Cancer Research

# Angiogenesis Inhibition by Minocycline

Rafael J. Tamargo, Robert A. Bok and Henry Brem

*Cancer Res* 1991;51:672-675.

[CANCER RESEARCH 51, 672–675, January 15, 1991]

# Angiogenesis Inhibition by Minocycline[1]

**Rafael J. Tamargo, Robert A. Bok,[2] and Henry Brem[3]**

*Department of Neurological Surgery, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

## ABSTRACT

**We describe a new inhibitor of angiogenesis, minocycline, a semisynthetic tetracycline antimicrobial with anticollagenase properties. Minocycline was incorporated into controlled release polymers and tested in the rabbit cornea against neovascularization in the presence of the VX2 carcinoma. Inhibition by minocycline was shown to be comparable to that of the combination of heparin and cortisone, a potent inhibitor of angiogenesis. Minocycline decreased tumor-induced angiogenesis by a factor of 4.5, 4.4, and 2.9 at 7, 14, and 21 days, respectively. At the end of the experiment, whereas the corneas with empty polymers had large, invasive, exophytic tumors, none of the corneas with minocycline had such vascular masses. Recently, studies of agents that disrupt collagen synthesis and deposition have yielded several new angiogenesis inhibitors. We suggest that investigation of agents that disrupt collagenolysis may similarly identify other angiogenesis inhibitors and further clarify the mechanisms of angiogenesis.**

## INTRODUCTION

Angiogenesis, the proliferation and migration of endothelial cells that result in the formation of new blood vessels, is an essential event in a wide variety of normal and pathological processes. For example, angiogenesis plays a critical role in embryogenesis, wound healing, psoriasis, diabetic retinopathy, and tumor formation (1–4). Indeed, because angiogenesis appears to be a prerequisite of exponential tumor growth in the brain, we became interested in agents that inhibit angiogenesis in attempts to inhibit tumor growth by this means. Moreover, identification of several agents that inhibit tumor angiogenesis has provided a conceptual framework for the understanding of angiogenesis in general. The inhibition of angiogenesis by certain steroids and heparin derivatives (5, 6) led to studies elucidating the crucial role of remodeling of the extracellular matrix in angiogenesis. These agents apparently prevent angiogenesis by specifically disrupting the deposition and cross-linking of collagen (6).

Theoretically, since remodeling of the extracellular matrix can also be disrupted by preventing collagen breakdown, angiogenesis could similarly be disturbed by inhibitors of collagenase. There is precedence for such a supposition since cartilage, the first inhibitor of angiogenesis to be described (7), was shown to contain an inhibitor of collagenase (8–10). We therefore chose to investigate minocycline, a semisynthetic tetracycline antimicrobial (11, 12) with secondarily discovered anticollagenase properties (13–16), to determine whether it could inhibit angiogenesis.

## MATERIALS AND METHODS

Putative inhibitors of angiogenesis, impregnated into controlled release polymers, were assessed in the rabbit cornea, which allows direct, quantitative observation of neovascularization.

**Animals.** New Zealand White rabbits weighing about 1.5–2.5 kg were obtained from Bunnyville Farm (Littlestown, PA), kept in standard animal facilities, 1 animal/cage, and given free access to food and water.

**Anesthesia.** For the corneal implantations, subsequent stereomicroscopic examinations, and serial transplantation of the VX2 tumor in the thigh, the animals were anesthetized with a mixture of xylazine, 15–17 mg/kg, and ketamine, 15–17 mg/kg, injected i.m.

**VX2 Rabbit Carcinoma.** The VX2 carcinoma (17), a serially transplantable tumor syngeneic to the New Zealand White rabbit, was propagated by serial transplantation in the flank of New Zealand White rabbits.

**Test Substances and Polymer Preparation.** Ethylene-vinyl acetate copolymer (18) (40% vinyl acetate by weight, Elvax 40P) was obtained from the Du Pont Co., Wilmington, DE. The polymer was washed extensively in absolute ethyl alcohol, with total volume changes every 24 h, to extract the inflammatory antioxidant butylhydroxytoluene. The presence of butylhydroxytoluene in the wash was monitored spectrophotometrically at 230 nm, and the washes were continued until the absorbance fell below 0.03 unit. The polymers were then dried in a vacuum desiccator for 5 days.

