Local factors essential to the development of periodontal diseases include the accumulation of biofilms (plaque) on the root surfaces. The biofilms form complex ecosystems, which contain a broad variety of microorganisms and a secreted matrix and may calcify to form calculus. Bacterial endotoxins, cytotoxins, and other pathogenic substances are released from the plaque and calculus and diffuse into the adjacent soft tissues where they elicit an inflammatory response that results in tissue disruption and degradation. Therefore, the removal of calculus, plaque, and plaque-derived products by scaling and root planing has long been a key component of the treatment for periodontal disease. Although scaling and root planing eliminate a great deal of the root surface deposits and promote tissue healing, high magnification microscopic analyses have demonstrated that a “smear layer” containing residual calculus, remnants of cementum, bacteria and bacterial matrix, and structural proteins are left on the root surface. It has been proposed that an effective removal of the smear layer would enhance the potential for periodontal regeneration.

Register and Burdick studied periodontal healing in dogs and cats after root surface demineralization by different acids, including hydrochloric, lactic, citric, phosphoric, trichloroacetic, and formic acids. The results were promising and stimulated the interest in this type of root surface modification. The
structural benefits of root surface demineralization on the regeneration of the periodontal ligament were addressed by electron microscopy, and it was shown that newly formed collagen fibers interdigitated with existing collagen fiber extending from the demineralized root surface.\(^9\) In addition, Poolson and Proye proposed that the exposed collagen fiber remnants might link with fibrin during periodontal wound healing to serve as an initial healing complex and barrier, which could inhibit epithelial cell downgrowth.\(^10\) Contrary to the healing results around teeth in dogs,\(^7,8,11\) the benefits of citric acid treatment have been less convincing for periodontal treatment in human.\(^12,13\)

The demineralizing acidic agents mentioned above remove toxins from root surfaces as the smear layer is dissolved and thereby promote the migration and attachment of reparative cells. However, the potentially injurious effects of the acids also have gained attention. Although some investigators suggested that acidic demineralization does not induce irreversible, long-term effects,\(^14\) histological studies by others found a reduced cell density during initial stages of healing in experimental wounds.\(^15\) Furthermore, when assaying lactate dehydrogenase as a marker of cell vitality, Blomlof and Lindskog\(^16\) demonstrated that periodontal cells serve as an initial healing complex and barrier, which linked with fibrin during periodontal wound healing to pose the exposed collagen fiber remnants might aid in improving the healing in periodontal defects.\(^22\) However, TCN could have similar negative effects on cells seen with other acidic agents.

An additional issue pertinent to treatment of periodontal diseases is the increased activity of collagen degrading enzymes in the periodontium of patients with periodontitis.\(^23,24\) One essential group of enzymes with collagenolytic properties consists of the matrix metalloproteinases (MMPs, collagenases).\(^25,26\) In health, the MMPs serve in natural maintenance of tissue development and integrity. Yet, they also are key to excessive tissue degradation in a number of diseases, including the chronic inflammatory conditions of periodontal disease and arthritis, as well as tumor expansion and metastasis in cancer.\(^25,27\) The MMPs are produced by a variety of cell types and have the important capacity to degrade collagen fibrils in the periodontal connective tissues and at the root surface leading to epithelial downgrowth, increased periodontal probing depth, and loss of periodontal ligament attachment.\(^23\) MMPs are secreted at greater levels by inflammatory and other cell types of the periodontium when these are exposed to pro-inflammatory cytokines, bacterial endotoxins, and lipopolysaccharides typical of periodontal diseases.\(^23,28\) Importantly, TCNs as well as the related minocycline and doxycycline have the capacity to inhibit MMP-1, -2, -8, -9, -12, -13, and -14, several of which have been implicated in periodontal diseases.\(^29\) In addition, treatments with TCN or derivatives of TCN that inhibit MMP have reduced the bone loss in experimental periodontitis in animal models and among patients with periodontal diseases.\(^30,31\) Of special clinical importance, TCNs possess substantivity;\(^32\) that is, they have the ability to adsorb to surfaces such as dentin from which they are released slowly for a period of up to 4 days or longer, depending on the concentration with which the dentin was treated.\(^33,34\) This is of interest in the long-term treatment of periodontal disease, since continuous administration of doxycycline may extend the anti-collagenolytic activities of the drug.\(^31\)

