J Periodont Res 2006; 41; 124–131

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2005.00843.x

Effects of minocycline and doxycycline on cell survival and gene expression in human gingival and periodontal ligament cells

Ayako Suzuki, Junko Yagisawa, Shin-ichi Kumakura, Takeki Tsutsui
Department of Pharmacology, The Nippon Dental University, School of Dentistry at Tokyo, Tokyo, Japan

Suzuki A, Yagisawa J, Kumakura S, Tsutsui T: Effects of minocycline and doxycycline on cell survival and gene expression in human gingival and periodontal ligament cells. J Periodont Res 2006; 41: 124–131. © Blackwell Munksgaard 2006

Objectives and Background: Periodontitis is an infectious disease in the gingival crevice caused by periodontopathic bacteria, including *Porphyromonas gingivalis*, *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans*, and *Tennerella forsythensis*, and antibacterial agents are directly administered to the site of infection to treat it. To maximize the therapeutic effects while reducing the adverse effects, the antibacterial agents should be administered at concentrations greater than their MIC_{90} doses required to inhibit the growth of 90% of periodontopathic bacteria and the administration should not damage the periodontal tissue. One approach for estimating cellular damage in the periodontal tissue caused by the administration is to assay cytological damages following exposures of cultured human cells derived from periodontal tissues to antibacterial agents. In the present study, we investigated the cytotoxic effect of minocycline (MINO) and doxycycline (DOX) by using a human gingival fibroblast cell line, a human gingival epithelial cell line, and a human periodontal ligament fibroblast cell line. We also used these cell lines to study the effect of MINO or DOX on the mRNA and protein expressions of genes associated with the differentiation of fibroblasts and the proliferation, differentiation, or cellular adhesion important to the epithelial regeneration of the periodontal attachment.

Methods: The cytotoxic effect of MINO or DOX was measured as a decrease in cell survivals. The effects of these antibiotics on the mRNA and protein expressions in the cell lines were studied by reverse transcription–polymerase chain reaction (RT–PCR) and western blot analyses, respectively.

Results: The maximum concentration of MINO or DOX that has little effect on the cell survivals and the mRNA and protein expressions of genes for alkaline phosphatase, type I procollagen, keratinocyte growth factor receptor, keratin 18 or 8/18, integrin β1, integrin β4, and laminin 5γ2 was 10 or 30 μM, respectively, which are greater than their MIC_{90} doses against periodontopathic bacteria described above.

Conclusions: These findings suggest that little, if any, cellular damage would be expected with topical administration of MINO or DOX 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.

Takeki Tsutsui, Department of Pharmacology, School of Dentistry at Tokyo, 1-9-20 Fujimi, Chiyoda-ku, Tokyo 102–8159, Japan
Tel: +81 33261 8771
Fax: +81 33263 5452
e-mail: takeki@tokyo.ndu.ac.jp

Key words: cytological effect; human gingival cells; human periodontal ligament cells; tetracyclines

Accepted for publication July 28, 2005

Periodontitis is an infectious disease in the gingival crevice caused by periodontopathic bacteria, including *Porphyromonas gingivalis*, *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans*, and *Tennerella forsythensis*, and antibacterial agents are directly administered to the site of infection as chemotherapy to treat it. Because of their efficacy against periodontopathic bacteria, tetracyclines such as minocycline (MINO), doxycycline (DOX), and tetracycline are widely used as topical therapy (1). Administered directly to the periodontal pocket, these antibiotics should inflict little or no damage on the host cells, otherwise they might adversely affect recovery from periodontitis. Therefore, it is important to estimate cellular damage in the periodontal tissue where the antibiotics are topically administered. The estimation would facilitate maximizing therapeutic effects of these antibiotics, while reducing their adverse effects. One approach for estimating the cellular damage in the periodontal tissue is to assay cytological damages following exposures of cultured human cells derived from periodontal tissues to antibacterial agents. For this purpose, we previously measured quantitatively the cytotoxic effects against human periodontal ligament fibroblasts of four tetracyclines, seven macrolides, and five fluoroquinolones that are used or intended for direct administration to the periodontal pocket (2–4).

The periodontal tissue consists of epithelial, soft and mineralized connective tissues and has a unique system called epithelial attachment and connective tissue attachment where the three tissues come together to form a junction at the tooth interface. As loss or regeneration of this system closely correlates with progress or healing of periodontitis, it is important to estimate cellular damage arising from topical administration of antibacterial agents not only by using fibroblasts derived from human periodontal ligaments but by using epithelial cells and fibroblasts, both of which are derived from human gingivae.