The agents to be tested for angiogenesis inhibition were incorporated into the EVAc[4] matrix by a modification of the fabrication procedure described by Rhine *et al.* (19). Minocycline and heparin crystals were ground to a fine powder and sieved through a 200 mesh (74 $\mu$m) screen in a Cellector tissue sieve (Bellco Glass, Inc., Vineland, NJ), to obtain a uniform sample consisting of particles less than 75 $\mu$m in diameter. The final concentrations (w/w) of the angiogenesis inhibitors in the polymers were: (*a*) minocycline hydrochloride (Sigma Chemical Co., St. Louis, MO), 10 and 20%; (*b*) cortisone acetate (Sigma Chemical Co.), 7.5 and 27%; (*c*) and heparin (Hepar Inc., Franklin, OH) and cortisone acetate, 15 and 30% combined loading, with a fixed 1:8 heparin:cortisone ratio. No significant differences were observed in the degree of angiogenesis inhibition between the two loading levels for each drug.

**Rabbit Cornea Angiogenesis Assay.** The inhibition of angiogenesis was determined by assaying the degree of angiogenesis in the rabbit cornea in the presence of specified inhibitors (8, 9, 20). The cornea provides an avascular matrix into which blood vessels grow and can be quantitated. A total of 116 corneas were implanted as follows: 50 corneas with VX2 carcinoma and empty polymer; 16 with VX2 carcinoma and minocycline polymer; 14 with VX2 carcinoma and heparin/cortisone polymers; and 36 with VX2 carcinoma and cortisone polymers. Five corneas were excluded from the study: three became infected, one lost its polymeric implant, and one was lost when the rabbit died prior to the first reading on day 7. The corneas were examined with a Zeiss slit lamp stereomicroscope (Carl Zeiss, Inc., Thornwood, NY) on days 7, 14, and 21 after implantation. A total of 111 corneas were evaluated on days 7 and 14, and 77 corneas were assessed on day 21. The angiogenesis response was quantitated by measuring both vessel length and vessel number to provide an angiogenesis index. For vessel length, the span of the blood vessels from the corneo-scleral junction to the leading edge of the new blood vessel front was measured with an ocular microscale eyepiece. The number of blood vessels present was designated, based on the following 4-level scale: 0, 0 vessels; 1, 1–10 vessels; 2, >10 vessels, loosely packed so that details of the iris could

Received 6/15/90; accepted 10/24/90.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by The Association for Brain Tumor Research Fellowship in Memory of Steven Lowe, by NIH Grant NS01058, and by the Andrew W. Mellon Foundation Johns Hopkins University Faculty Development Award.

[2] Present address: Department of Medicine, University of Minnesota, 420 Delaware Street, Southeast, Minneapolis, MN 55455.

[3] To whom requests for reprints should be addressed, at Johns Hopkins Hospital/Meyer 7-113, 600 N. Wolfe Street, Baltimore, MD 21205.

[4] The abbreviation used is: EVAc, ethylene-vinyl acetate copolymer.

be observed through the gaps between the vessels; and 3, >10 vessels, tightly packed so that the iris could not be seen through the gaps between the vessels. An angiogenesis index was then determined as follows:

$$\text{Angiogenesis index} = \text{vessel length} \times \text{vessel density}$$

**Histological Examination of Corneas.** The rabbits were sacrificed after the last stereomicroscopic examination on day 21 by the i.v. administration of T-61 Euthanasia Solution (Taylor Pharmacal Co., Decatur, IL). Representative corneas were removed and placed in phosphate-buffered formalin for 10–14 days, embedded in paraffin, sectioned with a microtome, and stained with hematoxylin and eosin for histological examination.

**Statistical Analysis.** The angiogenesis indexes for the four groups were compared by using the Kruskal-Wallis test for nonparametric single factor analysis of variance and the Newman-Keuls nonparametric analogue for multiple comparisons (21). Independent analyses were carried out for group values on days 7, 14, and 21.

## RESULTS

Tumor angiogenesis was significantly inhibited ($P < 0.05$) by the controlled release of minocycline, cortisone alone, and the combination of heparin and cortisone at 7, 14, and 21 days after implantation (Fig. 1). The degrees of inhibition obtained with the three agents were comparable; there were no statistically significant differences among the three inhibitors at any time. Minocycline decreased tumor-induced angiogenesis by a factor of 4.5, 4.4, and 2.9 at 7, 14, and 21 days, respectively. At the end of the experiment, whereas the corneas with empty polymers had large, invasive, exophytic tumors, none of the corneas with minocycline had such vascular masses (Fig. 2). When implanted alone in the cornea, the polymers containing minocycline, cortisone, and the combination of heparin and cortisone did not induce angiogenesis. Polymers containing heparin alone, however, were noted to promote a mild angiogenic response in the cornea.

