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SELECTIVE ENDOTHELIAL GROWTH INHIBITION BY TETRACYCLINES THAT INHIBIT COLLAGENASE+

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The potential of angiogenesis inhibitors as therapy for human diseases is limited by a lack of clinically available agents. We investigated the mechanism of the anti-angiogenesis effects of minocycline, a commonly used drug, and several derivatives. Endothelial cell proliferation was inhibited by several of these compounds. We found that inhibition was associated with inhibition of collagenase, did not require antibiotic activity, and was not related to cytotoxicity. Other microvessel-associated cells were unaffected. This endothelial antiproliferative effect is a potential mechanism of the anti-angiogenic activity of minocycline.

Angiogenesis, the proliferation of new blood vessels, is a prominent component of several human diseases and therefore inhibition of this process has been advocated as a possible means of arresting such disorders (1). Nevertheless, although many angiogenesis inhibitors have been discovered, the development of a clinically useful compound has generally been hindered either by limited availability or by potential toxicity. Minocycline, a semisynthetic tetracycline derivative, is a clinically available compound that we recently found to inhibit tumor-induced angiogenesis in the rabbit cornea (2). In this report, we investigate the effects of minocycline on several of the major cell types implicated in microvascular structure and regulation. Chemically modified tetracyclines (CMTs) that vary in their biochemical properties were also used to help define the mechanism underlying the anti-angiogenic effects of minocycline.

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Abbreviations: CMT, chemically modified tetracycline; DiI-acyl-LDL, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate acetylated low density lipoprotein.

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METHODS

Preparation of Microvessel Cells. Bovine retinal endothelial cells were isolated as previously described (3). Third to fourth passage cells used in experiments were verified as 99% endothelial by immunofluorescence with DiI-acyl-LDL. Porcine aortic endothelial cells were isolated from collagenase treated aortas (incubated 15 min at 37°C, rinsed, centrifuged at 200g, resuspended and plated), used at seventh to eighth passage, and were 99% endothelial by DiI-acyl-LDL staining. Endothelial cell medium consisted of 80% minimal essential medium (containing d-valine, 16 U/ml heparin, 200 mM l-glutamine, nonessential amino acids, MEM vitamins, 200 U/ml penicillin, and 200 µg/ml streptomycin), 20% fetal bovine serum, and 50 µg/ml of endothelial mitogen (Biomedical Technologies). Pericytes were isolated by a modification of the method of Orlidge and D'Amore (4). Briefly, fresh retinas were washed, minced, and digested with 0.2% collagenase/0.2% bovine serum albumin for 40 min at 37°C, dispersed by vigorous pipetting, filtered through 118 micron mesh, centrifuged at 800g, resuspended in medium and plated. First passage cells used in experiments were 95-99% pericytes by immunofluorescence for alpha-smooth muscle actin. Astrocytes were isolated as previously described (3) and first passage cells used in experiments were verified as 90-95% pure by immunofluorescence for glial fibrillary acidic protein. C6 astroglial cells were obtained from American Type Culture Collection (Rockville, MD). Astrocytes, pericytes, C6, and F98 cells were grown in 90% Dulbecco's modified Eagles' medium (containing 200 mM L-glutamine, 200 U/ml penicillin, and 200 µg/ml streptomycin) and 10% fetal bovine serum (except C6 cells where nonfetal serum was used).

Determination of Growth Rates. Cells were passaged onto 24-well tissue culture plates (fibronectin-coated for endothelial cells) in the presence of solvent (water) only or minocycline. Medium was replenished daily and parallel plates were trypsinized and the cells were counted daily until control cells reached confluence (3-4 days). The exponential growth rate (population doublings per unit time) was calculated as the inverse of doubling time. In other experiments, cells were allowed to attach overnight, then treated once and counted when controls reached confluence (3-4 days).

