Tetracycline and Doxycycline Inhibit Pleural Fluid Metalloproteinases

A Possible Mechanism for Chemical Pleurodesis

Adam N. Hurewitz, M.D., F.C.C.P.; Chien Liang Wu, M.D.; Raul Mancuso; and Stanley Zucker, M.D.

We hypothesized that inhibition of matrix-degrading metalloproteinases (MMPs) accounts for a portion of the pleural fibrosis and adhesions of tetracycline pleurodesis. MMPs recently have been described in pleural fluid from patients with both exudative and transudative effusions. Since tetracyclines are recognized inhibitors of other metalloproteinases, we investigated their inhibitory capacity in pleural fluid. High concentrations of several different tetracyclines reduced MMP activity of pleural fluid by more than 75 percent. Lower concentrations (<1 mg/ml) had only modest inhibitory effects. High concentration of tetracyclines also inhibited cell synthesis of MMPs, in vitro, but other measures of vital cell function were also impaired. We conclude that tetracyclines are effective inhibitors of MMP activity in pleural fluid and may also reduce synthesis of MMPs via non-specific cell injury. These data suggest a possible mechanism to account for tetracycline pleurodesis; i.e., an inhibition of MMP activity in pleural fluid.

(Chest 1993; 103:1113-17)

Intrapleural tetracycline produces an inflammatory response, the outcome of which is injury to pleural mesothelial cells, thickening of the subpleural connective tissue, and adhesions between the visceral and parietal pleural surfaces. These changes in the pleural space are of value in the treatment of patients with recurrent effusions. How tetracycline produces these changes and why the clinical efficacy varies from 40 to 90 percent, however, remains unclear. Animal studies indicate that intrapleural adhesions arise primarily at sites of mesothelial cell destruction.1 In these animal studies as well as in several poorly controlled human studies, higher concentrations of tetracycline produced proportionally greater pleurodesis.2,3 The acidity of tetracycline was initially believed to be an essential factor for successful pleurodesis though this has more recently been brought into question in animal studies by Sahn and Good.4 One mechanism of tetracycline pleurodesis that has not been explored is the inhibition of intrapleural matrix-degrading metalloproteinases (MMPs) resulting in unopposed collagen and fibrin deposition in the inflamed pleural space.

Matrix-degrading MMPs recently discovered in pleural fluid degrade primarily type IV collagen and gelatin.5 Both MMP-2 and MMP-9 are synthesized within the pleura and are present in high concentrations in both transudates and exudates.6 These enzymes possibly contribute to the integrity of the pleural space by dissolving adhesions and fibrosis following injury to the pleura. Activity of these enzymes is dependent on the presence of Zn+2 in the center of the molecule. As a result, metal chelators such as 1,10-phenanthroline and ethylene diaminetetraacetic acid (EDTA) are effective inhibitors of MMPs. Tetracycline is also a metal chelator and has been proven to effectively inhibit MMPs.6 Metalloproteinase inhibition is the basis for tetracycline therapy in several clinical conditions, including corneal ulcers, periodontitis with gingivitis, and epidermolysis bullosa.7,8 Although the types of cells within the pleural space that synthesize MMPs have not as yet been identified, several possibilities exist. Immunologically similar MMPs are produced by fibroblasts,9 macrophages,10 neutrophils,11 tumor cells,12 and synovial (mesothelial-like) cells,13 each of which is found in pleural tissue or fluid in health or in diseases associated with pleural effusions.

We investigated the capacity of tetracyclines to inhibit both the activity of MMPs in pleural fluid and the synthesis of MMPs by human fibrosarcoma cells (known to produce substantial amounts of MMPs similar to those previously found in pleural fluid). These studies were carried out using several different members of the tetracycline family. Tetracycline hydrochloride (TCN-HCl) was selected because it has been the standard agent used for tetracycline pleurodesis. Doxycycline was also selected, in part because it may be a more effective MMP inhibitor than TCN-HCl and also because TCN-HCl is no longer commer-
cially available. Finally, we selected a tetracycline that had been chemically modified to eliminate its antibacterial properties (CMT-1) to help determine if the antibiotic characteristics of these agents are necessary for their inhibition of MMPs.