In the present study, we examined the cytotoxic effect of MINO and DOX by using the human gingival fibroblast cell line Ayt, the human gingival epithelial cell line NDUSD-1, and the human periodontal ligament fibroblast cell line Pelt, which had been established as described previously (5–7). The cytotoxic effect was determined by survival of cells treated with these antibiotics. We also used these cell lines to study the effect of MINO and DOX on the mRNA and protein expressions of genes associated with the differentiation of fibroblasts and the proliferation, differentiation, or cellular adhesion important to the epithelial regeneration of the periodontal attachment, such as alkaline phosphatase (*ALP*), type I procollagen (*COL*), keratinocyte growth factor receptor (*KGFR*), *keratin 18* or *8/18*, *integrin* β1, *integrin* β4, and *laminin* 5γ2. Ayt and NDUSD-1 cells have the same level of lethal sensitivity to MINO or DOX as early passage, normal fibroblasts and epithelial cells derived from human gingivae (8, 9). In addition, there are little differences in the effects of MINO or DOX on cell survivals and the mRNA and protein expressions of *ALP* and *COL* between Pelt cells and the early passage, normal counterparts derived from human periodontal ligaments (Suzuki A *et al.* unpublished data). Here we report that the maximum concentration of MINO or DOX that had little cytotoxicity and little adverse effects on the mRNA and protein expressions of growth-, differentiation-, and cell-adhesion-related genes in the three cell lines was greater than their MIC_{90} doses required to inhibit the growth of 90% of periodontopathic bacteria described above, suggesting that little, if any, cellular damage would be expected with the topical administration of MINO or DOX to the periodontal pocket at a dose equivalent to the MIC_{90}.

Material and methods

Cells and tetracyclines

The human gingival fibroblast cell line Ayt and the human periodontal ligament fibroblast cell line Pelt used in this study and the culture conditions have been described previously (7). Both cells are cell lines immortalized by infection of normal adult gingival fibroblasts (Ay) at 8 population doublings or normal adult periodontal ligament fibroblasts (Pel) at 9 population doublings with a retrovirus vector encoding human telomerase catalytic subunit (*hTERT*) cDNA, as described previously (7). The human gingival epithelial cell line NDUSD-1, which was a cell line immortalized by infection of normal gingival epithelial cells in secondary culture with origin (–)SV40 DNA and human c-*fos*, and the culture conditions have been described previously (5, 6). NDUSD-1 cells grew for more than 1000 days. Southern blot analysis demonstrated that both transfected DNAs were integrated into cellular DNA in the cells. NDUSD-1 cells exhibited persistent production of large T antigen at similar levels to WI38VA13 cells, a SV40 virus transformed cell line, and expressed *KGFR*, *keratin 8/18*, *integrins* β1 and β4, and *laminin* 5γ2. The number of chromosomes in NDUSD-1 cells was mainly distributed in the hypodiploid range (42–46) with a modal number of 44 (63%), and the cells were not tumorigenic in nude mice (5, 6). Minocycline hydrochloride (MINO) and doxycycline hydrochloride (DOX) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), dissolved in complete medium at 1 mM, and filter-sterilized.

Cytotoxicity

Cytotoxicity of MINO and DOX was determined by survivals of cells treated with MINO or DOX. Two hundred Ayt or Pelt cells or one thousand NDUSD-1 cells were plated in triplicate on 60-mm dishes, incubated overnight, and exposed to different concentrations of MINO or DOX for 48 h. The cells were then washed twice with 2 ml complete medium and incubated to form colonies for 6 days for Ayt and Pelt cells or 12 days for NDUSD-1 cells. 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 survivals was

calculated as the number of colonies in the treated plates divided by the number in control plates and multiplied by 100. All experiments were carried out under yellow lights. Statistical analysis was performed by Student's *t*-test to examine the significant difference between control and treated groups. The level of significance in the statistical analysis was determined at $p < 0.05$.

Reverse transcription–polymerase chain reaction analysis

The effect of MINO or DOX on the mRNA expressions of genes for *ALP*, *COL*, *KGFR*, *keratin 18*, *integrins* β1 or β4, and *laminin* 5γ2 was assessed by RT–PCR analysis. Ayt, Pelt, and NDUSD-1 cells (5×10^5) were plated into 25-cm^2 flasks, incubated overnight, and exposed to the desired concentrations of MINO or DOX for 48 h. After harvesting, total cellular RNA was isolated and reverse-transcribed as described previously (10). PCR was performed in a reaction mixture (25 μl) containing 1 μl of the RT reaction products, 0.025 units of *Taq* polymerase (Ampritag 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 *ALP* (11), *COL* (11), *KGFR* (12), *keratin 18* (13), *integrins* β1 (14) or β4 (15), *laminin* 5γ2 (16), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (17). The reaction mixtures were subjected to 35 cycles of amplification under the conditions described previously (11–17). The PCR products were resolved on a 2% agarose gel, stained with SYBR Green (Biowhittaker Molecular Applications, Rockland, ME, USA), and analyzed with a fluorescence imaging analyzer.

