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Proceedings of a symposium held in
Garden City, New York
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TETRACYCLINES INHIBIT CONNECTIVE TISSUE BREAKDOWN BY MULTIPLE NON-ANTIMICROBIAL MECHANISMS

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Abstract—A seminal experiment involving a *germ-free* rat model of connective tissue breakdown (followed soon thereafter by a series of *in vitro* studies) identified an unexpected non-antimicrobial property of tetracyclines (TCs). This ability of TCs to inhibit matrix metalloproteinases (MMPs) such as collagenase was found to reflect multiple direct and indirect mechanisms of action, and to be therapeutically useful in a variety of dental (*e.g.*, adult periodontitis) and medical (*e.g.*, arthritis, osteoporosis, cancer) diseases. The site on the TC molecule responsible for its MMP-inhibitory activity was identified which led to the development of a series of chemically modified non-antimicrobial analogs, called CMTs, which also have therapeutic potential but do not appear to induce antibiotic side-effects. Longitudinal double-blind studies on humans with adult periodontitis have demonstrated that a sub-antimicrobial dose of doxycycline (previously reported to suppress collagenase activity in the periodontal pocket) is safe and effective and has recently been approved by the FDA as an adjunct to scaling and root planing.

Key words: Non-antimicrobial tetracyclines, doxycycline, collagenase, matrix metalloproteinases, host modulation.

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As recently reviewed, the early science (1972-1982) that led to the discovery, by Golub and his "Stony Brook" group, of tetracycline's anti-collagenolytic properties was originally directed at explaining how diabetes increases the severity of periodontal disease, and why diabetics are often (not always) refractory to periodontal therapy (Golub *et al.*, 1996). In their initial studies on this systemic disease/local disease interaction, they observed that alloxan-induced type I (insulin-deficient) diabetes in the rat increased the urinary excretion of hydroxyproline, an amino acid "marker" of collagen and its degradation fragments, and reduced the solubility (at 4°C) of collagen in the gingival tissues (Ramamurthy *et al.*, 1972). These and a series of subsequent experiments led to the conclusion that experimental diabetes produced a cascade of abnormalities in collagen metabolism in the periodontal and other tissues, including: enhanced collagen breakdown (both intracellularly following synthesis of the procollagen precursors, and extracellularly after incorporation of the secreted and processed collagen into the matrix); suppressed intracellular collagen biosynthesis; and increased formation of intermolecular covalent crosslinks in the extracellular matrix fibrils (premature "aging" of collagen), the latter mediated both enzymatically by lysyl oxidase, and non-enzymatically following binding of glucose to collagen to form advanced glycosylation end-products, or "AGEs" (Golub *et al.*, 1978a,b; Schneir *et al.*, 1982; Ramamurthy *et al.*, 1983; Leung *et al.*, 1986; Ryan *et al.*, 1995). One oral manifestation of these abnormalities was the detection of osteoporosis in the jaws of diabetic rats (Ramamurthy *et al.*, 1973a), with type I collagen constituting 90% of the organic matrix of bone. Only recently has the relationship between osteoporosis (such as that seen in estrogen-deficient post-menopausal women as well as in diabetics) and accelerated periodontal breakdown begun to be clarified (Ryan *et al.*, 1995; Grodstein *et al.*, 1996; Payne *et al.*, 1997).

Although inducing diabetes in the rat with either of two β-cell-destructive agents, alloxan and streptozotocin, produced the complex series of collagen abnormalities described above, the observation which eventually led to the seminal tetracycline experiment was our finding in the 1970's that gingival tissues from hyperglycemic diabetic rats produced dramatically elevated collagenase activity compared with the low activity of this specific neutral proteinase generated by non-diabetic controls (Ramamurthy *et al.*, 1973b; Golub *et al.*, 1978b). Interstitial collagenase was the first-discovered (early 1960's) of a family of Ca^{++}-dependent Zn^{++}

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endopeptidases (Gross and Lapiere, 1962), now called the matrix metalloproteinases (MMPs). These MMPs are largely responsible for degrading the various constituents of connective tissues during normal remodeling and pathologic tissue breakdown (Greenwald *et al.*, 1998; Konttinen *et al.*, 1998). Since collagenase has long been thought to be the only proteinase able to degrade the triple-helical collagen molecule under physiologic conditions (although recently, Aimes and Quigley [1995] demonstrated that another MMP, gelatinase A, also possesses "triple helicase" activity), we concluded that the excess urinary excretion of hydroxyproline exhibited by diabetic rats (see above), and the unusually severe periodontal breakdown exhibited by many (not all) diabetic patients and diabetic rats, could be related to increased expression and activity of collagenases (Golub *et al.*, 1978b). Currently, at least three collagenases are recognized, MMP-1, MMP-8, and MMP-13, although MMP-1 could not have been responsible for our observations in diabetic rats, since this collagenase is not produced by rodent tissues (Greenwald *et al.*, 1998).

Seminal tetracycline experiment

As outlined in Fig. 1, we next questioned whether the elevated collagenase activity in the gingiva of the diabetic rat could be explained by a shift toward a more anaerobic Gram-negative microflora in the gingival crevice, thus generating elevated endotoxin levels which could penetrate the diabetes-damaged crevicular epithelium/basement membrane into the subepithelial connective tissue and stimulate host cells, such as fibroblasts and/or macrophages, to produce excessive collagenase (McNamara *et al.*, 1982). Our alternative hypothesis was that diabetes increased gingival collagenase activity and, as a result, accelerated periodontal breakdown by a mechanism *independent* of microbial factors and due only to altered host responses (*e.g.*, Vlassara *et al.* [1988] proposed that elevated expression of MMPs by macrophages in the diabetic tissues was induced by binding of AGEs to surface receptors on these cells). To identify whether microbial or host factors were causative, Golub *et al.* (1983) reported that treatment of the hyperglycemic diabetic rats with minocycline, a semi-synthetic tetracycline (TC), "normalized" the excess collagenase activity generated by the gingival explants in culture, even though the drug did not affect the severity of hyperglycemia. To determine whether the TC therapy was *secondarily* reducing mammalian collagenase activity in the gingiva by *first* suppressing the Gram-negative microflora (the latter would presumably reduce bacterial endotoxin levels in the gingival crevice and, as a result, suppress gingival collagenase activity; Fig. 1), the investigators carried out a second experiment, using *germ-free* rats which were rendered diabetic by streptozotocin injection and treated with a TC, to eliminate any microbial factors. To the amazement (and delight) of the investigators, minocycline treatment produced the same reduction in collagenase activity in the germ-free rats, which lacked a microflora, just as it did in conventional rats which exhibit a complex oral microflora (Golub *et al.*, 1983, 1984). These and a series of subsequent experiments clearly demonstrated

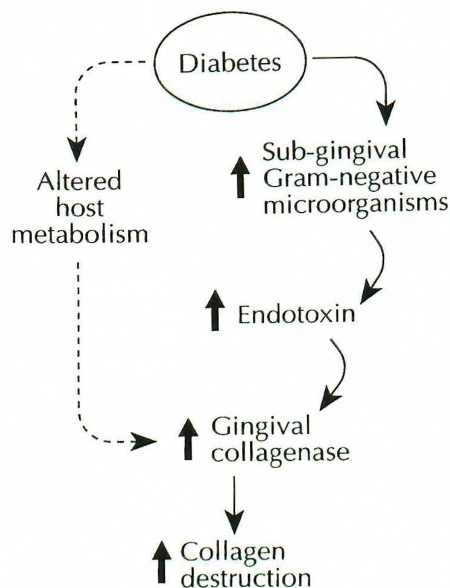

Fig. 1—Experimental diabetes induces excess gingival collagenase activity in tissue culture: mediated by microbial or host factors?

that TCs possess an unexpected property and led us to propose the following: (i) that TCs can inhibit mammalian collagenolytic activity by mechanisms *independent* of the antimicrobial property of these antibiotics, and (ii) that this newly discovered property of these drugs could provide a novel therapeutic approach to the treatment of a variety of dental (*e.g.*, periodontitis, root caries, intra-oral ulcers, and arthritis of the temporomandibular joint) and medical diseases (*e.g.*, the arthritides, different types of osteoporosis, cancer invasion and metastasis) characterized by excessive collagen breakdown. A number of papers at this symposium addressed both of these working hypotheses.