**Methods**

Pleural fluids were collected by thoracentesis using standard clinical techniques. Samples were centrifuged at 1,000 g for 15 min and supernatants were stored at −70°C until ready for use; 5 mM sodium azide was added to all samples to prevent bacterial overgrowth.

**Reagents**

Trypsin-N-tosyl-L-phenylalanlychloromethyl ketone (trypsin-TOPCK), 5-alpha-p-tosyl-L-lysine chloromethylketone (TLCK), soybean trypsin inhibitor (SBTI), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldtetrazolium bromide, and EDTA were obtained (Sigma Chemical Co, St Louis, Mo). Hydrofluor was also obtained (National Diagnostics, South Somerville, NJ) as was human placental 1H-type IV collagen (NEN-Dupont, Boston, Mass), gelatin-sephrose (Pharmacia-LKB, Piscataway, NJ), dimethyl sulfoxide (Fisher Scientific, Pfairlawn, NJ), mouse monoclonal antibody to human 92 kDa type IV collagenase (Dr. U. Moll, SUNY at Stony Brook, NY), biotinylated affinity purified goat antimouse IgG antibody and alkaline phosphatase conjugated streptavidin (Bethesda Research Laboratories, Bethesda, Md), and rabbit polyclonal antibodies to 66 kDa (a-MMP-2) and 92 kDa (a-MM-P-9) type IV collagenase (Dr. W. Stetler-Stevenson, Bethesda, Md). The chemically modified tetracycline (CMT-1) we used inhibited MMP activity but had no antibacterial activity (provided by Drs. Golub and McNamara, SUNY at Stony Brook, NY). Doxycycline hydrochloride was purchased (ESI Pharmaceuticals, Cherry Hill, NJ), as was TCN-HCL buffered in ascorbic acid (Acromycin, Lederle, Pearl River, NY).

**Substrate Degradation Assay for Collagenase Activity**

Type IV collagenase activity was measured using a 1H-proline-radiolabeled type IV procollagen substrate as described previously.8 Samples consisted of unmodified pleural fluid that had been incubated for 1 h with one of the tetracyclines solubilized in Tris buffer (50 mM Tris base, 0.2 M NaCl, 5 mM CaCl₂, 0.05 percent Brj 35, 0.02 percent NaN₃). All samples, assayed in duplicate, were then incubated with TLCK (100 mg/L) to inhibit nonspecific serine and cysteine proteinase activity and then incubated with radiolabeled substrate for 18 h at 35°C.

Inhibition of bioactivity was examined using three different tetracyclines: TCN-HCL, DOXY, and CMT-1. The highest concentrations of these tetracyclines (5 to 10 mg/mL) were selected to approximate the concentrations of tetracycline recommended for clinical pleurodesis in which approximately 1,500 mg is injected into a pleural space containing less than 100 mL of residual fluid. Several lower concentrations were also studied. The normally acidic pH of these tetracycline samples was titrated to 7.6 with 1N NaOH. The high concentrations of drug selected for this study produced a mild turbidity at this pH that was minimized by first raising the pH to 10 to 12 and then lowering the pH with 1N HCL to 7.6.

Collagenase activity is reported as the micrograms of radiolabeled substrate degraded per hour of incubation per milliliter of pleural fluid sample. Inhibition of type IV collagenase in pleural fluid by 25 mM EDTA and 1 mM 1,10-phenanthroline was studied to document the metal dependency of the pleural fluid MMPs.