Effects of Tetracycline Derivatives on Endothelial Cell Growth. Bovine retinal endothelial cells were passaged onto fibronectin-coated plastic and allowed to attach overnight. Medium was then replaced by fresh medium containing either 20 µM test derivative or an equivalent volume of solvent (50 mM Tris, pH 8.5) only, and subsequently replenished daily until control cells were confluent (3-4 days). Plates were then trypsinized and cells were counted. Percentage growth inhibition was calculated as {(control - experimental)/control} x 100. Minocycline, doxycycline, and tetracycline were obtained from Sigma Chemical. CMT-1 is produced from tetracycline by removal of the C-4 dimethylamino group, lacks antimicrobial activity, and is a potent collagenase inhibitor (5); CMT-5 lacks the C-11 carbonyl oxygen and C-12 hydroxyl groups, does not significantly inhibit collagenase, and is predicted to lack antimicrobial action by structure (5).

Tritium-labelling Studies. Cells were passaged at subconfluent densities and allowed to attach overnight. Medium was then replaced by fresh medium containing solvent (water) only or minocycline. After 12 h for thymidine and 18 h for leucine, medium was again replaced by medium with solvent or drug plus 1 µC/ml of [methyl-^{3}H]-thymidine or L-[4,5-^{3}H]-leucine (Amersham). Cells were labelled for 4-6 h , rinsed, fixed with trichloroacetic acid, and solubilized with NaOH. Aliquots were used for protein and radioactivity determination by the method of Bradford (6) and liquid scintillation spectroscopy, respectively. Counts per minute were normalized to total protein levels.

Morphological and Viability Studies. Endothelial cells were passaged at subconfluent densities and allowed to attach overnight. Medium was then replaced by fresh medium containing either solvent (water) only or minocycline. After 18 h, cells were fixed with 3.7% buffered formaldehyde and photographed by phase contrast microscopy. Cytotoxicity was assessed in simultaneous cultures by either: 1) 0.5 ppm ethidium bromide exposure for 5 min, fixation as above and counting fluorescent nuclei, or 2) trypsinization, exposure to 0.2% trypan blue for 5-10 min and counting blue cells.

RESULTS

Effect of Minocycline on Microvessel Cells. We first investigated whether the *in vitro* proliferation of cells comprising the microvascular wall, including endothelial cells, pericytes, and astrocytes, was inhibited by minocycline. Pericytes are major cellular components of essentially all

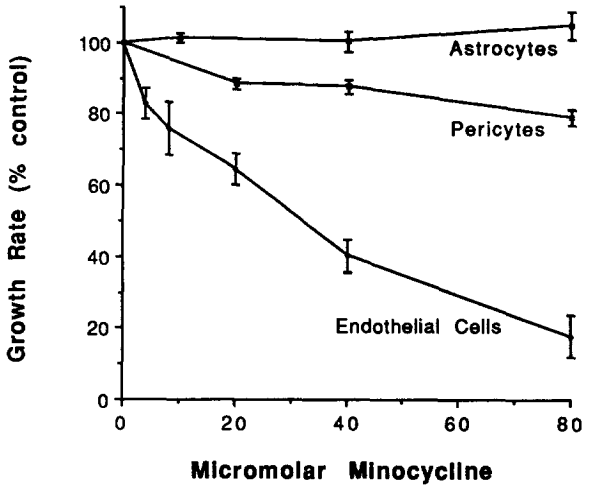

Figure 1. **Selective inhibition of endothelial cell growth by minocycline.** Endothelial cell growth is significantly inhibited by minocycline at concentrations that minimally affect astrocytes and pericytes. (Values are means ± standard errors; n = 4 for each point).

microvessels and are known to modulate endothelial proliferation (4). Astrocytes ensheathe brain microvessels and regulate microvessel morphogenesis and endothelial differentiation (3,7,8). Cultured cells were treated daily with minocycline and their growth rates were determined. The proliferation of bovine retinal microvascular endothelial cells was significantly reduced by minocycline at concentrations as low as 10 μM (Figure 1). In contrast, the growth of neonatal rat brain astrocytes was completely unaffected and that of bovine retinal pericytes was only minimally affected by concentrations up to 80 μM. Multiple microvascular endothelial cell isolates were consistently inhibited by minocycline in a concentration-dependent fashion, with an IC_{50} (concentration that decreased the growth rate to 50% of control) between 20 and 40 μM. Endothelial cells from porcine aorta were also inhibited at nearly identical concentrations, indicating that this effect was not limited to endothelium of microvascular or central nervous system origin, and was not species specific. Under similar experimental conditions, growth of the C6 and F98 glioma cell lines was not affected. Thus, minocycline selectively inhibits the proliferation of endothelial cells.

