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Minocycline reduces gingival collagenolytic activity during diabetes

Preliminary observations and a proposed new mechanism of action

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Diabetes increases gingival collagenase activity, an effect that may be mediated by endogenous tissue changes and exacerbated by an overgrowth of Gram-negative organisms in the gingival crevice (see Ramamurthy & Golub 1983, McNamara et al. 1982). In an attempt to reverse this collagenolytic abnormality, we administered an appropriate antibiotic, minocycline (a semi-synthetic tetracycline), to diabetic rats and humans. Adult male conventional or germfree rats were made diabetic with streptozotocin, and half of these animals were administered minocycline (20 mg per day) by tube feeding for 3–4 weeks prior to sacrifice. The buccal gingiva, entire skins, and mandibles were dissected and tested for collagenolytic enzyme activity, collagen content, and alveolar bone loss, respectively. In a preliminary study, minocycline (200 mg per day) was administered for 7 days to an insulin-dependent diabetic adolescent human and an adult non-diabetic human; the twin brother of the diabetic was treated with penicillin. Gingival fluid collagenase activity was measured (using [^{3}H-methyl] collagen as substrate in a new microassay) in 8 periodontal pockets in each subject before and after antibiotic therapy. Examination of collagenase digestion products by SDS-polyacrylamide gel electrophoresis and fluorography was also carried out. In rats, minocycline treatment: (1) suppressed the abnormally elevated collagenolytic enzyme activity in gingiva of diabetic rats, *even under germfree conditions;* (2) inhibited PMN leukocyte collagenase activity *in vitro*, an effect that was reversed by the addition of calcium ions (penicillin-streptomycin had no effect on the activity of this enzyme); and (3) retarded the abnormal loss of skin collagen and alveolar bone in diabetic rats. In a preliminary study on humans, minocycline therapy reduced the collagenase activity of gingival crevicular fluid, an effect not produced by penicillin.

Our data suggests that (1) tetracycline therapy inhibits tissue collagenolytic enzyme activity by a mechanism at least in part unrelated to its antibacterial efficacy, and (2) this mechanism may provide a new therapeutic approach for suppressing excessive collagen resorption which occurs during periodontal disease and which can occur during other pathologic conditions.

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Introduction

We have found that diabetes in rats and humans increases tissue collagenase activity. Evidence for this effect was seen in extracts of gingiva and skin (Ramamurthy & Golub 1983), in cultures of gingival tissue (Ramamurthy et al. 1974, Golub et al.

1978a, Kaplan et al. 1982), and in gingival crevicular fluid (McNamara et al. 1979, Kaplan et al. 1982). Unusually severe periodontal disease, which occurs during diabetes in man (Finestone & Boorujy 1967, Cianciola et al. 1982) and experimental animals (Bissada, Shaffer & Lazarow 1966), reflects accelerated collagen breakdown which could be mediated by the excessive collagenase generated during this systemic disease (see Ramamurthy & Golub 1983). We have suggested that the overgrowth of Gram-negative organisms in the gingival crevice of the diabetic rat could, by generating excessive endotoxin in the area, be the cause of the abnormally high collagenase levels in the gingiva (McNamara et al. 1982). To test this hypothesis, we determined (a) whether an antibiotic effective against oral Gram-negative organisms (minocycline; see Genco 1981) reduces gingival collagenase activity during diabetes; and (b) whether diabetes stimulates gingival collagenase activity under germfree conditions. During the course of these investigations, minocycline (a semi-synthetic tetracycline) administered to rats was found to inhibit collagenase activity, at least in part, by a mechanism(s) unrelated to its antimicrobial efficacy. This preliminary report describes our initial studies using minocycline in diabetic rats and humans.

Materials and Methods

Animal experiments

Preparation of animals. In the experiment using conventional animals, five-month-old (mean weight, 445 g) male Sprague Dawley rats were made diabetic by I.V. injection of streptozotocin (70 mg per kg body weight), after a 12 h fasting period, as described by us previously (Golub et al. 1978b, Golub et al. 1982). Age matched uninjected rats served as controls. Some of the diabetic rats were administered the tetracycline, minocycline (20 mg Minocin® per day; Lederle Laboratories, Pearl River, N.Y.), by gavage on a daily basis beginning 3 days after diabetes was induced. After a 25 day experimental period, a blood sample was taken for glucose analysis, and subgingival plaque samples were taken for assessment of the microflora in the region (McNamara et al. 1982). The animals were then sacrificed, gingival samples were taken for tissue culture, and the jaw bones dissected (see below).