**Gelatin Gel Electrophoresis**

Gelatin zymography was performed using a 7 percent sodium dodecyl sulfate polyacrylamide gel that had been cast in the presence of gelatin (1.5 mg/mL) as described by Heussen and Dowdle.19 After electrophoresis, gels were washed three times in 50 mM Tris-HCl, 5 mM CaCl₂, 0.001 mM ZnCl₂, 2.5 percent octoxynol-9 (Triton-X-100) (v/v), pH 7.6 and incubated in the above buffer containing 1 mM aminophenylmercuric acetate (APMA). Zones of gelatinolytic activity were characterized by negative Coomassie brilliant blue staining. Protein standards were run concurrently and approximate molecular weights of sample proteins were determined by plotting the relative mobilities as compared with those of the protein standards.19

Two identical gels were run concurrently to study the effect of doxycycline on gelatinase activity of pleural fluid. One of the gels was incubated with the standard incubation buffer, described above. The other was incubated with buffer containing DOXY at concentrations of 0.5 and 5 mg/mL at a pH = 7.6. The sample in the first lane of both gels contained unmodified, raw pleural fluid; the second lane of these gels contained pleural fluid that had been preactivated with trypsin (1 mg/L) for 5 min followed by a fivefold greater concentration of trypsin inhibitor (5 mg/L). Gels were also run with the pleural fluid samples preincubated for 2 h with doxycycline at the same concentrations as described above.

**Culture of HT1080 Human Fibrosarcoma Cell**

HT1080 cells were grown to confluence in conditioned medium (RPMI 1640) containing 10 percent fetal calf serum (FCS). Each 35-mm plate contained 4×10⁴ cells. After washing with phosphate-buffered solution (PBS), HT1080 cells were incubated over the next 2 h at 37°C in serum-free medium (SFM) to which various concentrations of doxycycline (5, 1, 0.2, 0.04, 0.008 mg/mL; pH 7.6) had been added. Each of these concentrations as well as control plates not containing any doxycycline was studied in triplicate. Emetine, at a concentration of 60 mg/L, was used as a positive control for nonselective cell injury. At the end of this 2-h incubation, all plates were washed three times with PBS and then reincubated in SFM containing radiolabeled 1H-cysteine (1 µCi/mL, Radiochemical Center, England) at 37°C. At the end of 2 days, cells were scraped from the plates and the plates and cell media were separated by centrifugation at 7,000g×10 min. A portion of the supernatant was stored at −70°C for later collagenase immunoassay. Another portion of supernatant was concentrated (10-centrifrip, Amicon, Calif) 20-fold for subsequent gelatin gel zymography.

The cell pellet was resuspended in 0.9 percent NS. A portion of this suspension was filtered through Whatman paper (3 mm), and washed under vacuum suction (Sampling Manifold, Millipore, Mass) with 10 percent and 5 percent trichloroacete. The filter paper was allowed to dry overnight and the radioactivity was measured using a β-counter.

**MTT Assay**

HT 1080 cells were grown to confluence (2×10⁴ cells/well) in 96-well microtiter plates (Costar, Cambridge, Mass) and then incubated at 37°C with doxycycline or emetine at the concentrations described above. This medium was then removed by washing with PBS and the cells were recultured with SFM. At the end of 48 h, MTT uptake was studied as described by Mosmann.20 In brief, 0.02 mM MTT (5 mg/ml in PBS) was added to each well and incubated at 37°C for 4 h. All the unreacted dyes and media were removed by shaking and then acid-isopropanol (100 µl 0.4N HCL isopropanol) was added to each well and mixed thoroughly. After 30 min, the plate was read on an enzyme-linked immunosorbent assay (ELISA) plate reader at a wavelength of 570 nm. Percent cytotoxicity was then calculated as: (1−[ODₓₐₓ-Doxy treated/ODₓₐₓ control])×100.

**Results**

In Vitro Inhibition of MMPs in Human Pleural Fluid

Type IV collagenase activity was analyzed in pleural fluid...
fluids incubated with and without tetracyclines. As shown in Figure 1, activity of the untreated samples ranged from 0.17 to 0.22 μg of substrate degraded per hour of incubation per milliliter of pleural fluid sample. Incubation of the pleural fluid samples for 1 h with either TCN HCl, DOXY, or CMT (neutralized to a pH of 7.6) reduced the type IV collagenolytic activity in pleural fluid. At the highest drug concentrations (10 mg/ml for TCN and DOXY; 4 mg/ml for CMT), the type IV collagenase activity was reduced to 0.02 to 0.11 μg/h/ml. With increasing concentrations of the various tetracyclines, the collagenase activity became progressively diminished with excellent correlations for TCN-HCl (r = 0.9), DOXY (r = 0.93), and CMT-1 (r = 0.71).

