposure to the eight test agents did not correlate with their cytocidal effects. These findings suggest that the differences between the cytocidal effects of the eight test agents on NDUSD-1 cells may not be explained by their ability to induce apoptosis.

The MNCCs of eight test agents were: 0.3 μM for minocycline, 1 μM for tetracycline, 3 μM for ofloxacin and erythromycin, 10 μM for enoxacin, clarithromycin, and azithromycin, and 100 μM for roxythromycin. The MIC_{90} concentrations of these test agents, except enoxacin, for periodontopathic bacteria, including *P. gingivalis*, *P. intermedia*, and *A. actinomycetemcomitans*, range from 0.031 to 2 μg/ml (0.06–4.0 μM) for minocycline, from 0.25 to 4 μg/ml (0.5–8.3 μM) for tetracycline, from 0.063 to 1 μg/ml (0.2–2.8 μM) for ofloxacin, from 0.125 to 16 μg/ml (0.2–21.8 μM) for erythromycin, from 0.125 to 4 μg/ml (0.2–5.3 μM) for clarithromycin, from 0.5 to 2.0 μg/ml (0.7–2.7 μM) for azithromycin, and from 0.25 to 4 μg/ml (0.3–4.8 μM) for roxythromycin (3, 29–31). The results suggest that there would be little or no growth inhibitory effect on basal epithelial cells if ofloxacin, azithromycin, clarithromycin, and roxythromycin were topically administered to the periodontal pocket at the MIC_{90} doses.

Next we examined the effect of these four agents (clarithromycin, roxythromycin, ofloxacin, and azithromycin) on the mRNA and protein expressions of genes involved in the growth, differentiation, and adhesion of epithelial cells when NDUSD-1 cells were exposed to them at their MNCCs. The results are summarized in Table 2. None of these agents affected the mRNA expression of five genes, *KGFR*, *keratin 8*, *integrin* β1, *integrin* β4, and *laminin 5*γ2. However, the protein expression of some genes was affected by clarithromycin, roxythromycin, and ofloxacin. clarithromycin and ofloxacin slightly decreased the protein expression of *integrin* β4, and roxythromycin markedly decreased the protein expression of *integrin* β4 and *laminin 5*γ2. azithromycin had little inhibitory effect on the protein expression of any of the genes. These results suggest that few, if any, adverse effects on growth, differentiation and adhesion of basal epithelial cells would occur as a result of topical administration of clarithromycin, ofloxacin, or azithromycin to the periodontal pocket at the doses equivalent to the MIC_{90}. It is important

Table 2. Summary of mRNA and protein expressions in NDUSD-1 cells exposed for 48 h to clarithromycin (CAM), roxythromycin (RXM), ofloxacin (OFLX) and azithromycin (AZM) at the maximum non-cytocidal concentrations (MNCCs)

		KGFR		*Keratin 8* or *8/18*		*Integrin* β1		*Integrin* β4		*Laminin 5*γ2		
Antibacterial agent	MNCC (μM)	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein	MIC_{90} (μM)
CAM	10	→	→	→	→	→	→	→	↘	→	→	0.2–5.3
RXM	100	→	→	→	→	→	→	→	↓	→	↓	0.3–4.8
OFLX	3	→	→	→	→	→	→	→	↘	→	→	0.2–2.8
AZM	10	→	→	→	→	→	→	→	→	→	→	0.7–2.7

→, Little effect; ↘, 20% decrease as compared with the control cells; ↓, 40% decrease as compared with the control cells. MIC_{90}, the concentration required to inhibit the growth of periodontopathic bacteria, including *P. gingivalis*, *P. intermedia*, and *A. actinomycetemcomitans* [see (3)].

to note, however, that extrapolation of these findings to *in vivo* conditions has yet to be undertaken. The bactericidal effect of fluoroquinolones is attributable to their inhibition of prokaryotic DNA gyrase, a type II DNA topoisomerase (32). Although eukaryotic topoisomerase II is about two orders of magnitude more resistant to inhibition by fluoroquinolones than the prokaryotic enzyme (33), fluoroquinolones not only inhibit eukaryotic topoisomerase II modifying DNA topology (34) but interfere with the other elements of eukaryotic DNA replication (35). Not only DNA synthesis but protein synthesis is inhibited by fluoroquinolones in eukaryotes because both DNA and protein syntheses are inhibited by exposure of Pel cells to ofloxacin at the MNCC (6). Macrolides are antibacterial agents that inhibit protein synthesis by reversibly binding to the 50S ribosomal subunits of sensitive microorganisms (26). Alternatively, macrolides may bind and cause a conformational change that terminates protein synthesis by indirectly interfering with transpeptidation and translocation (26). Exposure of Pel cells to roxythromycin or azithromycin inhibits protein synthesis in the cells, and each macrolide has its own inhibitory effect on macromolecule synthesis (Tsutsui *et al*., personal communication), suggesting that the differential effects of these macrolides on the protein expression of *integrin* β4 and *laminin* 5γ2 in NDUSD-1 cells are attributable to the differences in the concentrations used rather than in the cytocidal effects of the agents. Exposure of NDUSD-1 cells to clarithromycin, roxythromycin, or ofloxacin at the MNCC unaffected the protein expression of *KGFR*, *keratin 8/18*, or *integrin* β1, but it did reduce the protein expression of *integrin* β4 or both *integrin* β4 and *laminin* 5γ2. Although the mechanisms of the differential effects on the protein expression among the genes examined are unknown, these findings suggest that the inhibitory effect of antibacterial agents on the protein expression in mammalian cells differs according to the genes examined.

Fluoroquinolones are used clinically as first-choice antimicrobial agents for combating a variety of bacterial infections. However, undesirable side-effects, such as digestive and central nervous system disorders or phototoxicity, have been reported (36, 37). clarithromycin and azithromycin have favorable pharmacokinetic properties, such as good tissue distribution and high intracellular drug concentrations (including in phagocytes) (26). Although the adverse effects encountered with these macrolides are rarely serious (26), the most frequently reported adverse effects have been diarrhea, nausea, dysgeusia, and dyspepsia (26). Topical application of ofloxacin, clarithromycin, or azithromycin to the periodontal pocket may require the development of a slow-release, long-life matrix, not only to decrease their side-effects, but to increase their clinical efficacies.

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