Histological examination of the corneas with tumor and minocycline-EVAc polymers confirmed the presence of viable tumor adjacent to the polymer, surviving in an avascular state by day 21 (Fig. 2).

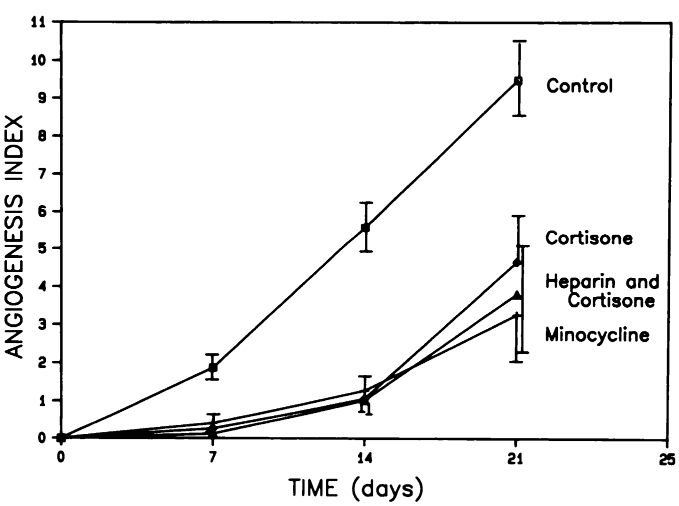


Fig. 1. Inhibition of tumor angiogenesis in the rabbit cornea in the presence of minocycline, cortisone, and the combination of heparin and cortisone. *Points*, mean; *bars*, SEM.

## DISCUSSION

The present study was designed to determine whether minocycline, a semisynthetic tetracycline with anticollagenase activity, was an inhibitor of tumor angiogenesis in the rabbit cornea. We found that minocycline inhibited angiogenesis to an extent comparable to that of the combination of heparin and cortisone. Histological evidence of viable tumor adjacent to the minocycline-EVAc matrix at 21 days suggests that minocycline itself, as previously reported, is not directly cytotoxic to mammalian cells (22). Preliminary data from our laboratory shows that *in vitro* minocycline significantly prolongs the doubling time of bovine retinal endothelial cells but not C6 glioma, F98 glioma, or 9L gliosarcoma tumor lines. We are currently exploring the optimal route of administration. In the present study, the combination of heparin and cortisone was only slightly better than cortisone alone at the three time points and the difference was not significant. This result is consistent with published reports describing the variability in the antiangiogenesis synergism between heparin and steroids, which may be a result of the chemical heterogeneity among the different types of heparin. Whereas several groups have reported that heparin enhanced the antiangiogenesis effect of steroids, other groups have not noted this synergism (23).

The original description of angiogenesis inhibition in the presence of cartilage (7–9) led to the isolation and purification from bovine cartilage of a protein fraction that not only inhibited angiogenesis but inhibited protease activity (10). Subsequently, an extract derived from the vitreous of rabbits was shown also to inhibit tumor angiogenesis (24). The demonstration that heparin alone enhanced the angiogenesis response buttressed the hypothesis that heparin produced by mast cells that had migrated to the tumor site facilitated the development of new capillaries (25).

Recent studies on inhibition of angiogenesis have highlighted the importance of enzyme-mediated remodeling of the extracellular matrix in capillary growth and proliferation (5, 6, 23, 26–28). It has been suggested that the steroid-heparin combination is involved in the dissolution of the capillary basement membrane by inhibiting the deposition or cross-linking of collagen (6, 27). The isolation of a collagenase inhibitor from cartilage (8–10), the first source of an angiogenesis inhibitor (7), was of interest since it suggested that angiogenesis may be inhibited not only by disrupting collagen deposition, but also by interrupting collagen breakdown. It is possible that a combination of agents that interfere with both the anabolic and catabolic phases of collagen metabolism will prove even more effective in halting tumor angiogenesis. For these reasons we considered that it would be of interest to examine the effects of minocycline on angiogenesis.