Western blot analysis

Ayt, Pelt, and NDUSD-1 cells (1×10^6) were plated into 75-cm^2 flasks, incubated overnight, and exposed to the desired concentrations of MINO or DOX for 48 h. Western blots were performed as described previously (7). Cells were lysed in buffer [0.1% Nonidt 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/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% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel for ALP, COL, 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). ALP, integrin β1, and integrin β4 were observed with the ALP, integrin β1, and integrin β4 polyclonal antibodies, respectively. The ALP antibody was purchased from Biomeda (Foster City, CA, USA) and the integrin β1 and integrin β4 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A horseradish-peroxidase-conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnology) was used for the ALP, integrin β1, and integrin β4 blottings, followed by detection with enhanced chemiluminescence (Amersham Pharmacia, Tokyo, Japan). X-ray films (RX-U, Fuji Film, Tokyo, Japan) were exposed to the blot. COL and laminin 5γ2 were observed with the COL and laminin 5γ2 polyclonal antibodies, respectively (Santa Cruz Biotechnology), a horseradish-peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology), and detection by enhanced chemiluminescence. KGFR and keratin 8/18 were observed with the KGFR and keratin 8/18 monoclonal antibodies, respectively (Santa Cruz Biotechnology), a horseradish-peroxide-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology), and detection by enhanced chemiluminescence. The probe for extracellular-signal-related kinase 1 (ERK 1) was used as an internal control.

Results

The effects of MINO or DOX on cell survivals and expressions of mRNA and protein in the human gingival fibroblast cell line Ayt, the human gingival epithelial cell line NDUSD-1, and the human periodontal ligament fibroblast cell line Pelt were examined. Morphology of each cell line was different from those of the others and shapes of colonies formed by each cell line were different from those formed by the others (Fig. 1). The effect of MINO or DOX on survivals of Ayt cells is shown in Fig. 2(A). Survivals of the cells were not decreased by treatment with MINO at 10–100 μM or DOX at 10 or 30 μM, but decreased by treatment with MINO at 300 or 1000 μM or DOX at 100–1000 μM in a concentration-dependent manner. Survivals of NDUSD-1 cells were not decreased by treatment with MINO at 3 or 10 μM or DOX at 3–30 μM, but decreased by treatment with MINO at 30–300 μM or DOX at 100 or 300 μM (Fig. 2B). Survival curves of Pelt cells treated with MINO or DOX showed similar sensitivity of the cells to MINO or DOX (Fig. 2C). The cell survivals were not decreased by treatment with MINO or DOX at 10–100 μM, but decreased by treatment with these antibiotics at 300 or 1000 μM. These findings indicate that MINO and DOX were little cytotoxic when the three cell lines were treated with MINO at ≤ 10 μM or with DOX at ≥ 30 μM. Thus, the maximum concentration of MINO or DOX that had little cytotoxic effect all over the three cell lines was 10 or 30 μM, respectively. Meanwhile, treatment with MINO at ≥ 30 μM or with DOX at ≥ 100 μM was cytotoxic in the cell lines and the cytotoxicities were greater in NDUSD-1 cells than in Ayt and Pelt cells. In addition, the cytotoxicity of MINO in NDUSD-1 cells was greater than that of DOX.

Next we examined the effect of MINO or DOX on the mRNA expressions in the three cell lines with RT–PCR analysis (Fig. 3). The mRNA expression of *ALP* in Ayt cells was not decreased by treatment with MINO or DOX at 30–300 μM, but decreased by treatment with MINO or DOX at 1000 μM. Conversely, the mRNA expression of *COL* in Ayt cells was not decreased, even in the cells treated with MINO or DOX at 1000 μM (Fig. 3A). The same results were obtained from RT–PCR analysis conducted with 25 cycles of amplification (data not