To begin to identify mechanisms, we examined the effects of TCs *in vitro* on the collagenase activity in extracts of polymorphonuclear leukocytes (PMNLs) and in conditioned media of cultured macrophages (both types of inflammatory cells were isolated from glycogen-induced peritoneal exudates of systemically normal adult rats) and found that: (i) the addition of minocycline (but not other antibiotics such as penicillin, streptomycin, and metronidazole) to the incubation mixture, in concentrations ranging from 2-25 μg/mL (levels found in the serum and GCF of humans orally administered this or other TCs), inhibited the activity of collagenase from both cell types in a dose-dependent fashion (Golub *et al.*, 1983, 1994); and (ii) adding increasing concentrations of Ca^{++} to the incubation mixture progressively blocked the ability of minocycline to inhibit collagenase activity *in vitro* (Golub *et al.*, 1983). More recent *in vitro* studies showed that μM concentrations of Zn^{++} (L. Yu *et al.*, 1991; Lee *et al.*, 1992) could produce the same "block" of this anti-MMP

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property of TCs as mM concentrations of Ca^{++}. These early studies were rapidly followed by wide-ranging efforts by a number of research teams to confirm this non-antimicrobial property of TCs, to elucidate the mechanisms involved, and to explore the immense therapeutic potential of this discovery (Golub *et al.*, 1987, 1991, 1997; Tamargo *et al.*, 1991; L. Yu *et al.*, 1991; Sotomayor *et al.*, 1992; Cole *et al.*, 1994; Masumori *et al.*, 1994; Brandt, 1995b; Paeman *et al.*, 1996; Williams *et al.*, 1996; Greenwald *et al.*, 1998).

TCs PLEIOTROPIC THERAPEUTIC EFFECTS: ADDITIONAL NON-ANTIMICROBIAL MECHANISMS OF COLLAGENOLYTIC INHIBITION

TC-mediated EXTRACELLULAR anti-collagenolytic mechanisms

In a series of experiments addressing mechanisms, in addition to the early approach which was directed at inhibition of extracellular active forms of collagenase *in vitro* (see above), Golub and his colleagues investigated the specificity of the anti-collagenolytic activity of TCs and located the site on the TC molecule responsible for this host-modulating, non-antimicrobial property of the drug (Golub *et al.*, 1987, 1991, 1992). Regarding the former mechanism, TCs were found to directly inhibit collagenases from a wide variety of human and animal cells, including (but not limited to) PMN leukocytes, macrophages, osteoblasts, osteoclasts, chondrocytes, and malignant melanoma cells (Zucker *et al.*, 1985; Golub *et al.*, 1987, 1992; Ramamurthy *et al.*, 1990). Other investigators confirmed this effect using additional human and animal sources of collagenase (Maehara *et al.*, 1988; Burns *et al.*, 1989; McCulloch *et al.*, 1990; Cole *et al.*, 1994; Masumori *et al.*, 1994). It is now recognized that collagenase-3 (MMP-13) is more sensitive to TC inhibition ($IC_{50} < 1\ \mu M$) than collagenase-2 (MMP-8; $IC_{50} \sim 30\ \mu M$) and that collagenase-1 (MMP-1) is the least sensitive ($IC_{50} > 200\ \mu M$) to the inhibition of enzyme activity by these drugs *in vitro* (Golub *et al.*, 1995; Smith *et al.*, 1996; Greenwald *et al.*, 1998), although the mechanisms are more complex than originally thought (see below). Other MMPs—namely, gelatinase A (MMP-2) and gelatinase B (MMP-9) and macrophage metalloelastase (MMP-12) and other types of MMPs—are also inhibited by these drugs, whereas other classes of proteinases such as serine proteinases (*e.g.*, PMN elastase and plasminogen activator) and acid proteinases (*e.g.*, cathepsins B and L) are not (Greenwald *et al.*, 1987; Golub *et al.*, 1991, 1992; Paemen *et al.*, 1996). One exception to this pattern of proteinase inhibition was identified by Simon *et al.* (unpublished results): A highly lipophilic non-antimicrobial TC analog, 6-demethyl 6-deoxy 4-de-dimethylamino tetracycline (now called CMT-3; see below), but *not* other TCs, appears to inhibit PMN elastase, a serine proteinase, competitively.

Regarding the site on the TC molecule responsible for its anti-MMP activity, Golub *et al.* (1987) produced the first of their chemically modified TCs, now called CMTs, by removing the dimethylamino group from the carbon-4 position [this side-chain is required for the antimicrobial activity of TCs (Mitscher, 1978)] and found that the resulting compound, 4-de-dimethyl amino TC, or CMT-1, had lost its antimicrobial property but retained (actually showed enhanced) anticollagenase activity both *in vitro* and *in vivo* (Golub *et al.*, 1987; Z. Yu *et al.*, 1991). Currently, 10 different non-antimicrobial chemically modified TCs, namely, CMT-1 through CMT-10, have been generated, with different side-chains removed and/or added to the TC molecule, and 9 of the 10 compounds were found to be non-antibacterial MMP inhibitors (see reviews by Greenwald *et al.*, 1998; Golub *et al.*, 1992; Ryan *et al.*, 1996b). However, when the carbonyl oxygen at C-11 and the hydroxyl group at C-12 were replaced by nitrogen atoms, thus producing the pyrazole analog of TC (Valcavi *et al.*, 1963), now called CMT-5, this compound was found to be the only CMT which lost its MMP inhibitory activity (Golub *et al.*, 1991). These studies demonstrated that this important (but not the only) Ca^{++} and Zn^{++} binding site (the β-diketone moiety at C-11 and C-12) was responsible, at least in part, for inhibiting the extracellular activity of MMPs (intracellular mechanisms of MMP regulation by TCs are described below). Recent enzyme kinetic studies have indicated that doxycycline, the most potent MMP inhibitor of antimicrobial TCs (Burns *et al.*, 1989; Golub *et al.*, 1995), acts as a non-competitive inhibitor of collagenase (Sorsa *et al.*, 1994), whereas CMT-3, among the most potent collagenolytic inhibitors of the non-antimicrobial TC analogs (Rifkin *et al.*, 1994; Ryan *et al.*, 1996b; Greenwald *et al.*, 1998), appears to exhibit this, plus more complex mechanisms of inhibition, at least for cancer cell MMP-2 (gelatinase A) and is a more potent inhibitor than doxycycline *in vitro* (Seftor *et al.*, 1998). In this regard, (i) the relative efficacy of TCs and CMTs appears to be positively correlated to the Zn^{++} binding efficacy of these compounds (Usman *et al.*, 1996), and (ii) converting anti-microbial TCs, such as doxycycline, to their nonantimicrobial analogs (such as CMT-8, the non-antimicrobial analog of doxycycline), appears to increase the MMP-inhibitory efficacy and some other therapeutic, matrix-sparing, activities of these drugs (Paemen *et al.*, 1996; Zernicke *et al.*, 1997; Greenwald *et al.*, 1998; Ohyori *et al.*, 1998).