In summary, tetracycline is a therapeutic agent of special interest that may promote periodontal healing by its ability to remove the smear layer and by its anti-collagenolytic and antibacterial properties. Unfortunately, recent research reports on cytotoxic effects of acids used for modification of root surfaces have questioned its use in clinical practice.\(^15-17\) Therefore, this project was designed to test and establish conditions for the topical use of tetracyclines in periodontal therapy.

**MATERIALS AND METHODS**

**Cell Culture**

Primary cultures of human gingival (hGF) and periodontal ligament fibroblasts (hPDL) were generated from biopsies of gingiva and periodontal ligaments from the middle third of root surfaces of extracted teeth according to previously detailed methods.\(^35\) Briefly, immediately upon procurement, tissues were placed into serum-free 50% Dulbecco’s minimal essential medium/50% Ham F-12 (w/w) (DF)\(^†\) at room temperature and rinsed several times with DF supplemented with 10% newborn calf serum (NCS),\(^7\) 2.5 µg/ml fungizone, 100 units/ml penicillin, and 100 µg/ml streptomycin.\(^§\) Explants were sliced into small pieces and immobilized in tissue culture plates for 3 days. Once extensive cell outgrowth from the tissues was evident, cells were trypsinized and passed to large flasks for expansion and subsequent storage in liquid nitrogen. Cells from

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\(^1\) Gibco, Rockville, MA.  
\(^†\) Gibco, Rockville, MA.  
\(^‡\) Summit Biotechnology, Fort Collins, CO.  
\(^§\) Sigma, St. Louis, MO.
We utilized a WST-1 assay system \( \text{¶} \) to measure the hGF and the hPDL.

**Cell Viability and Cytotoxicity Studies**

We utilized a WST-1 assay system \( \text{¶} \) to measure the effects of different treatments on the cells. This colorimetric assay quantifies cell proliferation and cell viability and is based on the cleavage of the tetrazolium salt WST-1 to soluble formazan dye by mitochondrial dehydrogenases. Since only viable, metabolically active cells cleave the WST-1, an increase in the cytotoxicity of the tested compound will correlate inversely with the intensity of the measurable dye. In preparation for the assays, preliminary assays were designed to determine the optimal cell number and incubation time with the cell proliferation reagent (WST-1) for each cell line. According to the manufacturer’s instructions, 10 µl of concentrated WST-1 reagent was added to each of a range of duplicate wells in 96-microwell plates. Each microwell contained 100 µl serum-free culture medium (MEM) and a serial dilution of cells (\( 1 \times 10^4 \) to \( 1.5 \times 10^2 \) cells/well). The color reaction was quantified directly from the wells containing cells at 450 nm using a plate reader \( \# \) after incubation in the tissue culture incubator (37°C, 5% CO₂) for 0.5, 1, 2, 4, and 6 hours. From cell number versus absorbance response curves (data not shown), it was determined that a cell number of \( 2.5 \times 10^2 \)/well and an incubation time of 4 hours yielded an optimal response in the linear range of the assay for all tested cell lines. Cell viability was verified by trypan blue exclusion, and only single cell suspensions of >90% viability were used. To eliminate the potential for confounding interference from cell proliferation when testing the different treatments, we determined from serum concentration versus WST-1 response curves (data not shown) that 0.25% serum was the minimal concentration required to maintain the cells viable and quiescent.