In a second experiment, germfree rats (purchased from Taconic Farms, Germantown, N.Y.) were maintained in inflatable vinyl isolators (Standard Safety Equipment Co., Palatine, Illinois) until a weight of 360g ± 15 was reached. At this time (6 weeks after purchase), diabetes was induced in some of the animals, as described above, and the control and diabetic animals maintained under germfree conditions for an additional 6 weeks prior to sacrifice. Half of the diabetic animals were administered Minocin® (20 mg/day) by gavage for the last 4 weeks of the protocol. Blood and tissues were then obtained to assess gingival collagenolytic enzyme activity and the loss of skin collagen and bone.

The maintenance of germfree conditions was tested by swabbing various surfaces of the isolators (and its contents) and the rat's oral cavity and fecal material with sterile cotton-tipped applicators. These were immediately placed into Brain Heart infusion broth (Difco Laboratories, Detroit, Michigan) to detect the presence of aerobic organisms; separate samples were placed into pre-reduced PYG broth (Anaerobe Laboratory, Virginia Polytechnical Institute) to detect anaerobes. All samples were incubated at 37 °C for 7 days and examined daily for evidence (turbidity) of growth. The isolator was tested for sterility twice before the rats were introduced. Thereafter, the equipment and animals were monitored mi-

crobiologically once per week, including the day of sacrifice. At this time, the aerobic and anaerobic samples were Gram-stained and examined microscopically for the presence of microorganisms.

Assaying collagenolytic enzyme activity. The techniques used to measure the collagenolytic enzyme activity of gingival tissue in culture, and in polymorphonuclear, leukocytes (PMNLs), are those described by us previously (Golub et al. 1978a, Nicoll et al. 1981) with minor modifications. Briefly, the labial gingiva of maxillary incisors was excised, pre-incubated in Gey's media containing penicillin and streptomycin, and dissected under aseptic conditions into fragments weighing 4–6 mg wet weight. Two fragments from each rat were cultured on a gel of reconstituted ^{14}C-glycine labeled collagen fibrils (32,264 DPM per 0.5 ml gel). The acid-soluble rat skin collagen was prepared according to the method of Glimcher and Krane (1964); the animals were injected with ^{14}C-glycine as described by us previously (Golub et al. 1976). After incubation (3 days, 35°C, in a humid atmosphere of 95% air and 5% CO_2), the contents of each culture dish were centrifuged (10,000 × g, 1 h, 22°C) and aliquots of the supernatant were analyzed for ^{14}C-labeled collagen fragments in a liquid scintillation spectrometer (In this system, 96–98% of the solubilized collagen is recovered as dialyzable breakdown products, Golub et al. 1978a).

To test the effect of physiologic levels of minocycline (the concentration in human serum during a therapeutic regimen is approximately 2 μg/ml, that in the gingival crevicular fluid is 4–5 times greater; Ciancio, Mather & McMullen 1980) on collagenase activity *in vitro*, we collected peritoneal exudate PMNLs from 15 male control rats 4 h after I.P. injection of 0.15% glycogen. An extract of PMNLs (in 0.05 M Tris-0.2 M NaCl-5 mM $CaCl_2$) was prepared, 250 μl aliquots were incubated with ^{14}C-gly labeled collagen fibrils (12,720 DPM per 50 μl gel) for 14 h at 35°C (in the presence or absence of minocycline, EDTA, or added $CaCl_2$), and collagenolytic activity was measured as described by us previously (Nicoll et al. 1981).

Measuring the loss of alveolar bone and skin collagen. Alveolar bone loss was assessed in the right and left mandibles of the conventional and germfree rats (control and diabetic) using a modification of a previously described technique (Stralfors, Thilander & Bergenholtz 1967, Heijl et al. 1980). The mandibles were defleshed (autoclaving followed by soaking in 2 N NaOH for 2–3 h) and dried, and the distance (expressed as units; 1 unit = 0.05 mm) between the cemento-enamel junction (CEJ) and the crest of the alveolar bone was measured in the long axis of the root surface at 10 specified sites on lingual surfaces of the 3 molar teeth (see Crawford, Taubman & Smith 1978) using an eye-piece micrometer in a dissecting microscope (20 × magnification).