Minocycline is one of the semisynthetic tetracyclines, first described in 1967, derived from the naturally produced parent compounds chlortetracycline and oxytetracycline (11, 12). The tetracyclines are effective against a broad range of pathogens (29). They bind to the bacterial 30S ribosome, block access of the aminoacyl tRNA to the binding site on the mRNA-ribosome complex, and thus inhibit protein synthesis by preventing the addition of amino acids to the growing peptide chain. The tetracyclines usually spare protein synthesis in mammalian cells, however, since these cells lack the active transport system found in bacteria (22).

Minocycline inhibits collagenase activity directly by a mechanism unrelated to its antimicrobial properties (13–16). Sub-

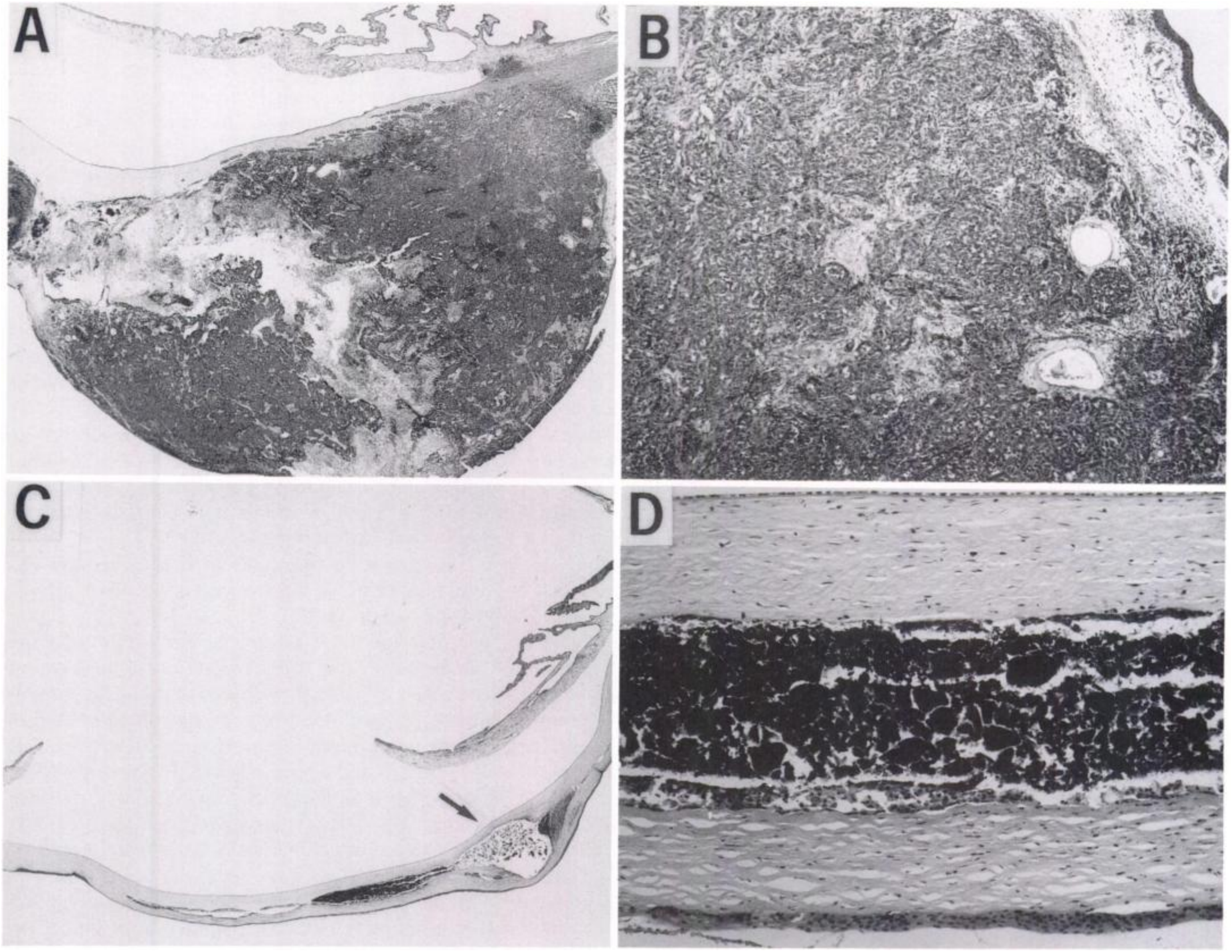


Fig. 2. Sections of rabbit corneas, prepared as described in the text and stained with hematoxylin/eosin, showing extent of tumor growth and neovascularization 21 days after implantation. *A* and *B* (×12 and ×65, respectively), show extensive tumor growth and vascularization in the presence of an empty polymer. *C,* (×16) shows minimal tumor burden and vascularization in the presence of minocycline. *D,* at higher magnification (×115) demonstrates that the tumor cells adjacent to minocycline-impregnated polymer are viable, although avascular. Thus despite the prevention of neovascularization through the cornea, the minocycline does not appear to be directly toxic to the tumor cells.