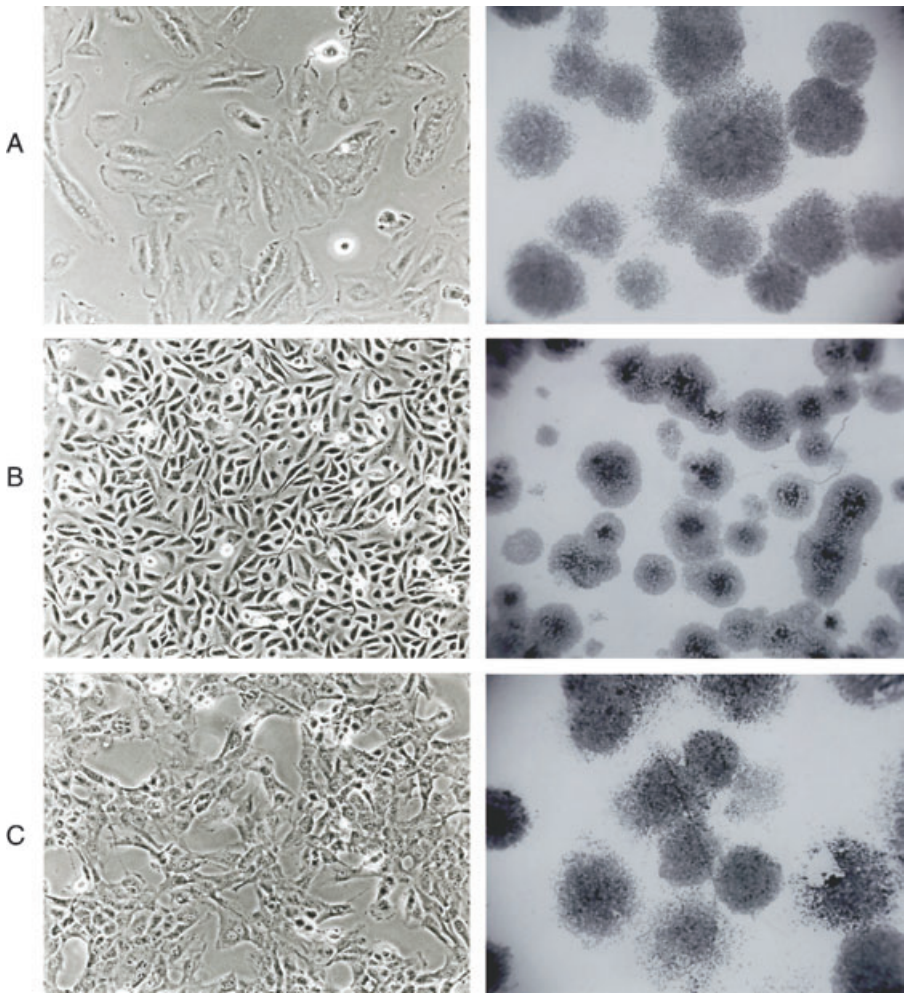

Fig. 1. Phase-contrast micrographs of three cell lines and their colonies stained with Giemsa solution. (A) Ayt cells; (B) NDUSD-1 cells; (C) Pelt cells. Each photograph was taken with the same magnification.

shown). The mRNA expression of *KGFR* in NDUSD-1 cells was not decreased by treatment with MINO at 10 or 30 μM, but decreased by treatment with MINO at 50 or 100 μM. NDUSD-1 cells treated with DOX at 30 or 100 μM retained the constitutive level of mRNA expression of *KGFR*, but the expression level was decreased by treatment with DOX at 200 or 300 μM. The mRNA expressions of *keratin 18* and *integrin* β1 in NDUSD-1 cells were not affected by treatment with MINO at 10–100 μM or DOX at 30–300 μM. The mRNA expression of *integrin* β4 in NDUSD-1 cells was not decreased by treatment with MINO at 10–50 μM or DOX at 30–200 μM, but decreased by treatment with MINO at 100 μM or DOX at 300 μM. The mRNA expression of *laminin* 5γ2 in NDUSD-1 cells was not decreased by treatment with any concentration of MINO or DOX (Fig. 3B). The mRNA expression of *ALP* in Pelt cells was not decreased by treatment with MINO at 30–1000 μM and DOX at 30 or 100 μM, but decreased by treatment with DOX at 300 or 1000 μM. Treatment of Pelt cells with MINO or DOX at any concentration examined did not reduce the constitutive level of mRNA expression of *COL* (Fig. 3C). These findings indicate that the maximum concentration of MINO or DOX that did not decrease any mRNA expression all over the three cell lines was 30 or 100 μM, respectively.

The effect of MINO or DOX on the protein expressions of these genes in the three cell lines was examined with western blot analysis. In this experiment, each cell line was treated with MINO or DOX at the concentrations that had little effects on the survivals and mRNA expressions. All the treatments had little inhibitory effect on the protein expressions of genes examined (Figs 4 and 5).

Discussion

We investigated the effects of MINO or DOX on cell survivals and expressions of mRNA and protein in the three cell lines. Cell survivals were not decreased when the cells were treated with MINO at $\leq$ 10 μM or with DOX at $\leq$ 30 μM, but decreased when treated with MINO at $\geq$30 μM or with DOX at $\geq$100 μM. The maximum concentrations that did not affect the survivals of any of the three cell lines were 10 μM