Because uncontrolled diabetes in the rat results in the massive resorption of skin collagen (Schneir & Golub 1981) associated with increased collagenase activity in this tissue (Ramamurthy & Golub 1983), we also measured the collagen content of entire skins from control and diabetic rats (both germfree and conventional), including those treated with minocycline. The techniques used were described by Schneir and Golub (1981).

Initial human study

Gingival crevicular fluid (GCF) samples were collected on filter paper strips inserted into 8 interproximal pockets of selected teeth in the maxillary arch of two 19 year-old twin brothers with juvenile-onset (insulin-dependent) diabetes mellitus, as described by us previously (Golub et al. 1976). Fluid volume was immediately determined

on a modified Periotron (model 6000 [Harco Electronics Ltd., Winnipeg, Canada]; see Hinrichs & Bandt 1983). The filter strips were stored frozen for 1 h in plastic microfuge tubes then incubated with 10 μl [^{3}H-methyl] collagen (20,675 DPM), prepared as described by Bhatnagar and Decker (1981), and 70 μl buffer (50 mM Tris-HCl, pH 7.8, containing 0.2 M NaCl and 5 mM $CaCl_2$). The GCF samples (or reagent blanks or trypsin controls) were incubated at 27°C (Birkedal-Hansen & Dano 1981) for 18 h with gentle shaking. The reaction was stopped and undigested collagen precipitated by adding 10 μl 0.1 M phenanthroline in dioxane/water (1:1, v/v), 10 μl of non-radioactive methylated carrier collagen (2 mg/ml) in 50 mM Tris-HCl buffer containing 1 M NaCl (pH 7.0), and 100 μl dioxane (Bhatnagar & Decker 1981). After mixing, the radiolabeled collagen degradation products were collected by filtration and counted in a liquid scintillation spectrometer (Golub et al. 1976). Using this technique, the reaction blank (no enzyme or GCF added) released about 10% of the total substrate counts, 2–20 ng bacterial collagenase produced a linear increase in the release of radioactive counts from the ^{3}H-collagen substrate (the 20 ng level of enzyme degraded 60% of the collagen substrate), and 50 ng trypsin released less than 1% of the counts above blank values. GCF collagenase activity was expressed as units of equivalent activity of vertebrate collagenase (obtained from New England Nuclear, Cat. No. NEK-016); 1 unit was operationally defined as the amount of enzyme that degrades 1 μg of collagen per hour at 27°C.

Four additional GCF samples were collected from other maxillary pockets of the same diabetic twin brothers on filter paper strips, their volume determined (see above), and collagenase activity assessed using the same procedure described above but with a different substrate (10 μl of [^{3}H-propionate] collagen, NET-660 [New England Nuclear Corp., Boston, MA.], 302,000 DPM). Half of the 12 GCF samples per subject were activated by pretreatment with 1.04 μM trypsin followed by the addition of a 5-fold molar excess of soybean trypsin inhibitor (Hassell 1982). Samples were selected to be treated or not treated with trypsin by matching them for Gingival Index (Löe & Silness 1967), GCF flow, and for pocket depth; the clinical parameters (GI, PD) were measured immediately after GCF collection. All measurements were carried out immediately before antibiotic coverage and after 7 days of treatment with either penicillin G (1 g per day; patient P.D.) or minocycline (200 mg per day; patient K.D.).

The same protocol using minocycline therapy was carried out on a 41-year-old non-diabetic male (R.R.), in 12 selected sites in the maxillary arch (GI = 1.7 ± 0.2; PD = 4.2 mm ± 0.3), and GCF collagenolytic activity was measured as described above. This patient exhibited deeper periodontal pockets than the 19 year-old diabetic twin brothers. Two additional GCF samples were collected from the same 4 mm pocket, in this patient, before and after minocycline therapy. These samples were incubated with 10 μl ^{3}H-collagen for 18 h (22°C) then thermally denatured, and the collagen subunits and collagenase digestion products were separated by SDS-polyacrylamide gel electrophoresis (Nicoll et al. 1981). The gels were processed for fluorography (see Sodek, Hurum & Feng 1981) and exposed to Kodak XAR-5 film for 5 days.