Inhibition of endothelial cell growth was also seen after a single exposure to minocycline. Growth of pericytes and astrocytes was unaffected at minocycline concentrations of less than 320 μM, while microvascular endothelial cell growth was inhibited in a concentration-dependent fashion by a single exposure to as little as 10 μM minocycline. Minocycline also inhibited endothelial DNA and protein synthesis at concentrations that had only minimal effects on C6 astroglial cells (Figure 2).

Endothelial cells exposed to 40 μM minocycline (the IC_{50} for growth) retained their normal morphology. Morphological changes in endothelial cells were noted within 18 hours of exposure to higher concentrations (Figure 3). At 100 μM and above, cells developed increasing numbers of clear vacuoles. Nevertheless, endothelial cells were viable in up to 320 μM minocycline as determined by both trypan blue and ethidium bromide exclusion. Thus, the

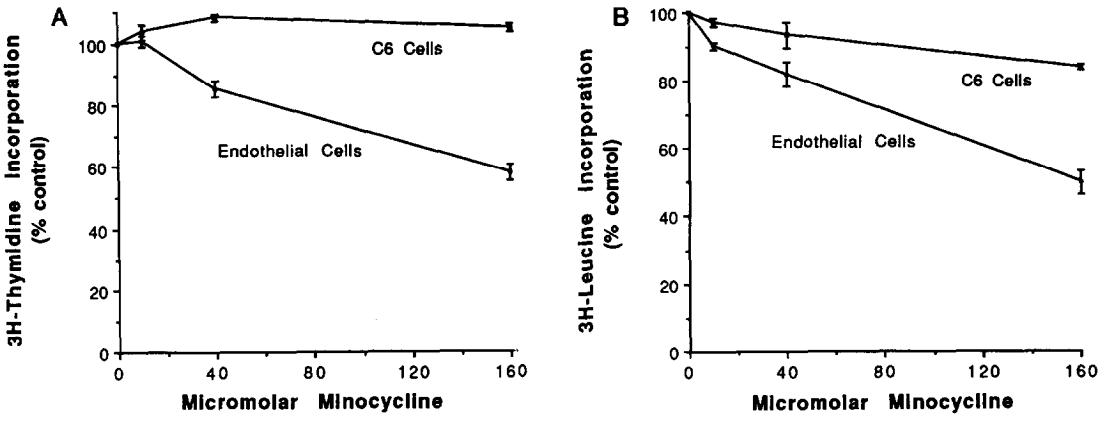

Figure 2. **Selective inhibition of endothelial cell DNA and protein synthesis by minocycline.** A, Incorporation of tritiated thymidine; B, Incorporation of tritiated leucine. (Values are means ± standard errors; n = 3 for each point).

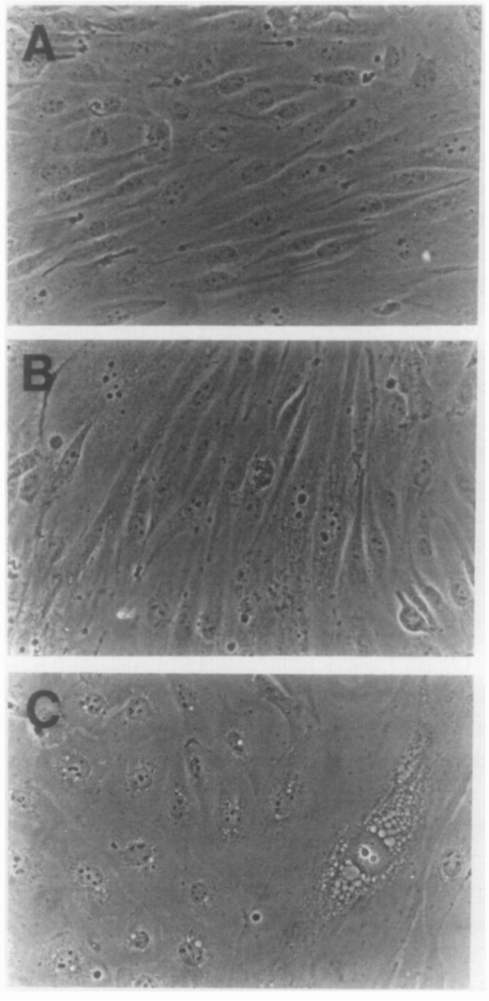

Figure 3. **Morphological changes in endothelial cells exposed to minocycline for 18 hours.** A, Control; B, 40 μM minocycline; C, 320 μM minocycline. Cells exposed to 40 μM minocycline are indistinguishable from controls. At 320 μM concentrations, cells appear vacuolated.