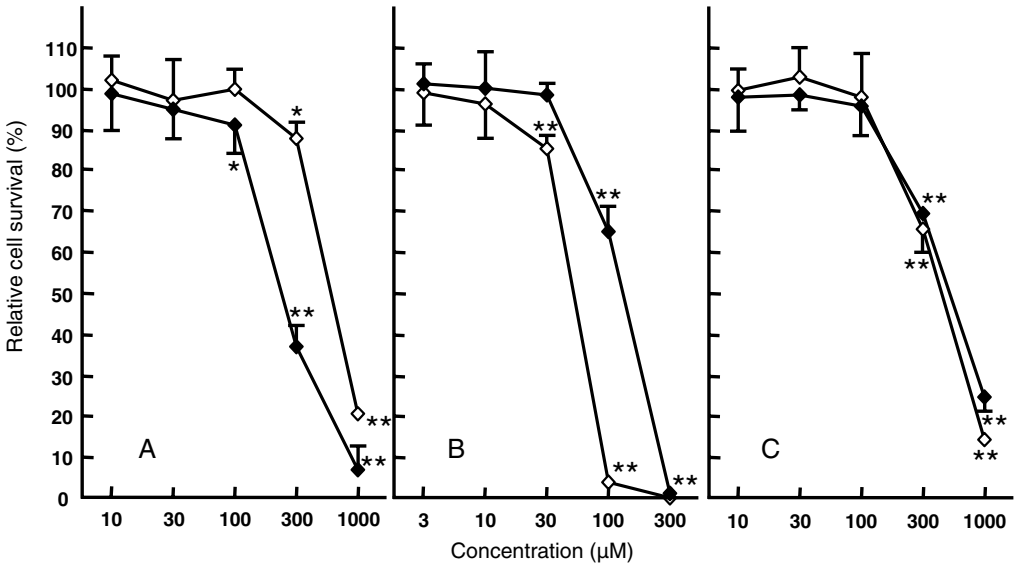

Fig. 2. Relative survivals of three cell lines treated with minocycline (MINO) or doxycycline (DOX) at varying concentrations for 48 h. (A) Ayt cells; (B) NDUSD-1 cells; (C) Pelt cells. ◇, MINO; ◆, DOX. The actual colony-forming efficiency in control cultures was 80.2 ± 5.5% (SD) for Ayt cells, 37.1 ± 1.1% for NDUSD-1 cells, and 73.4 ± 3.2% for Pelt cells. Bars denote SD. When not indicated, SD are within symbols. Significantly different from the control: $*p < 0.05$; $**p < 0.01$, *t*-test.

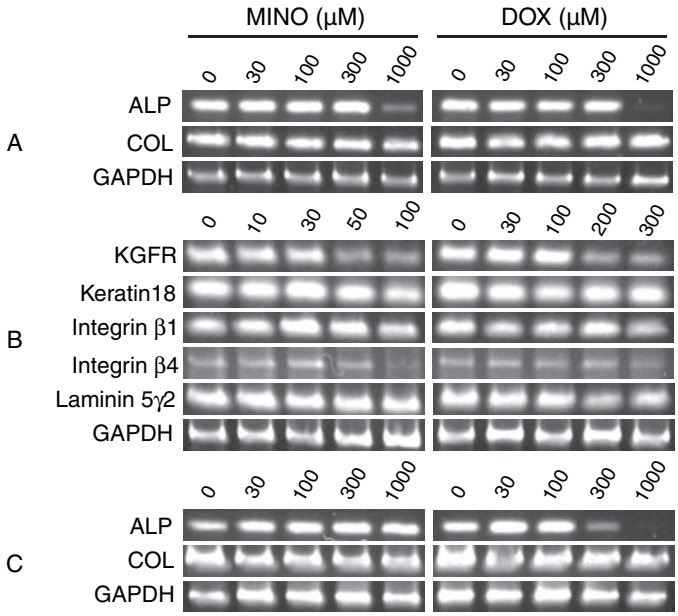

Fig. 3. Reverse transcription–polymerase chain reaction analysis to detect the mRNA expressions of various genes in three cell lines treated with minocycline (MINO) or doxycycline (DOX) at the indicated concentrations for 48 h. (A) Ayt cells; (B) NDUSD-1 cells; (C) Pelt cells. Alkaline phosphatase (ALP; 475 bp); type I procollagen (COL; 599 bp); keratinocyte growth factor receptor (KGFR; 141 bp); keratin 18 (86 bp); integrin β1 (265 bp); integrin β4 (369 bp); laminin 5γ2 (452 bp). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expressions are shown as the internal controls.

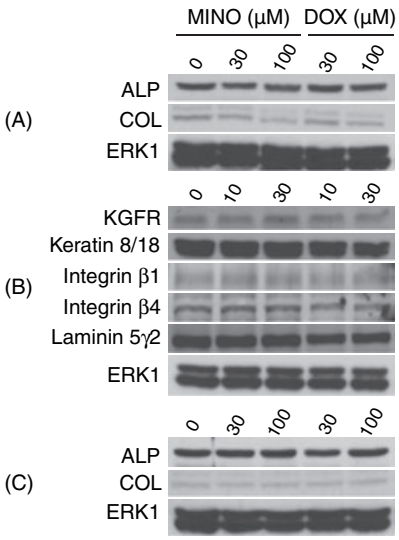

Fig. 4. Western blot analysis to detect the protein expressions of various genes in three cell lines treated with minocycline (MINO) or doxycycline (DOX) at the indicated concentrations for 48 h. (A) Ayt cells; (B) NDUSD-1 cells; (C) Pelt cells. Alkaline phosphatase (ALP; 58 kDa); type I procollagen (COL; 190 kDa); keratinocyte growth factor receptor (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.