sequently, minocycline was shown to inhibit collagenolysis and cytolysis induced by melanoma-produced metalloproteases *in vitro* (30) and also the collagenase activity in the synovial fluid of patients with rheumatoid arthritis (31). The mode of action of minocycline on collagenase is not well understood. Chelation of calcium may be involved (32), but this cannot explain all the inhibitory activity of minocycline. Direct complex formation between minocycline and the protease has been postulated (31).

Minocycline is ideally suited for studying inhibition of angiogenesis since it is readily available commercially, is innocuous to mammalian cells, and has been in clinical use for several years. Theoretically, one of the appealing features of anticollagenase agents as potential antineoplastic agents is that they could be used to inhibit pathological collagenolytic processes, while leaving physiologically necessary proteolytic processes unaffected. If specific collagenase inhibitors with low systemic toxicity can be identified, they may be very useful as modulators of tumor growth.

## ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of Michael L. Pinn and review of the manuscript by Dr. Pamela Talalay.

## REFERENCES

1. Folkman, J. Angiogenesis and its inhibitors. *In:* V. T. DeVita, S. Hellman, and S. A. Rosenberg (eds.), Important Advances in Oncology, pp. 42–62, Philadelphia: J. B. Lippincott Co., 1985.
2. Brem, H., Tamargo, R. J., Guerin, C., Brem, S. S., and Brem, H. Brain tumor angiogenesis. *In:* P. L. Kornblith and M. D. Walker (eds.), Advances in Neuro-Oncology, pp. 89–101. Mount Kisco, NY: Futura Publishing Co., 1988.
3. Folkman, J. Tumor angiogenesis: therapeutic implications. N. Engl. J. Med., *285:* 1182–1186, 1971.
4. Folkman, J. Successful treatment of an angiogenic disease. N. Engl. J. Med., *320:* 1211–1212, 1989.
5. Folkman, J., Langer, R., Linhardt, R. J., Haudenschild, C., and Taylor, S. Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone. Science (Washington DC), *221:* 719–725, 1983.
6. Ingber, D., and Folkman, J. Inhibition of angiogenesis through modulation of collagen metabolism. Lab. Invest., *59:* 44–51, 1989.
7. Brem, H., and Folkman, J. Inhibition of tumor angiogenesis mediated by cartilage. J. Exp. Med., *141:* 427–439, 1975.
8. Brem, H., Arensman, R., and Folkman, J. Inhibition of tumor angiogenesis by a diffusible factor from cartilage. *In:* H. C. Slavkin and R. C. Greulich (eds.), Extracellular Matrix Influences On Gene Expression, pp. 767–772. New York: Academic Press, 1975.
9. Langer, R., Brem, H., Falterman, K., Klein, M., and Folkman, J. Isolation of a cartilage factor that inhibits tumor angiogenesis. Science (Washington DC), *193:* 70–72, 1976.