**Short-Term Exposure of Cells to Tetracycline**

To assay for cellular effects of short-term TCN exposure, hGF, hPD, and WI-38 cells were plated in 96-microwell plates at \( 2.5 \times 10^3 \) cells/well in 100 µl DF with 0.25% NCS and maintained overnight at 37°C. Prior to the treatment, the medium was carefully removed. Then, 100 µl TCN \( \text{**} \) dissolved in physiological saline (0.9% NaCl) was added to duplicate wells at a concentration range of 27 to 0.1 mg/ml. Of note, preliminary experiments had demonstrated that TCN had propensity for precipitation in the presence of divalent cations, whereas it remained dissolved for the experimental times in saline. After 3 minutes of treatment at 37°C, cells were rinsed once with 0.9% NaCl after which 100 µl DF containing 0.25% NCS and 10 µl WST-1 reagent was added per well. Wells with saline only served as no treatment controls. The dye conversion was measured after 4 hours incubation at 37°C. The TD₅₀, which indicates the treatment concentration at which there was 50% cell death, was determined from plots of cell viability versus TCN concentration.

**Long-Term Exposure of Cells to Tetracycline**

For long-term treatment, hGF, hPD, and WI-38 cells were initially prepared as for the short-term treatment experiments. The TCN was then diluted and added over a concentration ranging from 3 to 0.01 mg/ml in pre-warmed DF containing 0.25% serum. After 24-hour treatment at 37°C in the tissue culture incubator, the cells were rinsed gently and the WST-1 added in DF with 0.25% NCS. The cell viability was measured after 4 hours. Untreated control cells were included.

**Recovery of Periodontal Cells Following Tetracycline Treatment**

To analyze whether the periodontal cells had the capacity to recover after short-term exposure to TCN, hGF and hPD were initially plated in 96-microwell plates at \( 2.5 \times 10^3 \) cells/well in 100 µl DF containing 0.25% NCS and cultured overnight at 37°C. Based on critical concentration ranges established in preceding short-term treatment experiments, cells were then treated with 27, 9, 3, or 1 mg/ml TCN for 3 minutes. The cells were rinsed twice with serum-free medium to remove the treatment medium and then incubated for recovery with DF containing 10% NCS. After 2 days, the cell recovery was quantified with the WST-1 assay as detailed above. For comparison, separate wells were assayed prior to treatment and immediately following treatment. An increase in the absorbance would signify that the mitochondrial machinery was only temporarily inactivated, whereas absence of dye conversion would indicate that the treatment induced irreversible damage to the cells.

**Expression of Recombinant MMP-2 and MMP-9**

To define the concentration of TCN that inhibited matrix metalloproteinase-2 and -9, we expressed and purified recombinant MMP-2 and MMP-9 as detailed previously. \( \text{36,37} \) Briefly, the cDNA coding for the two full-length MMPs minus the pro-domain, to ensure constitutive activity, were amplified by polymerase chain reaction (PCR) from the MMP-2 plasmid p186.2

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\( \text{¶} \) Roche Applied Science, Indianapolis, IN.

\( \text{**} \) Fisher Scientific, Pittsburgh, PA.
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Enzyme Activity Assays

Gelatin enzymography. To assess rMMP-2 and -9 gelatinolytic activities, samples were separated under non-reducing condition on 10% SDS-PAGE minislab gels39 co-polymerized with 150 µg/ml type I gelatin§§ and processed as detailed previously.42,43 Briefly, after electrophoresis gels were thoroughly washed in 5% Triton X-100, equilibrated with collagenase assay buffer (50 mM Tris, 200 mM NaCl, 5 mM CaCl2, pH 7.2), and incubated at 37°C for appropriate times. Counters- staining of gels with Coomassie brilliant blue R-250 revealed MMP-2 and -9 as cleared bands against a blue background. Conditioned culture medium from ROS 17/2.8 cells that contains high level MPP-2 served as a positive control.