Table 1. Effects of tetracycline derivatives on endothelial cell growth

Derivative (20 μM)	Collagenase inhibition	Endothelial cell growth inhibition (% of control)
Tetracycline (parent)	+	32.5±3.1
Doxycycline	++	47.2±2.8
Minocycline	++	49.6±1.8
CMT-1	++	75.6±1.3
CMT-5	-	9.4±2.9

Details are described under "Methods." Values are means ± standard errors; n = 3 for each point except for minocycline, where n = 6.

antiproliferative effects of minocycline could not be attributed to endothelial cytotoxicity.

Effect of Other Tetracycline Derivatives. To gain further insight into the mechanism of the antiproliferative effects of minocycline, we tested the ability of other tetracycline derivatives to inhibit endothelial cell growth (Table 1). Like minocycline, the clinically available compounds doxycycline and tetracycline are also collagenase inhibitors, although tetracycline is the least potent (5,9). Doxycycline was found to be as effective as minocycline in inhibiting endothelial proliferation, whereas higher concentrations of tetracycline were required for equal inhibition, thus paralleling its potency for inhibiting collagenase.

Chemically modified tetracyclines that vary in their ability to inhibit collagenase have recently been described and provide a unique opportunity for investigating the roles of collagenase inhibition and antibiotic activity in anti-angiogenesis (5). We tested two chemically modified tetracyclines, one of which (CMT-1) lacks antibiotic activity but retains the ability to inhibit collagenase, and another (CMT-5) that lacks collagenase-inhibitory activity (CMT-5) (5). CMT-1 was more active than minocycline in inhibiting endothelial proliferation, whereas CMT-5 had minimal activity (Table 1). CMT-1 at 40 μM concentrations decreased endothelial proliferation by nearly 95%. Thus, inhibition of endothelial cell proliferation appears to be associated with the collagenase inhibitory properties of tetracyclines, and not with antibiotic activity.

DISCUSSION

Here we establish that minocycline, a clinically available inhibitor of both collagenase and angiogenesis, selectively inhibits endothelial cell growth. We also show that the growth of nonendothelial components of the microvascular wall, which modulate endothelial growth and differentiation, is relatively unaffected by minocycline. It is of particular interest that pericyte growth was unaffected, since these mural cells are believed to provide an endogenous anti-angiogenic influence *in vivo* (4). In addition, comparison of tetracycline derivatives shows that this antiproliferative activity segregates with collagenase inhibitory activity, and is independent of antibiotic activity.

The antiproliferative effect described here is likely to be one of the mechanisms whereby minocycline exerts anti-angiogenic activity. Previous

hypotheses on the role of collagenase inhibition have usually focused on the importance of degradation of the basement membrane to early angiogenic events (2,10-12). Endothelial proliferation is known to be regulated by matrix components and thus may also be affected by alterations in protease activity (13-15). Pericellular collagen degradation may be required for endothelial mitogens to have optimal exposure to cell surface receptors (16). Thus, collagenase inhibitors may affect multiple steps in the angiogenic process.

Tetracycline derivatives are potentially beneficial in the treatment of human diseases in which angiogenesis plays a major role, including neoplasia, diabetes, arthritis, and atherosclerosis (1). Several of these compounds are readily available, safe for human use, and standard doses decrease the collagenolytic activity of pathologically relevant human tissues and body fluids (17,18). Our finding that antiproliferative endothelial activity is independent of antibiotic activity indicates that derivatives without antimicrobial activity may increase the anti-angiogenic therapeutic index by eliminating side effects associated with antibiotic use. Additional investigations with such agents may advance our understanding of the relationships between collagenases, endothelial proliferation, and angiogenesis, and their relevance to human diseases.

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