for MINO and 30 μM for DOX. These results indicate that a high concentration of MINO or DOX is cytotoxic in mammalian cells. Tetracyclines inhibit bacterial protein synthesis by binding to the 30 S bacterial ribosome and preventing access of aminoacyl tRNA to the acceptor (A) site on the mRNA–

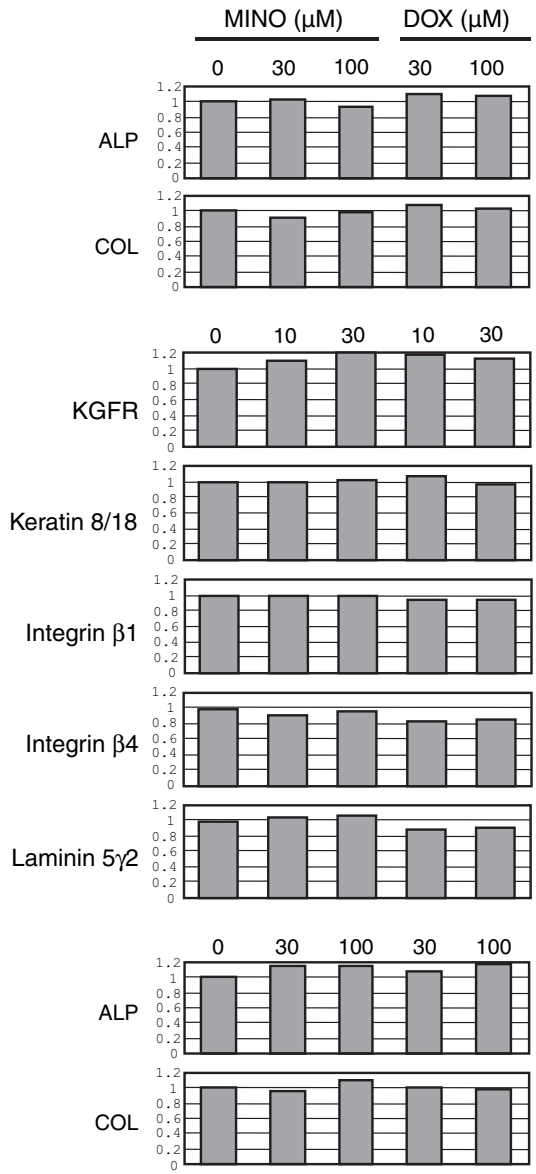

Fig. 5. Quantitative analysis of the protein expressions of various genes after treatment of three cell lines with minocycline (MINO) or doxycycline (DOX). The levels of protein expressions shown in Fig. 4 were quantified by densitometry, expressed relative to the protein levels in the control cells, and normalized to the extracellular-signal-related kinase 1 (ERK1) by using a Software LabWorks® (version 4.0) (UVP, Inc., Upland, CA, USA). ALP, alkaline phosphatase; COL, type I procollagen; KGFR, keratinocyte growth factor receptor.

ribosome complex (18). Tetracycline also impairs protein synthesis in mammalian cells at a high concentration (19). DNA and RNA syntheses are inhibited by tetracycline in mammalian cells as well (19). The inhibition of macromolecule syntheses may be one of the cytotoxic mechanisms of tetracyclines against mammalian cells. Mammalian cells lack the active transport system found in bacteria, and the ribosomal target is less sensitive (18), which is a possible mechanism of the differential sensitivity between mammalian cells and bacteria.

NDUSD-1 cells were more sensitive to the cytotoxic effect of MINO or DOX than Ayt and Pelt cells. The cytotoxic effect of tetracyclines in human cells has been shown to depend both on the intracellular concentration of tetracyclines and on the persistence of tetracyclines in the cells (9), suggesting that the difference in the cytotoxic effect between the cell lines might be attributed to the difference in the permeability of MINO or DOX into the cells and/or their retentivity in the cells. Meanwhile, MINO was more cytotoxic in NDUSD-1 cells than DOX. MINO and DOX are absorbed from the gastrointestinal tract to a considerable extent. When the stomach is empty, the percentage of an oral dose that is absorbed is 100% for MINO and 95% for DOX (18). In addition, the half-lives of MINO and DOX are almost same in the body (16–18 h) (18). These suggest that the differential sensitivity between MINO and DOX in NDUSD-1 cells might be due to mechanism(s) other than these pharmacokinetic properties. Persistence or retention in some macromolecules in the cells is considered as a candidate of the alternative mechanisms. After administration is stopped, MINO persists in the body possibly due to retention in fatty tissues (18).