Enzyme activity assay on fluorescent substrates. The TCN effects on rMMP-2 and -9 activities were quantified in reactions containing MMP-2 or MMP-9, fluorescent-labeled porcine type I gelatin substrate – DQ gelatin,III and a concentration range of TCN. Typically, enzyme activity assays were performed in 96-microwell platesIII with 2 µg DQ gelatin (22 pmol), 2 µg rMMP-2 or MMP-9 (0.15 µM), a concentration range of 500 to 8 µg/ml TCN, and assay buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl2, 0.2 mM NaN3, pH 7.6) in 200 µl reaction volumes at 22°C. The change in emitted fluorescence signal resulting from cleavage of the substrate was measured with λex at 495 nm and λem at 515 nm on a plate reader.III To assure that the inherent fluorescent signal of TCN did not interfere with the signal of the cleaved substrate, preliminary experiments determined the fluorescent interference from TCN was <4% of the background signal from the gelatin substrate at TCN concentrations of 50 to 100 µg/ml (data not shown). In the inhibition assays, the enzyme activities were recorded in relative fluorescent units (RFU) and converted to velocity (nmol gelatin hydrolyzed per minute) in the linear range of the assay. In our data interpretation, a concentration-dependent, reduced velocity compared to control reactions corresponded to inhibition of the enzyme activity. The concentrations of TCN that inhibited 50% of the velocities (IC50) were determined from inhibitor concentration versus enzyme activity plots.

Mechanical and Chemical Root Surface Modification

Treatment of root surfaces. Experiments were designed to analyze the root surface conditioning effects of TCN at concentrations that were tolerated by periodontal cells or were cytotoxic to the cells. Three freshly extracted teeth were collected for analysis of root surface modifications as a function of scaling and root planing alone or combined with TCN treatment. Experimental surfaces from teeth without periodontal disease were selected to eliminate confounding effects from calculus and modification due to periodontal disease. The roots were then sectioned longitudinally to accommodate subsequent electron microscopic analyses. Each of the two root sections from each tooth was divided into two zones by a bur groove in the surface, thereby yielding four experimental zones per tooth. Zone 1 was left untreated, whereas zones 2, 3, and 4 were root planed by an experienced periodontist (B.S.) using universal and area-specific curetes.*** To emulate root surface preparation in the clinic, the end goal of root planing was the point at which the root surface felt completely smooth with a Gracey EXD 11/12 periodontal explorer. After scaling and root planing, zone 2 was treated with saline control, whereas zones 3 and 4 were treated with either of two concentrations of TCN in saline selected after reviewing results from the preceding cytotoxicity studies. Zone 3 had a highly cytotoxic concentration of 75 mg/ml, which yielded some precipitation indicating that the solution was saturated, and zone 4 had a lower concentration of 1 mg/ml, which was tolerated well by the cells for short-term treatment and did not induce irreversible damage. The TCN was applied by continuous rubbing motions for a timed period of 3 minutes using a cotton pellet saturated with the TCN solutions, taking great care to not cross over between treatment zones. After treatment, the surfaces were washed extensively with water to remove all TCN and then processed for analysis by scanning electron microscopy.

Scanning electron microscopy. The teeth were main- tained under hydrating conditions before, during, and after root surface conditioning. Following treatments, the specimens were dehydrated through a concentration
series of ethanol and dried using hexamethyl disilazane (HDMS). The specimens were mounted on aluminum cylinders and sputter-coated with gold-palladium (60% to 40%) in a sputter coater. Coated root surfaces were analyzed and photographed in a scanning electron microscope at 1,500- or 4,000-fold magnifications.