Figure 2 shows the typical pattern of gelatinolytic activity in pleural fluid (lane 1). Prominent lysis is seen at a molecular weight of 66 kDa (MMP-2) with somewhat less activity at 92 kDa (MMP-9) and even fainter activity at 116 and 210 kDa. Preactivation of this sample with trypsin (lane 2) results in a new band of lytic activity at 74 kDa. Lanes 3 and 4 contain the unmodified and trypsin-activated pleural samples, respectively (as in lanes 1 and 2), after incubation overnight in buffer containing DOXY (5 mg/ml, pH 7.6).

Incubation in DOXY resulted in total inhibition of MMP activity in both the nonactivated and the trypsin-activated samples. At DOXY concentrations of 0.5 mg/ml in the incubation buffer (not shown), the MMP activity was not inhibited. Also, when the samples were preincubated with DOXY prior to loading onto the gels (and then incubated in buffer not containing DOXY), similar patterns of inhibition were seen to those shown for DOXY added to the incubation buffer (not shown).

**HT1080 Synthesis of MMP**

Gelatin zymography of the medium obtained from the HT1080 cells is shown in Figure 3. The first lane shows gelatinase activity from cells not exposed to DOXY; this activity is most evident at 66 kDa (MMP-2) and 92 kDa (MMP-9). The second lane shows minimal gelatinase activity in the sample obtained from cells exposed to the cytotoxic chemical, emetine. Lanes 3 to 5 show the gelatinase activity from cells exposed to DOXY at concentrations of 0.008, 0.04, and 0.2 mg/ml, respectively. The results did not differ, visually, from those seen in the control sample (lane 1). In those cells exposed to 1 mg/ml of DOXY (lane

![Figure 1. Type IV collagenase activity of pleural fluid and its inhibition by various concentrations of tetracycline hydrochloride (TCN), doxycycline hyclate (DOXY) and a chemically modified tetracycline (CMT-1), all at pH 7.6. Coefficients of correlation (r) are indicated on the graph.](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21669/)

![Figure 2. Typical gelatin zymogram of pleural fluid. Lane 1 contains unmodified pleural fluid and lane 2 contains the same sample preactivated with trypsin; lanes 3 and 4 are identical to lanes 1 and 2, respectively, but have been incubated overnight with doxycycline, 5 mg/ml, at a pH of 7.6. Molecular weight markers of 92 and 66 kDa are indicated to the left. The arrow points to a zone of lysis at 74 kDa seen in lane 2.](http://journal.publications.chestnet.org/pdfaccess.ashx?url=/data/journals/chest/21669/)
Concentration are cells 1116 ml) viability trations completely obliterated all evidence of gelatinase activity.

The concentration of MMP-2 in each of the above samples was quantitated by immunoblot. As shown in Figure 4, the amount of MMP-2 synthesized by the HT1080 cells never exposed to DOXY was 33.9 ± 3.4 ng/ml. In those exposed to DOXY concentrations below 1 mg/ml, the amount of MMP-2 produced was not altered from the control value. However, at DOXY concentrations of 1 and 5 mg/ml, the MMP-2 concentrations fell to 2.5 ± 0.6 and 3.3 ± 0.8 ng/ml, respectively.

These same HT1080 cells were tested for overall viability using both the uptake of MTT and the incorporation of 4C-leucine. The uptake of MTT was unaltered from control values at DOXY concentrations below 1 mg/ml. At DOXY concentrations of 1 mg/ml, the MTT uptake was reduced by 63 percent (p<0.05); at DOXY concentrations of 5 mg/ml, this uptake was reduced by 75 percent (p<0.05). Similar reductions were observed for 4C-leucine uptake at the higher doxycycline concentrations and these changes also reached statistical significance (p<0.05).