Numerous studies have found that TCs/CMTs can inhibit MMP activity and connective tissue breakdown *in vivo* and *in vitro* in various diseases in humans and experimental animals. As examples of *in vitro* efficacy, TCs and CMTs were found to inhibit bone resorption in both organ and cell culture, regardless of whether the resorption was induced by parathyroid hormone (PTH), PGE_2, or bacterial endotoxin (Golub *et al.*, 1984; Gomes *et al.*, 1984; Rifkin *et al.*, 1994). Doxycycline (DOXY) was the most potent MMP inhibitor of the antimicrobial TCs. Of the non-antimicrobial CMTs tested, CMT-1 and -3 and CMTs-6, -7, and -8 were effective inhibitors of bone resorption in culture (CMT-8 was the single most potent compound), whereas CMT-2, -4, and -5 were not. (Note: CMT-2 and -4 did block PMN collagenase but not bone cell collagenase, whereas CMT-5 was ineffective as an inhibitor in all active collagenolytic systems tested.) As other examples: minocycline and CMT-1

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TABLE

TETRACYCLINES (TCs) INHIBIT CONNECTIVE TISSUE BREAKDOWN: PLEIOTROPIC MECHANISMS

(A) Mediated by extracellular mechanisms
- Direct inhibition of active MMPs—dependent on Ca^{++} and Zn^{++} binding properties of TCs
- Inhibition of oxidative activation of Pro-MMPs—independent of cation-binding properties of TCs
- TCs disrupt activation by promoting excessive proteolysis of pro-MMPs into enzymatically-inactive fragments—dependent (?) on cation binding of TCs
- Inhibition of MMPs protects α_1-PI, thus INDIRECTLY ↓ serine proteinase (*e.g.*, PMNL elastase) activity

(B) Mediated by cellular regulation
- TCs ↓ cytokines, iNOS, PLA_2, prostaglandin synthase
- Effects on protein kinase C, calmodulin

(C) Mediated by pro-anabolic effects
- TCs ↑ collagen production
- TCs ↑ osteoblast activity & bone formation

inhibited malignant melanoma cell collagenase *in vitro* and blocked these cancer cells from lysing co-cultured host cells (Zucker *et al.*, 1985), and these same (and other) TCs *in vitro* inhibited MMPs derived from inflamed human gingiva and rheumatoid synovium (Golub *et al.*, 1987; Greenwald *et al.*, 1987), from human lung cancer cells (Zucker *et al.*, 1989), from diabetic rat skin and gingiva (Golub *et al.*, 1983, 1987), from rachitic rat cartilage (Greenwald *et al.*, 1988), from osteo-arthritic human cartilage (L. Yu *et al.*, 1991), from ulcerated rabbit cornea (Seedor *et al.*, 1987; Burns *et al.*, 1989), and from human gingival fluid (Golub *et al.*, 1983, 1991; Maehara *et al.*, 1988; McCulloch *et al.*, 1990). Moreover, when TCs or CMT-1 were administered p.o. *in vivo*, they were found to reduce pathologically excessive collagenase (and gelatinase) activity, and/or to inhibit matrix breakdown, in the gingival tissue and GCF of humans (TCs only) with adult periodontitis (Golub *et al.*, 1983, 1995, 1990a), in synovial tissue and fluid of humans with rheumatoid arthritis (Greenwald *et al.*, 1987), in skin and gingiva of rats with diabetes (Golub *et al.*, 1983, 1987), in cartilage of dogs with osteo-arthritis (Brandt, 1995b) and rats with rickets (Greenwald *et al.*, 1988), and in humans and rabbits with sterile corneal ulcers (Seedor *et al.*, 1987). (Note: CMTs have not yet been approved for human use by the FDA, although the National Cancer Institute has recently initiated preliminary studies, using CMT-3, on humans with cancer.) More recent studies have demonstrated the therapeutic potential of TCs' anti-MMP activity in *in vivo* and cell culture models of cancer invasion, metastasis, and angiogenesis (Zucker *et al.*, 1985; Tamargo *et al.*, 1991; Sotomayor *et al.*, 1992; Masumori *et al.*, 1994; Lokeshwar *et al.*, 1997; Seftor *et al.*, 1998).

As summarized in the Table, even the extracellular mechanisms (intracellular are discussed in a later section of this paper) of TC inhibition of matrix degradation are now recognized to be pleiotropic; additional examples now follow:

TC effects on pro-MMP activation

The initial experiments on this mechanism were carried out by means of an animal model of microbially induced periodontal breakdown. In brief, this model involved infecting either germ-free or pathogen-reduced rats with the human periodontopathogen, *Porphyromonas gingivalis* (*P.g.*), which results in elevated MMP (collagenase or MMP-8 and gelatinase or MMP-9) activity in the gingival tissues (as expected, MMP-1 was not found in the rat tissues) and severe alveolar bone resorption; *in vivo* administration of either doxycycline or CMT-1 by daily oral gavage reduced all of these "markers" of periodontal disease to essentially normal levels (Chang *et al.*, 1994; Golub *et al.*, 1994a). Recent experiments identified a two-week "window" of disease activity, characterized by elevated levels of active collagenase, active gelatinase, and rapid alveolar bone loss in this animal model of microbially induced periodontal disease (Ryan *et al.*, 1997). However, the initial series of studies with this model found that, at the end of a seven-week *in vivo* protocol, the elevated collagenase (and gelatinase) activity in the gingiva of the *P.g.*-infected rats was present as the inactive or latent form (pro-collagenase). These pro-MMPs required *in vitro* activation either by APMA or oxidatively by hypochlorous acid (HOCl) [*in vivo* activation of pro-MMPs is thought to be primarily mediated by limited proteolysis by neutral proteinases such as plasmin, or tumor-associated trypsin (TAT-2), or even other MMPs such as stromelysin (Sorsa *et al.*, 1997); however, Sorsa *et al.* (1992) have proposed that leukocyte-type pro-MMPs, MMP-8 and MMP-9, are primarily activated by reactive oxygen species (ROS), *e.g.*, HOCl, produced by inflammatory cells]. Based on the sequence of adding the following reagents—namely, CMT-1, excess Ca^{++} (50 mM instead of the usual 5 mM concentration in the buffer), and HOCl—to the incubation mixture containing extract of gingiva from the *P.g.*-infected rats, plus the radiolabeled collagen substrate, Golub *et al.* (1994a) found that the TC analog inhibited *both* active collagenase (converted from the pro-form by APMA) and the oxidative conversion of pro-collagenase into the active enzyme by HOCl. Based on these experiments and on the following *in vitro* observations, it was proposed that these two anti-MMP functions of CMTs (and other TCs) may