RESULTS

Tetracycline Toxicity for Periodontal Cells

Short-term treatments. To first investigate the response of hGF and hPDL cells, as well as the well-characterized WI-38 fibroblast cell line to TCN, cells were treated for 3 minutes with a concentration range of TCN. The period of 3 minutes was selected to simulate chemical root surface treatment in the clinic. TCN treatment concentration versus cell viability response curves demonstrated that the TD50 for short-term treatments were 4, 4, and 5 mg/ml for hGF, hPDL, and WI-38 cells, respectively (Fig. 1). For these cells, survival exceeding 90% (TD10) occurred with short-term treatment concentrations less than 0.2, 0.2, and 0.6 mg/ml TCN, respectively. By both measures, the three cell lines had similar sensitivities to TCN treatment. A technical issue encountered during preliminary experiments was precipitation of TCN, which occurred readily in the presence of divalent cations. The solubility was significantly improved in saline, although TCN precipitated even in this buffer at higher concentrations during cell culture experiments, presumably due to the release of divalent cations from lysed cells. The highest concentration of TCN that could be tested reliably for short-term treatments of cells was 27 mg/ml. While distinct minima of the response curves were not obtained with short-term treatments, the assay values at the highest TCN concentrations corresponded to those observed following ethanol treatments, which effectively killed the cells (data not shown).

Long-term treatments. Some clinicians recommend including TCN with bone replacement grafts. Therefore, we analyzed the effects of long-term treatments (24 hours) with TCN for the three cell lines. The TD50 were 70, 30, and 150 µg/ml for the hGF, hPDL, and WI-38 cells, respectively (Fig. 2). The concentrations of TCN that allowed for 90% cell survival (TD10) after 24-hour treatments were 20, 5, and 20 µg/ml for the three cell lines (Fig. 2). Thus, hGF, hPDL, and WI-38 displayed a similar level of cell survival also after 24-hour TCN treatments. There was a clear effect of exposure time as the tolerated concentrations of TCN were significantly lower after 24-hour compared to 3-minute treatments.

Cell Recovery After Tetracycline Treatment

Since a short-term treatment potentially could result in only reversible cytotoxic effects, the capacity of the cells to recover was investigated for hGF and hPDL cells. After treatment with a concentration range of TCN, cells were rinsed and incubated in regular cell culture medium without TCN and assayed for cell viability.
Figure 2. Cell viability of hGF, hPDL, and WI-38 cells in response to long-term treatments with TCN. The hGF (A), hPDL (B), and WI-38 (C) were treated for 24 hours with a concentration range (0 to 3 mg/ml) TCN and analyzed for cell viability with the WST-1 assay. Shown are the means and SD (bars) from duplicate samples representative of three separate experiments.

Figure 3. Capacity of periodontal cells to recover after short-term treatment with tetracycline. The hGF (A) and hPDL (B) were treated for 3 minutes with 0 (control), 1, 3, 9, or 27 mg/ml TCN. After rinses to remove all TCN, regular growth medium was added and the cell viability analyzed after 48 hours. Data points represent means and SD (bars) of duplicate samples from four repeated experiments.

after 4 (baseline) and 48 hours. The hGF treated with 27 mg/ml were damaged irreversibly (Fig. 3A), whereas the hGF that were treated with 9, 3, or 1 mg/ml TCN had the capacity to recover. The extent of recovery was concentration-dependent and treated cells never reached the level of the untreated control cells (Fig. 3A). In comparison to the hGF, the hPDL cells had a lower capacity to recover as illustrated by an unchanged cell number from 4 to 48 hours after treatment with 9 mg/ml or higher TCN (Fig. 3B). At concentrations lower than 9 mg/ml, a partial recovery was evident also for hPDL. Together, these experiments demonstrated that hGF and hPDL cells recovered only partially over a 48-hour period following the initial, deleterious effects of TCN treatments.

Inhibition of Recombinant MMP-2 and -9 by Tetracycline

Since a major biological effect of TCN is its capacity to inhibit MMP, an aim was to correlate the concentrations of TCN that were cytotoxic to hGF and hPDL cells to the concentrations of TCN that inhibited the MMPs. These studies used MMP-2 and -9, which both exert collagenolytic activities during periodontal disease.\textsuperscript{23}
To best control for variability in the assays, we expressed the MMP as recombinant proteins as detailed under Materials and Methods. The concentrations of TCN that yielded 50% inhibition (IC$_{50}$) of MMP-2 and MMP-9 were determined from dose-response curves within concentration ranges of 0 to 250 µg/ml TCN in the assays. The IC$_{50}$ were 25 µg/ml for both MMP-2 and -9 (Fig. 4). These results demonstrated that these two MMPs were inhibited by TCN at concentrations well tolerated by hGF and hPDL cells.