Results

Animal Experiments

The conventional and germfree control rats all exhibited relatively low blood glucose concentrations ranging from 85 to 186 mg% (mean = 141 mg% ± 14). As expected, the

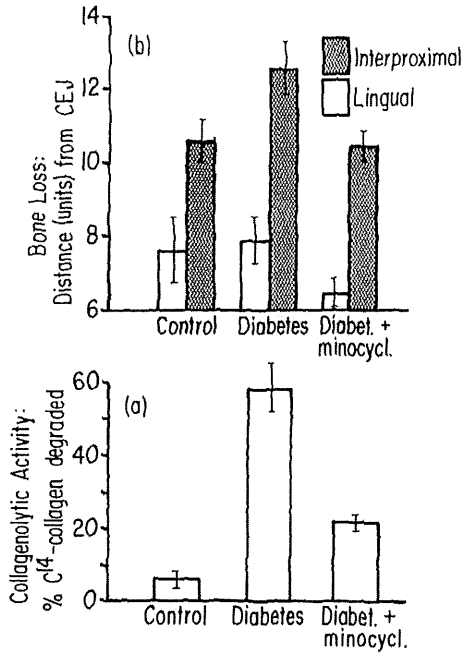

Fig. 1. The administration of minocycline to conventional diabetic rats: effect on (a) collagenolytic activity of gingiva in tissue culture, and (b) alveolar bone loss. Each value is the mean ± S.E.M. for 3–5 rats per group.

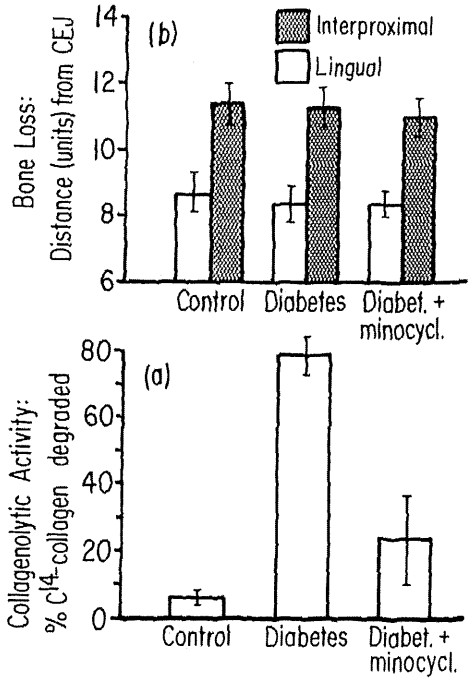

Fig. 2. The administration of minocycline to germfree diabetic rats; effect on (a) collagenolytic activity of gingiva in tissue culture, and (b) alveolar bone loss. Each value is the mean ± S.E.M. of 4–5 rats per group.

diabetic conventional and germfree rats, with or without minocycline therapy, were severely hyperglycemic; blood glucose values ranged from 310 to 832 mg%, with a mean of 503 mg% ± 42. The germfree rats and isolators showed negative results for aerobic and anaerobic cultures throughout the experimental period, and treatment of the conventional diabetic rats with minocycline eliminated detectable signs of a Gram-negative microflora in the gingival crevices.

The gingiva from the conventional and germfree control rats produced minimal collagenolytic activity in tissue culture; less than 10% of the ^{14}C-collagen fibrils were degraded after 3 days in culture (Figs. 1a and 2a). In contrast, collagenolytic activity was markedly increased in gingiva from the

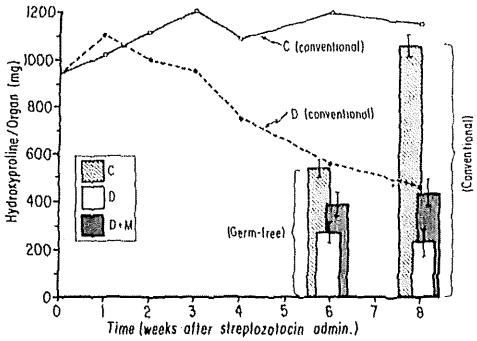

Fig. 3. The effect of streptozotocin-diabetes on skin collagen levels in germfree rats (see bar graphs at 6 weeks after streptozotocin administration) and conventional rats (see bar graphs at 8 weeks); half of the diabetic germfree and conventional rats were administered minocycline on a daily basis (see Materials and Methods). C = control rats; D = diabetic rats; D + M = minocycline treated diabetic rats. Each value is the mean ± S.E.M. of 4–5 rats per group. The line graphs for the C and D conventional rats were adapted from Schneir and Golub 1981.

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