**Discussion**

Although many patients with recurrent malignant pleural effusions or spontaneous pneumothoraces receive intrapleural tetracycline as an alternative to talc or to surgical pleurodesis, the mechanism for this effect remains poorly understood. Recently, Strange et al1 documented that intrapleural installation of tetracycline in rabbits induces formation of acellular fibrin strands, fibroblast proliferation, mesothelial injury, and fibrous thickening of the subpleural connective tissue. In an attempt to learn more about how these agents promote fibrosis in the pleural space, we studied the in vitro effects of three members of the tetracycline family of antibiotics on type IV collagenases in pleural fluid.

All three tetracyclines studied effectively reduced the bioactivity of type IV collagenases in pleural fluid, but only at concentrations of 5 to 10 mg/ml. We estimate that the 5 to 10 mg/ml concentration of tetracyclines is similar to the clinically recommended dose of 20 mg TCN-HCl per kilogram diluted in 50 ml of sterile water, assuming a further dilution in a 100 ml of residual pleural fluid. At drug concentrations of 1 mg/ml, inhibition of type IV collagenase was minimal. This is consistent with recent data indicating that some (ie, fibroblast) collagenases are more resistant to tetracycline inhibition than are other (ie, neutrophil) collagenases;18 much of the type IV collagenase in pleural fluid is similar to fibroblast (ie, 66 kDa) collagenase. The limited inhibition of bioactivity seen at lower concentrations is also consistent with clinical and laboratory animal studies showing that tetracycline concentrations of as much as 7 mg/kg produce only minimal pleurodesis.19

We chose to carry out the inhibitor studies at neutral pH because collagenases are inactivated by an acid milieu. In clinical practice, TCN-HCl is not neutralized prior to intrapleural instillation. However, in animal models of tetracycline pleurodesis, an acid pH persists for only the first hour after intrapleural instillation; after this the pH of the pleural fluid is neutral (Sahn 1981). This early acidity may provoke much of the initial pain associated with tetracycline and may or may not be necessary for inducing pleurodesis. Clearly, our data indicate that an acid pH is not necessary for in vitro collagenase inhibition by TCN-HCl, DOXY, or CMT.

Gelatin zymography revealed that latent as well as
activated MMP-2 and MMP-9 in human pleural fluid were effectively inhibited in vitro by concentrations of doxycycline at or above 5 mg/ml. At concentrations of 1 mg/ml, the inhibition was not as complete, and at concentrations at or below 0.5 mg/ml, there was no inhibition seen.

The synthesis of MMP-2 and MMP-9 by fibrosarcoma (HT1080) cells grown in culture was also inhibited by these higher concentrations of doxycycline (1 to 5 mg/ml). This was confirmed both by gelatin gel zymography and by immunobassay for MMP-2. However, at these same concentrations of doxycycline, tests for cell viability such as the incorporation of [14C]-leucine or the uptake of MTT were also reduced, both by more than 90 percent. At concentrations of doxycycline that did not inhibit these measures of cell function (≤0.2 mg/ml), the concentrations of MMP-2 were the same as those for the control samples and the gels showed baseline lysis of the gelatin substrate. From this we conclude that the doxycycline, at neutral pH, is capable of reducing cell synthesis of MMPs but only as a part of a generalized depression of protein synthesis and other vital cell functions.

This study demonstrates several important points: (1) TCN-HCl and DOXY are both effective inhibitors of pleural fluid type IV collagenase, even at neutral pH but only at high concentrations; (2) type IV collagenase inhibition is not dependent on the antibiotic properties of tetracycline since similar results were obtained with chemically modified tetracyclines that no longer have antibacterial properties; and (3) the capacity of certain cells to produce these collagenases is greatly diminished by high concentrations of doxycycline but general evidence of cell function is also depressed at these levels of doxycycline. Whether the clinical use of tetracycline for pleurodesis depends on its capacity to inhibit collagenase or is a consequence of direct cytotoxicity on pleural mesothelial cells is not, as yet, defined.

REFERENCES
10 Wilhelm SM, Collier IE, Marmer BL, Eisen AZ, Grant GA, Goldberg GI. SV 40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. J Biol Chem 1989; 264:17213-21