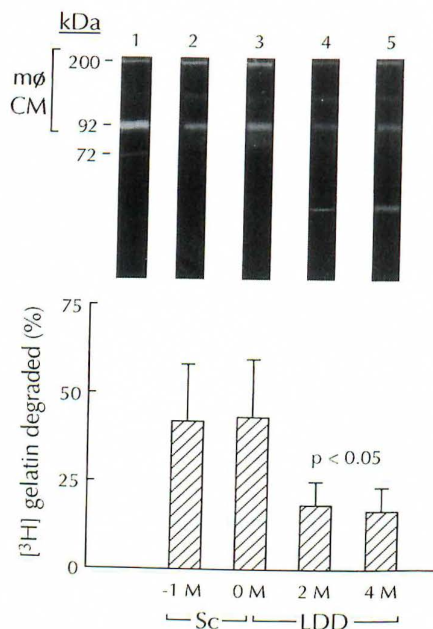

Fig. 2—Gelatinase in whole-mouth GCF (collected by mouthrinse technique) from four adult periodontitis patients 1 mo after scaling and prophylaxis (Sc) and after a four-month regimen of LDD: effects on molecular species (top) and activity (bottom) of this matrix metalloproteinase. (top) Enzymograph of whole-mouth GCF was carried out with the use of denatured type I collagen (gelatin; 1 mg/mL) co-polymerized with 10% SDS-polyacrylamide and electrophoresed at 3 m A/lane for 45 min. After overnight incubation, the gels were stained with Coomassie Blue, and the lytic zones in each lane show the different types of gelatinase. Lane 1 shows macrophage-conditioned media (m Ø CM) containing 92-kDa gelatinase (MMP-9) and its homodimer (Golub et al., *1995), plus 72-kDa gelatinase (MMP-2) obtained from Triple Point Biologics (Forest Grove, OR). Lanes 2-5 show predominantly 92 kDa and its homodimer, plus an intermediate band, presumably reflecting a heterodimer of MMP-9 complexed to lipocalin or TIMPs (Golub* et al., *1995). Lanes 4 & 5 show the different species of MMP-9, as in lanes 2 & 3, plus an additional smaller-molecular-weight band seen after 2 & 4 months' treatment with LDD. (bottom) Gelatinolytic activity (assessed with [^{3}H] methyl gelatin used as substrate) of whole-mouth GCF in the same patients and at the same time periods as shown for zymography lanes 2-5, above. Gelatinolytic activity was measured before treatment (-1M) and 1 mo after subjects received full-mouth scaling and prophylaxis (OM). Then, at time = OM, all subjects were administered LDD every day for 4 mos, and gelatinolytic activity was measured after 2 mos (2M) and 4 mos (4M) of drug therapy. Each value represents the mean of 4 determinations ± SEM. The reduction in gelatinase activity due to LDD therapy was statistically significant ($p < 0.05$) compared with the time = OM value.*

reside in different parts of the drug molecule: When CMT-1 and excess Ca^{++} were added to the partially purified gingival extract together with HOCl, CMT-1 was still able to inhibit the oxidative activation of the gingival pro-collagenase. However, when the pro-collagenase was activated by HOCl *prior* to the addition of CMT-1 and Ca^{++}, the excess metal ion prevented the drug from blocking already-active collagenase, as described previously. Consistent with these studies on gingival collagenase, (i) the addition of excess Ca^{++} (mM) or Zn (μM) did *not* prevent TCs from inhibiting the oxidative activation of pro-collagenase secreted by osteoblasts in culture (Ramamurthy *et al.*, 1993), and (ii) CMT-5, the pyrazole analog of TC, which is the only one of 10 CMTs to have *lost* the ability to inhibit already-active collagenase, presumably due to the loss of the Ca^{++} and Zn^{++}-binding site at the carbon-11 and carbon-12 positions, retained its ability to prevent pro-collagenase activation by HOCl (Sorsa *et al.*, 1998).

In addition to scavenging ROS, thus preventing pro-MMP activation, TCs appear to interact directly with pro-MMPs as well, thus affecting other mechanisms of activation. In this regard, Brandt and his group studied the effect of doxycycline on recombinant human pro-MMP-8 and detected two different mechanisms of inhibition (Brandt, 1995a; Smith *et al.*, 1996). As reviewed by Ryan *et al.* (1996b), when doxycycline was added *in vitro* after the activation of pro-MMP-8 (activation was achieved either by limited proteolysis or by the organomercurial agent, APMA), approximately 30 μM of the drug was required to inhibit the collagenase activity by 50%, an IC_{50} similar to that described by others (Sorsa *et al.*, 1994; Golub *et al.*, 1995). In contrast, when doxycycline was added during activation, the IC_{50} dropped to 5-12 μM. Based on Western blot analysis of the different molecular species of MMP-8, Brandt and his colleagues (Smith *et al.*, 1996) proposed that doxycycline binds to the pro-MMP (possibly complexing with Ca^{++} in the enzyme), thus altering the enzyme's conformation and resulting in excessive degradation of the proteinase precursor, during activation, to small-molecular-weight, enzymatically inactive fragments. This mechanism of MMP inactivation, although attractive, remains controversial, since Sorsa *et al.* (1994) were unable to detect any fragmentation of wild-type Pro-MMP-8 during activation by APMA in the presence of doxycycline. Of extreme interest, this mechanism may extend to other MMPs, since MMP-13 was recently found to be inhibited by very low concentrations (< 1 μM) of several CMTs (CMT-3, CMT-7, and especially CMT-8) as well as doxycycline (IC_{50} ~ 10 μM) *in vitro* when the TCs were added together with APMA during activation (Greenwald *et al.*, 1998). However, when the drugs were added *after* pro-MMP-13 was APMA-activated, this collagenase was found to be relatively resistant to TC inhibition (Lindy *et al.*, 1997). The relevance of this mechanism to human disease is suggested by preliminary data described later in this paper: When adult periodontitis patients were administered a four-month regimen of non-antimicrobial low-dose doxycycline, MMP activity in whole-mouth gingival crevicular fluid (GCF) samples was reduced coincident with the appearance, at the

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two- and four-month time periods, of small-molecular-weight MMP fragments and reduced pro-MMP levels (see Fig. 2).

TCs indirectly inhibit serine proteinases: α_1-PI protection

In vitro, cell culture, and *in vivo* approaches have all been used to determine the effects of TCs on α_1-PI inactivation (Sorsa *et al.*, 1993; Golub *et al.*, 1994a; Crout *et al.*, 1996; Lee *et al.*, 1997). The reasons? α_1-PI (also called α_1-proteinase inhibitor or α_1-antitrypsin) is the major endogenous inhibitor of another class of tissue-destructive enzymes, the serine proteinases (particularly PMN leukocyte elastase), and α_1-PI is known to be inactivated, *e.g.*, during inflammatory diseases, both by reactive oxygen species (ROS) and by MMPs including collagenase (Carp and Janoff, 1979; Michaelis *et al.*, 1990; Desrochers *et al.*, 1992).

When the animal model of periodontitis described above was used, in addition to the detection of severe alveolar bone loss and elevated MMPs in the gingiva—namely, collagenase and gelatinase (presumably MMP-8, MMP-13, and MMP-9, since MMP-1 is not found in rodent tissues and MMP-2 was not found to be elevated in this experiment)—elastase activity was also seen to be elevated in extracts of gingiva from these *P. gingivalis* (*P.g.*)-infected rats (Golub *et al.*, 1994a). This gingival elastase activity was characterized as a serine proteinase, not a metalloproteinase, based on its response to a series of proteinase inhibitors such as PMSF, EDTA, 1,10-phenanthroline, and CMT-1, and when the *P.g.*-infected rats were treated *in vivo* by daily oral gavage with CMT-1, the excessive elastase activity (like that of the MMPs) was reduced to essentially normal levels. Since gingival extracts from the untreated *P.g.*-infected rats were found to convert the 52-kDa α_1-PI to the inactive 48-kDa degradation fragment, which was *not* seen when these rats were treated with either doxycycline or CMT-1, it appeared that these TCs indirectly reduced elastase activity by protecting its host-derived (or endogenous) inhibitor, α_1-PI, from MMP or ROS attack.

This hypothesis is supported by two recent studies on humans with adult periodontitis (AP): Using Western blot analysis, Lee *et al.* (1997) found that (i) the intact 52-kDa α_1-PI, seen in the GCF of the healthy gingival crevice, was largely recovered as 48-kDa degradation fragments in the periodontal lesions (pockets) of the AP patients, and (ii) based on *in vitro* and *in vivo* studies, the primary source of this α_1-PI-degrading (serpinolytic) activity in the inflammatory exudate (gingival crevicular fluid, or GCF) of periodontal pockets was MMP-8 or PMN-type collagenase. In a longitudinal placebo-controlled double-blind study, Crout *et al.* (1996) administered a six-month regimen of low-dose doxycycline to AP patients and found that both collagenase and α_1-PI-degrading activity in the GCF (as well as severity of disease) were reduced by this therapy; of interest, the concentration of doxycycline *in vitro* required to inhibit 50% of the collagenase activity and the serpinolytic activity, with [^{3}H-] collagen and [^{3}H] α_1-PI as substrates, respectively, was about 30 μM for *both* (Lee *et al.*, 1997)—an IC_{50} essentially the same as that required for doxycycline to inhibit MMP-8 (Sorsa *et al.*, 1993; Golub *et al.*, 1995).