**Root Surface Conditioning by Tetracycline**

It has been reported that treatment with TCN following root instrumentation is effective for root surface modification, including removal of the smear layer. Analysis of scanning electron micrographs showed that scaling and root planing followed by treatment with saline control (Fig. 5B) or 1 mg/ml TCN (Fig. 5C) resulted in slightly smoother root surfaces compared to untreated controls (Fig. 5A). Yet, the surfaces were uneven and characterized by an amorphous smear layer of material containing residual calculus and other debris with no visible collagen fibrils or dentinal tubules. In comparison, demineralization with 75 mg/ml TCN after scaling and root planing effectively removed the smear layer and exposed both collagen fibrils and dentinal tubules (Fig. 5D, 5E). Thus, demineralization with 1 mg/ml TCN, which resulted in low cytotoxicity, did not remove the smear layer.

**DISCUSSION**

The present series of experiments investigated the potential for cytotoxic effects of tetracycline, which has been recommended for local application in periodontal therapy. In studies using human primary gingival and periodontal ligament fibroblasts, as well as the well-characterized WI-38 fibroblast cell line, a 90% cell survival was observed only with TCN concentrations below ~200 µg/ml for short-term treatments and below ~20 µg/ml for long-term treatments. These concentration levels were sufficient to inhibit two key MMPs, MMP-2 and -9, that are active in periodontal disease (IC$_{50}$ 25 µg/ml).

Although periodontal cells treated with a concentration of 1 mg/ml demonstrated significant recovery after short-term treatments, this concentration of TCN was insufficient to effectively remove the smear layer from root surfaces after scaling and root planing.

The successful regeneration of a functional periodontal ligament has been a rather evasive goal in the treatment of periodontal lesions. It has become apparent that the reconstitution of collagenous fibers that connect the tooth surface with the ligament connective tissue, gingiva, and alveolar bone is integral to a functional ligament. Clinical and electron microscopy studies have not only revealed that, although scaling and root planing reduces the amount of detectable calculus, pending such variables as tooth morphology, probing depth and operator experience, this treatment also leaves a residual smear layer on the root surface containing miscellaneous debris, bacteria and bacterial byproducts, and remnants of the periodontal ligament fibers. In vitro studies demonstrating that demineralization with different acids after the root instrumentation can secure the removal of this smear layer and expose remnants of the periodontal ligament fibers have provided the scientific basis for exploration of new treatment options in regenerative periodontal therapy involving root surface demineralization.

TCN is used widely for its antimicrobial activities and has also been recognized as an important anti-collagenolytic compound that may reduce the tissue
cleavage of the tetrazolium salt WST-1 to soluble formazan dye by mitochondrial dehydrogenases. To emulate the clinical mode of TCN application in our experimental design, we first applied TCN for a short period of time (3 minutes), which corresponds to that commonly used in the clinic for root surface demineralization with acidic agents. Fifty-percent survival of hGF and hPDL cells (TD50) was observed after treatment with ~4 mg/ml TCN. In addition, we tested survival after a 24-hour application (long-term). Although this treatment is shorter than the long periods of time that cells are exposed to TCN embedded into bone replacement graft materials, the experiments illustrated clearly that the duration of exposure is an essential factor for cell survival. Thus, the TD50 in 24-hour treatments were 30 to 70 µg/ml and corresponded to approximately 10% of the TD50 for short-term treatments. The corresponding concentrations resulting in 90% cell survival were ~200 and ~20 µg/ml for short- and long-term treatments, respectively, and were also subject to an effect of the treatment time.