The effects of MINO or DOX on the mRNA and protein expressions of the following genes associated with the differentiation of fibroblasts and the proliferation, differentiation, or cellular adhesion important to the epithelial regeneration of the periodontal attachment were studied by RT–PCR and western blot analyses, respectively. *ALP* and *COL* are expressed in fibroblasts capable of mineralization (3). *KGFR* is the specific receptor for keratinocyte growth factor which plays a major part in re-epithelialization in wound healing (12). Keratins are encoded by members of the 10 nm intermediate filament multigene family and their expressions vary with proliferation, differentiation, and the state of cell transformation and development. *Keratin 8* and/or *18* are expressed in normal, immortalized, and malignant oral epithelia in organotypic culture (20). Integrins belong to the family of cellular adhesion molecules. 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 (21). Integrins β1 and β4 are likely to be involved in apical migration of junctional epithelium during the periodontal attachment loss (22). Laminin-5, a laminin isoform specific for epithelial cells, is a major component of the internal basal lamina in both rodent and human tissues (23). Laminin-5 is composed of three truncated polypeptide chains, α3, β3, and γ2 (24). The *laminin* 5γ2 gene is expressed in the cells of the junctional epithelium but not in the other parts of the gingival epithelium (23).

The maximum concentration of MINO or DOX that did not decrease the mRNA expression of any gene examined was 30 or 100 μM, respectively. These findings indicate that the maximum concentration of MINO or DOX that had little inhibitory effects both on the cell survivals and on the mRNA expressions all over the three cell lines was 10 or 30 μM, respectively. Treatment with these concentrations of MINO and DOX also failed to decrease the constitutive level of the protein expressions in the cells. It is reported that the minimum inhibitory concentration required to inhibit the growth of 90% (MIC_{90}) against *P. gingivalis*, *P. intermedia*, and *A. actinomycetemcomitans* of MINO was 0.031 to 2 μg/ml (0.06 to 4 μM) (25) and the MIC_{90} of DOX against *P. gingivalis*, *P. intermedia*, *A. actinomycetemcomitans*, and *T. forsythensis* was 0.12 to 1.562 μg/ml (0.25 to 3.24 μM) (26). The maximum concentration of MINO or DOX that had little adverse effects in the three cell lines was 2.50- or 9.26-fold, respectively, the MIC_{90} values, suggesting that little, if any, cellular damage would be expected with topical administration of MINO or DOX 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. Periodontopathic bacteria form biofilms that are structured communities of bacteria encased in a self-produced polymeric matrix (27). Compared with bacteria in planktonic growth, biofilm bacteria are notoriously resistant to antibacterial agents (28) as well as natural and acquired immune responses (27). In addition, differential expression of a number of secreted surface-associated proteins of *A. actinomycetemcomitans* is demonstrated between the biofilm and planktonic cells (29), suggesting that periodontopathic bacteria in biofilms express different gene sets when compared with their planktonic counterparts. *In vitro* assessment using a system with biofilms remains to be done for estimating the antibacterial efficacy of antibacterial agents against periodontopathic bacteria.