However, the explanation of α_1-PI protection by TCs may extend beyond the ability of these drugs to inhibit MMP-8 activity. As described above, ROS (*e.g.*, O_2^-, H_2O_2, OH), particularly HOCl, generated by inflammatory cells, can also inactivate α_1-PI, and the ability of TCs to scavenge ROS provides another mechanism of α_1-PI protection by these drugs (Lee *et al.*, 1997). In this regard, Whiteman and Halliwell (1997) attributed nitric oxide (NO)-mediated tissue injury during inflammation, in part, to its rapid reaction with O_2^- to produce peroxynitrite (a reactive nitrogen species), which is also able to inactivate α_1-PI. As discussed in the next section, TCs can downregulate inducible NO synthase and decrease NO production, which could contribute not only to suppressing intracellular mechanisms associated with tissue breakdown (Note: NO can upregulate cytokine and PGE_2 production, as well as generating peroxynitrite which can damage DNA), but could also allow TCs to protect extracellular α_1-PI from inactivation by peroxynitrite, thus, again indirectly inhibiting serine proteinases such as PMN elastase.

TC-mediated INTRACELLULAR anti-collagenolytic mechanisms

In recent years, several studies have found that tetracyclines can downregulate the expression of pro-inflammatory and autoimmune mediators, including cytokines (TNFα and IL-1β), iNO synthase, and PGE_2. Such drug effects on these cell modulators would be expected to inhibit extracellular matrix breakdown, including bone resorption, in part by reducing MMP (collagenase and gelatinase) expression (Ralston and Grabowski, 1996; Milano *et al.*, 1997).

TC effects on cytokines

The lipopolysaccharide component of membranes of Gram-negative bacteria (endotoxin) induces the production of pro-inflammatory cytokines, TNFα and IL-1β, which can mediate septic shock systemically and connective tissue breakdown, including bone resorption, locally. The local effect is a likely host-mediated pathogenic pathway for microbially induced periodontal disease. In this regard, endotoxin-induced bone resorption and the excess collagenase activity secreted by endotoxin-stimulated macrophages were both inhibited by TCs added to the culture media (Golub *et al.*, 1984, 1994b).

Recent studies have shown that TCs can protect rodents from lethal endotoxemia and significantly reduce TNFα and IL-1 levels in their serum (Shapira *et al.*, 1996; Milano *et al.*, 1997). However, the efficacy of TCs as inhibitors of cytokine secretion by endotoxin-stimulated human monocytes in cell culture (Shapira *et al.*, 1996) was not confirmed in cultured mouse macrophages (Milano *et al.*, 1997)—this controversy possibly reflecting the relative degree of differentiation of these mononuclear inflammatory cells, or the different origin of these cells (humans *vs.* mice) in these two studies.

Since the inhibition of cytokine secretion by TCs appears to reflect a post-transcriptional effect (cytokine mRNA was not reduced by these drugs), the previously recognized ability of these drugs to inhibit MMP activity may play a role for the

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following reason: MMPs have been shown to convert membrane-bound pro-TNFα into its biologically active soluble form, a secretory process that can be inhibited by anti-collagenase agents such as hydroxamic acid derivatives (Mohler *et al.*, 1994). Clearly, the ability of TCs to inhibit cytokine production, by whatever pathway, provides another mechanism for these drugs to inhibit MMP activity in the extracellular matrix, thus protecting the latter from pathologic destruction.

TC effects on nitric oxide (NO) and arachidonic acid (AA) metabolism

One example of the complex interaction of these various cell regulators in tissue destruction was described by Ralston and Grabowski (1996), who stated that cytokines likely stimulate osteoclast-mediated bone resorption by altering "the balance between levels of NO and PGE_2". Nitric oxide (NO) is a short-lived messenger molecule with numerous functions, including (but not limited to) smooth muscle relaxation, neurotransmission, and tumor cell killing, as well as participation in the inflammatory process and tissue breakdown, and is produced from L-arginine by nitric oxide synthase, or NOS (Nathan and Xie, 1994). NOS_1 and NOS_3 are constitutive isoforms of the enzyme found in neuronal and endothelial cells, respectively, while the inducible form (NOS_2 or iNOS) is expressed by various cells such as macrophages and fibroblasts (Trachtman *et al.*, 1996).

Recently, a flurry of studies examined the effects of TCs on this multifunctional cell modulator. Amin *et al.* (1996) found that minocycline and doxycycline blocked NOS activity in IL-1β-stimulated human osteoarthritic cartilage explants and inhibited endotoxin-stimulated iNOS in murine macrophages in culture. These effects were *not* due to a direct inhibition by these TCs of NOS activity *in vitro*, but were found to reflect suppressed expression and translation of these enzymes. In subsequent studies by two separate teams, a series of CMTs exhibited the following efficacy as NOS inhibitors: CMT-3 and CMT-8 (IC_{50} ~ 10 μM and ~ 25 μM, respectively) > CMT-1 and CMT-2 > CMT-5 (the latter exhibiting no inhibitory activity) (Trachtman *et al.*, 1996; Amin *et al.*, 1997). These data suggested that the relative potency of these compounds as inhibitors of NO production was positively correlated to their ability to function as MMP blockers. Regarding mechanisms, the suppression of NO synthesis by CMTs was found to be associated with reductions in iNOS expression (Trachtman *et al.*, 1996), apparently reflecting enhanced degradation of this enzyme's mRNA (Amin *et al.*, 1997). Since NO is known to inhibit the synthesis of matrix constituents such as collagen and proteoglycans, as well as upregulate MMP expression (see Amin *et al.* [1996] for a review), the response of NOS to TCs provides an additional host-modulating non-antimicrobial therapeutic rationale for this family of drugs (see "Pro-anabolic effects of TCs", below).

Concerning arachidonic acid (AA) metabolism, the TCs have been found to have an impact at several steps in this pathway. Phospholipase A_2 (PLA_2) is a pro-inflammatory enzyme that participates early in the arachidonic acid cascade (it hydrolyzes glycerophospholipids in cell membranes → AA), and its levels in tissue fluids are correlated to connective tissue destructive activity during diseases such as rheumatoid arthritis. Pruzanski *et al.* (1992) measured the activity of PLA_2 *in vitro*, using ^{14}C-labeled membrane phospholipid as substrate, and found that both minocycline and doxycycline were inhibitory, exhibiting an IC_{50} of 18 μg/mL and 47 μg/mL, respectively. Recombinant PLA_2 showed lower IC_{50}s (*i.e.*, ~ 3-4 μg/mL) for both TCs. In contrast to their actions on MMPs, these TCs inhibited PLA_2 activity by a mechanism which did not involve interaction with metal ions, *i.e.*, calcium.

Earlier, ElAttar *et al.* (1988) reported that relatively high concentrations (~ 50 μg/mL) of minocycline were required to inhibit prostaglandin E_2 (PGE_2) synthesis by fibroblasts in culture. An intriguing mechanism is suggested by the ability of TCs to dampen reactive nitrogen as well as reactive oxygen species (Landino *et al.*, 1996): By inhibiting iNOS (and nitric oxide production) and scavenging ROS, TCs could suppress peroxynitrite levels (see previous section) which, in turn, could suppress cyclo-oxygenase activity and prostaglandin biosynthesis. PGE_2 and other arachidonic acid metabolites have long been recognized as mediators of connective tissue breakdown, including bone and cartilage destruction, in part by inducing MMP expression and activity (Golub *et al.*, 1984; Zhang *et al.*, 1997).

TC effects on other cell regulatory pathways—protein kinase C and calmodulin

Webster *et al.* (1994) demonstrated that TCs can inhibit protein kinase C (PKC). PKC mediates the transcriptional activation of several MMPs, such as stromelysin and collagenase. One possibility involves the activating protein factor-1 complex (AP-1); the latter binds to the MMP promoter, thus stimulating transcription of these enzyme proteins. However, Jonat *et al.* (1996) proposed that the inhibition of MMP transcription by TC was not due to a block of AP-1 activity, since this effect of the drug occurred upstream of the AP-1 binding site. To date, it is not clear which signaling pathway for MMP expression is down-regulated by TCs.