10. Murray, J. B., Allison, K., Sudhalter, J., and Langer, R. Purification and partial amino acid sequence of a bovine cartilage-derived collagenase inhibitor. J. Biol. Chem., *261:* 4154–4159, 1986.
11. Martell, M. J., and Boothe, J. H. The 6-deoxytetracyclines. VII. Alkylated aminotetracycline possessing unique antibacterial activity. J. Med. Chem., *10:* 44–46, 1967.
12. Zbinovsky, V., and Chrekian, G. P. Minocycline. *In:* K. Florey (ed.), Analytical Profiles of Drug Substances, pp. 323–339. New York: Academic Press, 1977.
13. Golub, L. M., Lee, H. M., Lehrer, G., Nemiroff, A., McNamara, T. F., Kaplan, R., and Ramamurthy, N. S. Minocycline reduced gingival collagenolytic activity during diabetes. J. Periodontal Res., *18:* 516–526, 1983.
14. Golub, L. M., Ramamurthy, N., McNamara, T. F., Gomes, B., Wolff, M., Casino, A., Kapoor, A., Zambon, J., Ciancio, S., Schneir, M., and Perry, H. Tetracyclines inhibit tissue collagenase activity. J. Periodontal Res., *19:* 651–655, 1984.
15. Golub, L. M., Wolff, M., Lee, H. M., McNamara, T. F., Ramamurthy, N. S., Zambon, J., and Ciancio, S. Further evidence that tetracyclines inhibit collagenase activity in human crevicular fluid and from other mammalian sources. J. Periodontal Res., *20:* 12–23, 1985.
16. Golub, L. M., McNamara, T. F., D'Angelo, G., Greenwald, R. A., and Ramamurthy, N. S. A non-antibacterial chemically-modified tetracycline inhibits mammalian collagenase activity. J. Dent. Res., *66:* 1310–1314, 1987.
17. Kidd, J. G., and Rous, P. A transplantable rabbit carcinoma originating in a virus-induced papilloma and containing the virus in masked or altered form. J. Exp. Med., *71:* 813–838, 1940.
18. Langer, R., and Folkman, J. Polymers for the sustained release of proteins and macromolecules. Nature (Lond.), *263:* 797–800, 1976.
19. Rhine, W. D., Hsieh, D. S. T., and Langer, R. Polymers for sustained macromolecule release: procedure to fabricate reproducible delivery systems and control release kinetics. J. Pharm. Sci., *69:* 265–270, 1980.
20. Gimbrone, M. A., Cotran, R. S., Leapman, S., and Folkman, J. Tumor growth and neovascularization: an experimental model using the rabbit cornea. J. Natl. Cancer Inst., *52:* 413–427, 1974.
21. Zar, J. H. Biostatistical Analysis. Englewood Cliffs, NJ: Prentice-Hall, Inc., 1984.
22. Sande, M. A., and Mandell, G. L. Tetracyclines, chloramphenicol, erythromycin, and miscellaneous antibacterial agents. *In:* A. G. Gilman, L. S. Goodman, T. W. Rall, and R. Murid (eds.), The Pharmacological Basis of Therapeutics, pp. 1170–1198. New York: Macmillan Publishing Co., 1985.
23. Folkman, J., Weisz, P. B., Joullie, M. M., Li, W. W., and Ewing, W. R. Control of angiogenesis with synthetic heparin substitutes. Science (Washington DC), *243:* 1490–1493, 1989.
24. Brem, S., Preis, I., Langer, R., Brem, H., and Folkman, J. Inhibition of neovascularization by an extract derived from vitreous. Am. J. Ophthalmol., *84:* 323–328, 1977.
25. Kessler, D. A., Langer, R. S., Pless, N. A., and Folkman, J. Mast cells and tumor angiogenesis. Int. J. Cancer, *18:* 703–709, 1976.
26. Crum, R., Szabo, S., and Folkman, J. A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science (Washington DC), *230:* 1375–1378, 1985.
27. Ingber, D., Madri, J. A., and Folkman, J. A possible mechanism for inhibition of angiogenesis by angiostatic steroids: induction of capillary basement membrane dissolution. Endocrinology, *119:* 1768–1775, 1986.
28. Ingber, D. E., and Folkman, J. Mechanochemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis *in vitro*: role of extracellular matrix. J. Cell Biol., *109:* 317–330, 1989.
29. Laskin, A. I. Tetracyclines. *In:* D. Gotlieb and P. D. Shaw (eds.), Antibiotics, pp. 331–359. New York: Springer-Verlag, 1967.
30. Zucker, S., Lysik, R. M., Ramamurthy, N. S., Golub, L. M., Wieman, J. M., and Wilkie, D. P. Diversity of melanoma plasma membrane proteinases: inhibition of collagenolytic and cytolytic activities by minocycline. J. Natl. Cancer Inst., *75:* 517–525, 1985.
31. Greenwald, R. A., Golub, L. M., Lavietes, B., Ramamurthy, N. S., Gruber, B., Laskin, R. S., and McNamara, T. F. Tetracyclines inhibit human synovial collagenase *in vivo* and *in vitro* . J. Rheumatol., *14:* 28–32, 1987.
32. Schlondorff, D., and Satriano, J. Interactions with calmodulin: potential mechanism for some inhibitory actions of tetracyclines and calcium channel blockers. Biochem. Pharmacol., *34:* 3391–3393, 1985.