The cells selected for the experiments were primary cell lines cultured from human gingival (hGF) and periodontal ligament (hPDL) explants. These same cell lines, which have been used in other studies by ourselves and others, behave predictably in our hands and are very valuable because they are primary in nature. However, since such primary cell lines may vary between cultures, we performed simultaneously experiments with the well-characterized WI-38 fibroblasts. This lung tissue fibroblast cell line has been characterized extensively and served here as a valuable internal standard to validate the consistency in our repeated experiments over time.

A key experimental question was whether and to what extent periodontal cells had the capacity to recover following short-term treatments with TCN. As a general rule, gingival fibroblasts displayed a slightly higher tolerance to TCN treatment compared to the periodontal ligament cells expressed by cell proliferation and viability and recovered to 60% of controls after short-term treatments with TCN at 9 mg/ml or less. By comparison, a similar level of recovery for periodontal ligament cells occurred only with treatment concentrations of 3 mg/ml or less. For both hGF and hPDL cells, a 90% recovery after short-term treatments occurred only with TCN concentrations <1 mg/ml.

An important quality of TCN is its capacity to effectively inhibit collagenases (MMP). To enable controlled studies of the functional characteristics of MMP, we have generated expression constructs for two of them, MMP-2 and MMP-9, which are known to be important in periodontal disease.

Figure 5. Root surface modification by instrumentation and tetracycline treatment. The root surfaces of three freshly extracted periodontally healthy teeth were each divided into four experimental zones: untreated control (A), scaling and root planing and saline treatment only (B), and scaling and root planing followed by treatment with either 1 mg/ml (C) or 75 mg/ml TCN (D, E). Original magnification: 1,500× A-D and 4,000× E.
and MMP-9. These two enzymes have activities on a series of other important extracellular structural matrix molecules, including several collagen types, elastin, fibronectin, and proteoglycan link protein as well as non-structural growth factors and cytokines. Importantly, both MMP-2 and -9 cleave the α-chains of denatured collagen as well as the initially cleaved and partially unwound triple helical collagen molecules during tissue breakdown in periodontal disease. Whereas MMP-2 is constitutively expressed by a number of cell lines in the periodontium, MMP-9 is primarily the product of polymorphonuclear leukocytes that are present at high levels under periodontal inflammatory conditions.

Having established the critical concentrations for cytotoxicity of TCN after short- and long-term treatments, we wished to understand to what extent these critical concentration levels related to the inhibition of MMPs. This was of particular interest relative to inclusion of TCN with bone replacement grafts. In our experiments, both MMP-2 and -9 were inhibited by 50% (IC₅₀) with ~25 µg/ml TCN and displayed virtually no activity in the presence of ~100 µg/ml TCN. Of note, results from our cytotoxicity experiments showed a 90% cell survival after 24-hour treatments of periodontal cells with ~20 µg/ml. This suggests that inclusion of TCN in grafts at a level which ensures a release at a concentration of 25 µg/ml would result in both a low cytotoxicity and a significant MMP inhibition.

An issue for consideration as we conjecture our in vitro results to the clinical situation pertains to the solubility of TCN. Assays performed in preparation for the experiments showed a greater solubility of TCN in either water or saline compared to Ca⁺⁺ or Mg⁺⁺-containing buffers. Likewise, when cells lysed in the experiments after exposure to high concentrations of TCN solubilized in saline, the TCN precipitated readily. On this basis, one can expect that high concentration solutions will also precipitate when exposed to blood in the periodontal wounds. The release of soluble TCN from the precipitate will then occur at lower concentrations pending the solubility. Yet, it is also likely that cell injury in the wound area will occur since the process does take time. For example, we found that the 3-minute cell toxic concentration of 10 mg/ml TCN solution in phosphate buffered saline containing Ca⁺⁺ or Mg⁺⁺ precipitated after 5 hours, which would provide ample time for cell damage to occur. The TD₅₀ of TCN for susceptible bacteria is in the range of 1 to 5 µg/ml and corresponds to the plasma level obtained with regular prescription of TCN. Therefore, any desired antibacterial effect would be well covered with 25 µg/ml TCN that was effective in inhibiting the MMP-2 and -9 as shown above. It is likely that TCN when implanted with graft materials precipitates and is slowly released over an extended period of time as it is solubilized.