Calmodulin is an intracellular calcium-binding protein which activates phosphodiesterase, enabling this enzyme to hydrolyze cAMP which, in turn, could affect MMP expression in at least some cells, *e.g.*, monocytes (Zhang *et al.*, 1997) and cartilage cells (Richard *et al.*, 1991). As one example, the cAMP second messenger system can modulate cytokine (IL-1)-induced PGE_2 production as a mechanism for enhanced tissue breakdown, and the inhibition of this Ca^{++}/calmodulin pathway may dampen this tissue-destructive cascade (Tatakis *et al.*, 1991). In this regard, Richard *et al.* (1991) reported that calmodulin antagonists, such as sulfonamide derivatives, downregulated interstitial collagenase at least in human osteoarthritic cartilage cells. Thus, the reported ability of TCs to bind to Ca^{++}/calmodulin, thus preventing its activation of phosphodiesterase (Schlondorff and Satriano, 1985), could provide yet another cell-regulatory (*i.e.*, cAMP-dependent) mechanism for inhibiting MMP-mediated connective tissue breakdown.

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TC-mediated pro-anabolic effects on connective tissues including bone

About a decade after the discovery of the MMP-inhibitory properties of TCs, it became evident that these compounds could also prevent extracellular matrix (ECM) degradation by additional mechanisms involving enhanced collagen production and increased bone formation, at least when these processes were suppressed during disease. In this regard, Schneir *et al.* (1990) reported that minocycline therapy increased the conversion of injected [^{3}H] proline into [^{3}H] hydroxyproline (the latter, an amino acid "marker" of collagen) in insulin-deficient diabetic rats, indicating that the suppressed rate of collagen synthesis in their skin (and, presumably, in other ECMs, *e.g.*, gingiva and bone) was returned to normal by this TC without affecting the severity of hyperglycemia (Golub *et al.*, 1990b). Since CMT-1 was subsequently found to have the same pro-anabolic effect on the synthesis of [^{3}H] hydroxyproline-labeled collagen, and increased the steady-state level of mRNA of type I procollagen, both effects observed *in vivo* (Craig *et al.*, 1998), the TCs clearly appear to enhance the expression of this major ECM constituent by a non-antimicrobial mechanism.

Additional studies, utilizing the techniques of ultracytochemistry, autoradiography, and dynamic histomorphometry, on the osteoporotic bones of both diabetic and ovariectomized rats, demonstrated similar increases in collagen production and bone formation by osteoblasts during TC and CMT therapy (Golub *et al.*, 1990b; Sasaki *et al.*, 1992; Williams *et al.*, 1996; Bain *et al.*, 1997). In the estrogen-deficient osteoporotic rat, the increased production of new bone appeared to "bridge the gaps" between, and increase "connectivity" of, the resorbed, discontinuous trabeculae in the metaphysis of long bones (Ohyori *et al.*, 1997). Moreover, this TC/CMT-induced newly synthesized bone [CMT-8 is the most potent of the TC compounds in preventing bone loss (Ohyori *et al.*, 1997; Ramamurthy *et al.*, 1997)] appears to increase the biomechanical strength of bone based on controlled fracture-type studies on the adjuvant arthritic rat model (Zernicke *et al.*, 1997). Although the studies just described address the production of type I collagen [and, perhaps, other ECM constituents, such as type II collagen and proteoglycans as well (Cole *et al.*, 1994)], Karimbux *et al.* (1997) reported that the expression of a minor but structurally important collagen constituent in ligaments, type XII collagen, was also upregulated during *in vivo* CMT-1 therapy. This collagen was lost in the periodontal ligament of rats with microbially induced experimental periodontitis but was regenerated when these animals were administered CMT-1.

Most experiments targeting the effect of TCs on the synthesis of ECM constituents have been carried out on animal models of diseases characterized by excessive collagenolysis and/or depressed collagen synthesis. However, one intriguing preliminary study suggested that TCs, administered over a prolonged time, could also increase bone formation in healthy animals (monkeys), which was assessed histomorphometrically in their mandibles (Polson *et al.*, 1986). One therapeutic implication raised by this observation is that bone density might be increased prophylactically in women who are deemed at risk for future development of osteoporosis.

The mechanisms by which TCs and CMTs exert this pro-anabolic effect on osteoblast activity and on collagen and bone formation are not yet known. However, several possibilities deserve investigation, including the following:

(1) Schneir *et al.* (1990) suggested that TCs could increase the rate of collagen production in connective tissues during experimental (and human) diabetes by increasing the production and/or biologic activity of insulin-like growth factor (IGF-1). In this regard, this proanabolic activity of TCs might reflect, at least in part, the earlier-recognized anti-catabolic (*i.e.*, MMP-inhibitory) properties of these drugs for the following reason: The increased proteinase activity in skin (and other tissues) of diabetic rats was recently associated with an increase in low-molecular-weight IGF-binding proteins—the latter suppress the biologic activity of this growth factor (Cechowska-Pasko *et al.*, 1996). Thus, the previously discussed ability of TCs/CMTs to inhibit proteinase activity in the diabetic rat could reduce the level of these low-molecular-weight "inactivating" binding proteins, thus "normalizing" IGF-1 activity.

(2) As described earlier, advanced glycosylation end-products (AGEs) are produced in elevated amounts during aging, osteoarthritis, and particularly during poorly controlled diabetes (see Ryan *et al.*, 1996b, for review). Recent studies indicate that AGEs can suppress osteoblast activity and bone formation (Katayama *et al.*, 1996), which could mediate osteoporosis systemically and unusually aggressive periodontal bone loss locally, as complications of longterm diabetes (Ryan *et al.*, 1996b; Bain *et al.*, 1997). In this regard, Ryan *et al.* (1995) observed that several CMTs can inhibit non-enzymatic glycosylation of proteins such as serum albumin and collagen, an effect in the diabetic rat which could prevent the inhibitory effect of AGEs on bone formation.

(3) As described above, pro-inflammatory cell regulators such as arachidonic acid metabolites (PGE_2), certain cytokines (*e.g.*, IL-1, TNFα), and nitric oxide (NO) are all believed to contribute to connective tissue breakdown not only by enhancing proteolytic cascades but also by suppressing the synthesis of ECM constituents. Thus, this last-described example by which TCs/CMTs could generate pro-anabolic effects is for these drugs to inhibit iNOS expression and activity, thus suppressing NO production (Amin *et al.*, 1996; Trachtman *et al.*, 1996), which would prevent NO inhibition of collagen and proteoglycan synthesis (Taskiran *et al.*, 1994).

THERAPEUTIC POTENTIAL OF THE NON-ANTIMICROBIAL PROPERTIES OF TCs AND CMTs

Several earlier and recent review articles have addressed the numerous diseases involving connective tissue abnormalities that show evidence, either from the use of experimental

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animal models or from clinical trials, of responding favorably to the newly discovered properties of TCs and CMTs described in the previous sections. Several of these disease conditions were discussed by other speakers at this symposium, and the reader is referred to these papers and to earlier reviews, particularly those by Ryan *et al.* (1996a,b) and Golub *et al.* (1991, 1992, 1994a,b). A partial listing of such TC-responsive diseases includes: various arthritides, including rheumatoid, osteo-, and reactive arthritis; several types of osteoporosis, including those associated with diabetes mellitus and estrogen deficiency; additional complications of diabetes, including impaired wound healing and nephropathy; sterile corneal ulcers; aortic aneurysms; bullous and ulcerative skin lesions; and cancer, including the inhibition by TCs and CMTs of invasion, metastasis, and angiogenesis.