References

1. Mashimo PA, Yamamoto Y, Slots J, Evans RT, Genco RJ. *In vitro* evaluation of antibiotics in the treatment of periodontal disease. *Pharmacol Ther Dent* 1981;**6:**45–56.
2. Omori N, Kobayashi H, Tsutsui T. Quantitative comparison of cytocidal effects of tetracyclines and fluoroquinolones on human periodontal ligament fibroblasts. *J Periodont Res* 1999;**34:**290–295.
3. Someya T, Tamura Y, Tsutsui T. Cytological and cytogenetic effects of the fluoroquinolones ofloxacin on human periodontal ligament fibroblasts. *J Periodont Res* 2000;**35:**352–360.
4. Maizumi N, Tamura Y, Kanai H, Tsutsui T. Quantitative comparison of the cytocidal effect of seven macrolide antibiotics on human periodontal ligament fibroblasts. *J Periodont Res* 2002;**37:**250–254.
5. Igarashi F, Uchida M, Tsutsui T. Establishment and characterization of cultured human gingival keratinocytes immortalized by transfection of origin (–) SV40 DNA and c-*fos* gene. *Odontology* 1998;**86:**37–47. (Text in Japanese with English abstract.)
6. Inoue K, Kumakura S, Uchida M, Tsutsui T. Effects of eight antibacterial agents on cell survival and expression of epithelial-cell- or cell-adhesion-related genes in human gingival epithelial cells. *J Periodont Res* 2004;**39:**50–58.
7. Tsutsui T, Kumakura S, Yamamoto A *et al.* Association of $p16^{INK4a}$ and pRb inactivation with immortalization of human cells. *Carcinogenesis* 2002;**23:**2111–2117.
8. Rokukawa A, Tsutsui T. Study of the ability of tetracyclines to induce chromosome aberrations in cultured human fibroblasts derived from gingival tissues. *J Japan Soc Periodont* 1997;**39:**339–347. (Text in Japanese with English abstract.)
9. Sato H, Tsutsui T. Effect of tetracyclines on cell survival of cultured human gingival keratinocytes and intracellular concentrations of incorporated tetracyclines. *J Japan Soc Periodont* 1998;**40:**1–8. (Text in Japanese with English abstract.)
10. Kumakura S, Yamamoto A, Yagisawa J, Uchida M, Tsutsui T. Telomere length and telomerase activity in the process of human fibroblast immortalization. *Odontology* 2002;**90:**13–21.
11. Rickard DJ, Kassem M, Hefferan TE, Sarkar G, Spelsberg TC, Riggs BL. Isolation and characterization of osteoblast precursor cells from human bone marrow. *J Bone Miner Res* 1996;**11:**312–324.
12. Das SJ, Olsen I. Up-regulation of keratinocyte growth factor and receptor: a possible mechanism of action of phenytoin in wound healing. *Biochem Biophys Res Commun* 2001;**282:**875–881.
13. Dimmler A, Gerhards R, Betz C *et al.* Transcription of cytokeratins 8, 18 and 19 in bone marrow and limited expression of cytokeratins 7 and 20 by carcinoma cells: inherent limitations for RT-PCR in the detection of isolated tumor cells. *Lab Invest* 2001;**81:**1351–1361.
14. Bouchet BY, Colon M, Polotsky A, Shikani AH, Hungerford DS, Frondoza CG. Beta-1 integrin expression by human nasal chondrocytes in microcarrier spinner culture. *J Biomed Mater Res* 2000;**52:**716–724.
15. Basora N, Herring-Gillam FE, Boudreau F *et al.* Expression of functionally distinct variants of the β_4A integrin subunit in relation to the differentiation state in human intestinal cells. *J Biol Chem* 1999;**274:**29819–29825.
16. Manda R, Kohno T, Niki T *et al.* Differential expression of the LAMB 3 and LAMC 2 genes between small cell and non-small cell lung carcinomas. *Biochem Biophys Res Commun* 2000;**275:**440–445.
17. Hirokawa I, Oshimura M, Barrett JC. Repression of the telomerase catalytic subunit by a gene on human chromosome 3 that induces cellular senescence. *Mol Carcinog* 1998;**22:**65–72.
18. Chambers HF. Antimicrobial agents, protein synthesis inhibitors and miscellaneous antibacterial agents. In: Hardman JG, Limbird LE, Gilman AG, eds. *Goodman and Gilman's the Pharmacological Basis of Therapeutics*. New York: Macmillan, 2001: 1239–1256.
19. Tsutsui T, Umeda M, Sou M, Maizumi H. Effect of tetracycline on cultured mouse cells. *Mutat Res* 1976;**40:**261–268.
20. Hansson A, Bloor BK, Haig Y, Morgan PR, Ekstrand J, Grasfström RC. Expression of keratins in normal, immortalized and malignant oral epithelia in organotypic culture. *Oral Oncol* 2001;**37:**419–430.

21. Gräber H-G, Conrads G, Wilharm J, Lampert F. Role of interactions between integrins and extracellular matrix components in healthy epithelial tissue and establishment of a long junctional epithelium during periodontal wound healing: a review. *J Periodontol* 1999;**70:**1511–1522.
22. Gürses N, Thorup AK, Reibel J, Carter GW, Holmstrup P. Expression of VLA-integrins and their related basement membrane ligands in gingiva from patients of various periodontitis categories. *J Clin Periodontol* 1999;**26:**217–224.
23. Hormia M, Sahlberg C, Thesleff I, Airenne T. The epithelium–tooth interface-a basal lamina rich in laminin-5 and lacking other known laminin isoforms. *J Dent Res* 1998;**77:**1479–1485.
24. Burgeson RE, Chiquet M, Deutzmann R *et al*. A new nomenclature for the laminins. *Matrix Biol* 1994;**14:**209–211.
25. Miyake Y, Tsuruda K, Okuda K, Widowati Iwamoto Y, Suginaka H. *In vitro* activity of tetracyclines, macrolides, quinolones, clindamycin and metronidazole against periodontopathic bacteria. *J Periodont Res* 1995;**30:**290–293.
26. Takemoto T, Kurihara H, Dahlan G. Characterization of *Bacteroides forsythus*. *J Clin Microbiol* 1997;**35:**1378–1381.
27. Kolenbrander PE. Oral microbial communities: biofilms, interactions, and genetic systems. *Ann Rev Microbiol* 2000;**54:**413–437.
28. Jorgensen MG, Slots J. Practical antimicrobial periodontal therapy. *Compend Contin Educ Dent* 2000;**21:**111–122.
29. Fletcher JM, Nair SP, Ward JM, Henderson B, Wilson M. Analysis of the effect of changing environmental conditions on the expression patterns of exported surface-associated proteins of the oral pathogen *Actinobacillus actinomycetemcomitans*. *Microb Pathog* 2001;**30:**359–368.