Further studies are needed to define the dissolution from such grafts, but our data suggest that as little as 70 and 30 µg/ml may adversely affect hGF and hPDL cells over 24-hour treatment periods, respectively. Finally, the dynamics of TCN solubility versus cell susceptibility may be cell type specific and a function of open wound versus epithelial barrier environment in intact pockets.

Returning to the issue of root surface demineralization, other investigators have presented data indicating that treatment with ~50 mg/ml or saturated solutions of TCN are effective for demineralization and smear layer removal. Extending these studies on the basis of our cytotoxicity data, we tested whether 1 mg/ml TCN, a concentration to which the periodontal cells recovered well, would be sufficient for smear layer removal. Scanning electron microscopy analyses of roots treated by scaling and root planing alone or in combination with TCN revealed that 1 mg/ml TCN did not remove the smear layer. That this lack of smear layer removal was an effect of the TCN concentration was evident from experimental samples where we successfully removed the smear layer using 75 mg/ml TCN after the scaling and root planing. It is noteworthy that 75 mg/ml was the highest concentration of TCN which remained in solution in saline for the duration of the short-term root surface treatment. In context of the TCN cytotoxicity, our results suggest that a concentration of TCN with low cytotoxic effects on periodontal cells is not effective for smear layer removal and, conversely, that the concentration of TCN required for effective root surface demineralization is not well tolerated by periodontal cells. On this basis, we conclude that TCN should not be the reagent of choice for root surface demineralization.

To seek an alternative agent for root surface demineralization, Blomlof et al. compared the acidic compounds with the chelator EDTA. In a series of studies on monkey and human teeth, these investigators demonstrated first that treatment of exposed root surfaces with ortho-phosphoric acid for 3 minutes during flap elevation and followed for 8 weeks resulted in significantly increased recession, reduced histological attachment, and less bone compared to controls. Subsequently, phosphoric and citric acids were found to induce significant cell death on the exposed flap surfaces as measured by cellular lactate dehydrogenase activity, whereas EDTA had no such effect on the cell vitality. Although these studies did not examine periodontal ligament cell vitality, our present results strongly suggest that fibroblasts from the gingiva and the periodontal ligament display a similar level of sensitivity to acidic agents. The investigators finally demonstrated that the smear layer may be efficiently removed by treatment with an EDTA gel preparation resulting in a surface morphology similar to that of...
acid-treated roots. On this basis, EDTA appears to present a safe alternative to acidic compounds for smear layer removal.

In practical terms according to our results, inclusion of one 250 mg capsule in 1 ml saline in a dappen dish corresponds to a dosage of ~10,000 times the cytotoxic level for periodontal cells and that required for inhibition of collagenases and antibacterial activities. A concentration of ~50 mg/ml, which has been shown to be effective for smear layer removal, is approximately 2,000 times the cytotoxic concentration defined in our short-term exposure experiments.

Collectively, our results have demonstrated that most periodontal cells will survive a 3-minute treatment with TCN at a concentration of ~1 mg/ml, whereas the cells survive continuous exposure for a period of 24 hours only in a concentration less than 25 μg/ml. Although 25 μg/ml TCN is sufficient to inhibit two MMP involved in periodontal disease, 1 mg/ml TCN is insufficient to demineralize root surfaces. The release of TCN placed with bone replacements grafts has the same potential for exerting cytotoxic effects on the surrounding tissues. On the basis of these data, we conclude that TCN is not recommended for root surface demineralization and caution should be exercised when integrating this compound with bone grafts.

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