However, because the initial discovery of the anti-collagenolytic property of TCs, independent of the antimicrobial activity of these drugs, was made with the use of animal models of periodontal disease, and because human clinical trials for this therapeutic target are at an advanced stage (not to mention the background of the first author), the therapeutic potential of non-antimicrobial formulations of the drugs in the treatment of this dental disease will now be addressed to conclude this paper.

Low-dose doxycycline (LDD) formulations: effect on biologic and clinical "markers" of ECM degradation in adult periodontitis (AP) patients

In their early studies, Golub *et al.* reported that regular doses of antimicrobial TCs, including minocycline, doxycycline, and TC itself, could inhibit collagenase activity in the GCF of AP patients during short-term (several weeks' duration) longitudinal studies (Golub *et al.*, 1983, 1991, 1994b). These observations were confirmed in humans with different types of arthritis (rheumatoid, osteo-, and reactive arthritis) who were administered regular doses of these TCs (Greenwald *et al.*, 1987; Golub *et al.*, 1991; Brandt, 1995b; Lauhio *et al.*, 1995; Ryan *et al.*, 1996b). More recently, these investigators focused on the therapeutic potential of specially formulated LDD capsules as an MMP inhibitor in humans. These LDD formulations (prepared by a collaborating pharmacist), each containing 20 mg doxycycline *per* capsule (in some experiments, 30 mg/cap), rather than the commercially available 50 mg or 100 mg/cap, were administered daily to AP patients in experiments which ranged from two weeks' to six months' duration; doxycycline was selected over other TCs for these experiments because it was found to be a more potent collagenase inhibitor than minocycline and TC itself (Maehara *et al.*, 1988; Burns *et al.*, 1989; Golub *et al.*, 1990a, 1994b).

In brief, these investigators found the following:

(1) The LDD regimen significantly reduced the collagenase activity not only in the GCF of these patients but also in extracts of their gingival tissue removed during periodontal surgery (Golub *et al.*, 1990a); placebo caps containing inactive filler had no effect on gingival collagenase activity in a separate group of AP subjects. Studies by others (Bouwsma *et al.*, 1992a,b) confirmed these results by demonstrating that a three-month regimen of LDD (in a double-blind, placebo-controlled study) significantly reduced GCF collagenase activity and, as an adjunct to supra- and subgingival calculus removal, reduced the severity of periodontal disease assessed by traditional clinical parameters, *e.g.*, periodontal attachment loss.

(2) The LDD regimen produced a dramatically lower blood level of this TC than regular-dose doxycycline (RDD) caps (McNamara *et al.*, 1990); the peak serum DOXY concentration, 4 hrs after the last LDD or RDD cap was taken, was found to be 0.29 ± 0.01 and 3.2 ± 0.2 μg/mL, respectively, based on a bioassay.

(3) Presumably because of the sub-antibiotic blood levels just described, LDD did *not* appear to result in the side-effects of RDD (see below), which include gastro-intestinal disturbance and the emergence of TC-resistant micro-organisms (Kornman and Karl, 1982; Eady *et al.*, 1990; Fiehn and Westergaard, 1990). In two early double-blind studies, one of two weeks' and the other of three months' duration, AP patients were administered LDD or placebo caps twice daily, and when selected periopathogens (*Fusobacterium nucleatum, Bacteroides* spp., *Actinomyces* spp.) were isolated from their subgingival plaque, they were found not to have developed either TC or pan-antibiotic resistance or any other side-effects (Schroeder *et al.*, 1990, 1992). These initial experiments have recently led to double-blind, placebo-controlled clinical trials which demonstrated that the daily use of LDD, over a one-year time period, showed efficacy as a therapeutic adjunct without any evidence of microbiological or other side-effects (Thomas *et al.*, 1995; Caton *et al.*, 1997). A one-year multi-center double-blind placebo-controlled study, on over 430 AP patients, was discussed in detail at this symposium by Ciancio and Ashley (this volume).

Two other recent studies on humans with AP indicated that LDD therapy improves both the biochemical and clinical "markers" of periodontal breakdown (Crout *et al.*, 1996; Lee *et al.*, 1997). In the first study, the effects of periodontal disease severity on the concentration and molecular form of α_1-PI (a "serpin" or serine-proteinase inhibitor) in the GCF of gingival crevices/periodontal pockets, and the *in vitro* effect of doxycycline on "serpinolytic" (or α_1-PI-degrading) activity in the GCF, were examined. The other study addressed the change in α_1-PI degradation (mediated by collagenase? see below) and the clinical response of the periodontal tissues resulting from a six-month "cyclical" regimen of LDD. In the first study (Lee *et al.*, 1997), GCF was collected from 51 human subjects, with either healthy gingiva, gingivitis, or adult periodontitis, on filter paper strips inserted into selected crevices and pockets. The strips were then extracted, and the α_1-PI in the GCF was quantified from a standard curve constructed from dot-blot analysis, and characterized by Western blot, with a rabbit anti-serum against human α_1-PI. Although the GCF α_1-PI concentration was increased as the severity of periodontal disease increased, the proportion of the α_1-PI, in the GCF, that was

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converted from the intact 52-kDa protein into the 48-kDa degradation fragment also increased. Two lines of evidence in this study indicated that mammalian collagenase (likely MMP-8) in the GCF was responsible for partially degrading and inactivating α_1-PI: (i) Both collagenase activity and "serpinolytic" (or α_1-PI-degrading) activity in the GCF from these patients increased in a parallel fashion with increasing severity of inflammation, and (ii) the "serpinolytic" activity in the GCF was characterized as a mammalian collagenase based on its *in vitro* response (or lack of response) to a panel of different proteinase inhibitors (*e.g.*, EDTA, 1,10-phenanthroline, PMSF, eriochrome black T); in this regard, doxycycline was also found to inhibit the α_1-PI-degrading activity of the GCF *in vitro*, and the concentration of this TC required to inhibit this serpinolytic activity was the same ($IC_{50} \sim 30$ μM) as that required to inhibit leukocyte-type collagenase (MMP-8), which is the dominant type of collagenase in the GCF of AP patients (Sorsa *et al.*, 1994; Golub *et al.*, 1997).

In the second, this time a longitudinal, study (Crout *et al.*, 1996), one group of AP human subjects was administered a "cyclical" regimen of LDD (Golub *et al.*, 1994b), *i.e.*, 2 months on the drug, the next 2 months no drug, and the final 2 months back on the drug. The control group was administered placebo capsules containing inactive filler according to the same schedule. Using the collagenase and α_1-PI-degrading ("serpinolytic") assays for GCF already described, plus various clinical measurements including periodontal attachment levels (PAL) with the computer-assisted, constant-pressure Florida disc probe, Crout *et al.* (1996) found: (i) that the LDD-treated group showed a 26-39% reduction in pocket depth at the two-, four-, and six-month time periods ($p < 0.01$), whereas the control group showed no change; (ii) that the control group tended to lose attachment, whereas the LDD group gained attachment, so that at the six-month time period the difference between the two groups was ~ 0.7 mm ($p < 0.05$); (iii) that no significant differences were seen between the two groups for clinical measures of severity of gingival inflammation and plaque formation at any time period; and (iv) that the LDD group showed the same 40-50% reduction in GCF collagenase activity and GCF "serpinolytic" (α_1-PI-degrading) activity during the six-month protocol, whereas the control group showed no change.

These studies strongly suggested that prolonged LDD therapy can protect the periodontal tissues in the AP patient from proteolytic attack by both direct and indirect mechanisms, which are summarized as follows: The inhibition of MMPs (collagenase and gelatinase) by non-antimicrobial TC formulations (LDD, CMTs) not only directly protects collagen fibers from degradation but also protects α_1-PI from inactivation by these proteinases as well. Since α_1-PI is a major "host-defense protein"—it is the major endogenous inhibitor of serine proteinases, particularly PMNL elastase—this would also enable TCs to indirectly inhibit the degradation of other ECM constituents—namely, the elastase-susceptible elastic fibers, proteoglycans and fibronectin, basement membranes, etc. Moreover, since elastase is also known partially to degrade and inactivate another "host-defense protein", α_2-macroglobulin (which inhibits all MMPs and other proteinases), this would allow TCs indirectly to inhibit still other MMPs, such as stromelysin (MMP-3), which may not be directly inhibited by these drugs.

THE EFFECT OF LDD THERAPY ON "MARKERS" OF BONE METABOLISM IN AP PATIENTS

To conclude this paper, we now address the topic of the effect of LDD therapy on "markers" of bone metabolism in AP patients, since bone loss is the signature event in the pathogenesis of periodontal disease. In this regard, a recent advance in the diagnosis of metabolic bone diseases (*e.g.*, different types of osteoporosis, Paget's disease) has been the development of techniques to assess "markers" of bone resorption in the serum and urine of patients [see Greenwald (1996) and Giannobile (1997) for reviews] based on the detection of pyridinoline-containing crosslink fragments of type I (and other types of) collagen. In collaboration with W. Giannobile at the Harvard School of Dental Medicine, Golub *et al.* (1997) measured these bone-type collagen degradation fragments (ICTP) in the GCF collected from inflamed periodontal pockets; the antibodies used in this radioimmunoassay assay were directed against a 12-20-kDa C-telopeptide pyridinoline-containing fragment of type I collagen released by proteolytic digestion of human bone matrix. This assay has been found to reflect elevated bone resorption during periodontal disease (Giannobile *et al.*, 1995) and during various metabolic bone diseases (Risteli *et al.*, 1993) when measured in the GCF and serum, respectively.

In a recently published study (Golub *et al.*, 1997) where this technology was applied, 18 human subjects with AP appeared to exhibit an abnormally elevated ratio of bone resorption (assessed by the ICTP "marker") relative to bone formation (the latter assessed by RIA measurement of osteocalcin in the GCF). However, during a two-month regimen of LDD, the bone resorption "marker" in the GCF (ICTP) was reduced by 50-60%, while no significant effect was seen on bone formation (GCF osteocalcin), indicating that this therapy restored a favorable balance in bone remodeling in these AP patients. The control subjects, who did not receive LDD during the two-month protocol, did not show any improvement in either GCF ICTP or osteocalcin. In the same study, collagenase activity, assessed functionally with [^{3}H-methyl] collagen as the substrate, was also significantly reduced in the GCF of the LDD-treated patients. At the one-month time period, both MMP-8 (which made up ~ 96% of the total collagenase protein in the GCF of these AP patients, based on Western blot analysis) and MMP-13 (also assessed by Western blot, and identified for the first time in the GCF) were reduced equally, by ~ 50%. However, at the two-month time period, the MMP-13, but not the MMP-8, was reduced by an additional 40%, so that this "bone-type" collagenase (also called collagenase-3) now

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constituted only ~ 1-2% of the total collagenase in the GCF, rather than the 3-4% seen at the beginning of the study. Thus, prolonged treatment with LDD appeared preferentially to suppress "bone-type" (MMP-13) rather than "leukocyte-type" (MMP-8) collagenase in the periodontal pocket, which may explain the sharp reduction in "bone-type" collagen breakdown fragments in the GCF of periodontal pockets, and the evidence of reduced alveolar bone loss diagnosed by subtraction radiography in AP patients on long-term (one-year) LDD therapy (Caton *et al.*, 1997).

Recent preliminary data, now discussed, confirmed and extended these observations. GCF samples were collected on filter paper strips, then extracted as described by Golub *et al.* (1997), from selected periodontal pockets in four additional human subjects with AP who were treated with a four-month regimen of LDD. Prior to this collection procedure, mouthrinse samples [which collects full-mouth GCF; Gangbar *et al.* (1990)] were obtained; the severity of periodontal disease, assessed by clinical parameters (GI, PlI, PAL, GCF flow), was determined after the GCF was collected on filter strips and at the same pocket sites. In brief, GCF ICTP levels were significantly ($p < 0.02$) reduced by 61% at the two-month duration of LDD therapy (consistent with the earlier study; see above), but the level of this bone resorption "marker" at the four-month time period appeared to be reduced to a lesser extent ($p > 0.05$), possibly reflecting reduced compliance of the patient with this daily treatment regimen at this longer time period. Of interest, the pro-inflammatory cytokine, IL-1β, which has been associated with bone resorption in humans systemically and locally in GCF (Uematsu *et al.*, 1996), was also reduced by this LDD therapy. The levels of this cytokine (expressed as pg/10-second collection $\pm$ SEM) at the two pre-treatment appointments (the second appointment was 1 mo after a 30-minute scaling and prophylaxis for the removal of dental plaque and calculus), then at 2 mos and 4 mos of LDD therapy, were 75.9 ± 11.1, 68.9 ± 14.0, 29.6 ± 8.3, and 37.8 ± 9.8, respectively. Thus, at two and four months' duration of LDD therapy, the levels of GCF IL-1β were reduced by 61% and 50%, respectively, whereas dental scaling and prophylaxis, in this study, were ineffective.

The data from this preliminary study on the mouthrinse samples, which represent pooled GCF for the entire mouth, provided additional interesting insights into the mechanisms of TC inhibition of MMP activity. As shown in the zymograms in Fig. 2, most of the gelatinase activity in the mouthrinse samples of the AP patients was characterized as MMP-9, *i.e.*, 92 kDa, which is consistent with the observations in GCF described previously (Golub *et al.*, 1995). Not surprisingly, the whole-mouth GCF gelatinase activity, assessed functionally with [^{3}H-methyl] gelatin used as the substrate, was reduced at both the two- and four-month time periods of LDD therapy by about 50% ($p < 0.05$). However, based on an examination of the different molecular species of gelatinase assessed by zymography, although the 92-kDa pro-MMP-9 bands were seen to decrease slightly in intensity at the two- and four-month LDD treatment time periods, additional small-molecular-weight bands, ~30-35 kDa, appeared at these times. Western blot analysis with monoclonal antibodies to MMP-9 confirmed this loss of 92-kDa gelatinase and the appearance of small-molecular-weight MMP-9 fragments (< 50 kDa) during this prolonged LDD therapy (data not shown). Interestingly, this "autodegradation" of Pro-MMP-9 was *not* seen for Pro-MMP-8. These *in vivo* data on humans support the hypothesis proposed by Brandt and his colleagues based on *in vitro* studies (Brandt, 1995; Smith *et al.*, 1996) and are summarized as follows: Doxycycline (or other TC analogs) reacts with pro-MMP-9, possibly binding to Ca^{++} in the structure of this enzyme protein, which is needed for conformational stability. Then, during extracellular activation of this zymogen, possibly mediated by bacterial proteinases in the saliva or GCF (Sorsa *et al.*, 1992), this partially denatured Pro-MMP is excessively degraded to small-molecular-weight enzymatically inactive (or less active) fragments, contributing to the suppressed MMP activity in the periodontal pockets of these patients.

CONCLUSIONS

Since the first demonstration in germ-free rats that these drugs can inhibit MMPs and other tissue-destructive pathways, dramatic progress has been made toward realizing the enormous therapeutic potential of the non-antimicobial, matrix-sparing properties of TCs and their chemically modified analogs. The mechanisms identified to date are pleiotropic and complex, involving the previously unrecognized ability of these antibiotics to modulate intra- and extracellular anabolic and catabolic processes in connective tissues. Sites on the TC molecule, responsible for some of these beneficial metabolic effects, are now known, and, based on this new knowledge, new TC analogs and formulations, devoid of antimicrobial activity (thus avoiding antibiotic side-effects), are being developed. Human clinical trials have already demonstrated their safety and efficacy for periodontal disease; studies on patients with various types of cancer have just begun. The expectation is that additional trials involving chronic degenerative diseases such as osteoporosis, arthritis, and respiratory and cardiovascular diseases will soon follow.

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