The Combination of Antiangiogenic Agents to Inhibit Primary Tumor Growth and Metastasis

By Harold Brem, Ion Gresser, Jeffrey Grosfeld, and Judah Folkman
Boston, Massachusetts and Villejuif, France

● **Neovascularization is a critical component for the growth of tumors and is a dominant feature in diseases such as diabetic retinopathy and hemangiomas in infancy. Angiogenesis inhibition is a potentially important therapeutic modality. We have previously reported that AGM-1470 is a fungal-derived angiogenesis inhibitor that suppresses primary tumor growth and metastases and is also nontoxic. α-Interferon, an angiogenesis inhibitor, is effective in the treatment of life-threatening hemangiomas. We therefore attempted to treat murine primary tumors and metastases with a combination of AGM-1470 and α/β-interferon. Treatment began after solid tumors formed. Six-week-old syngeneic C57Bl/6 mice were treated for 21 days with either AGM-1470, or α/β-interferon or AGM-1470 + α/β-interferon. The combination of the angiogenesis inhibitors AGM-1470 and α/β-interferon suppressed tumor growth by 80% compared with controls ($P \leq .001$). AGM-1470 and α/β-interferon inhibited pulmonary metastatic tumor growth greater than sevenfold ($P \leq .001$) compared with controls. These effects were better than either inhibitor alone, and the combined effect was additive. Combination of angiogenesis inhibitors may be useful in the treatment of tumors and other angiogenesis-dependent diseases.**

INDEX WORDS: Angiogenesis, metastasis; interferon; AGM-1470; TNP-470; antiangiogenic therapy; Lewis lung carcinoma.

THE ABILITY of a primary tumor to grow beyond a few millimeters in size depends on its ability to stimulate angiogenesis, ie, new blood vessel growth.[1-6] Metastatic activity also appears to be angiogenesis-dependent. Successful metastasis requires that the primary tumor[7,8] and subsequent implants be vascularized.[6,9,10] Therefore, potent angiogenesis inhibitors may be useful to control both primary tumor growth and metastasis.

AGM-1470, also known as TNP-470, is a synthetic analogue of fumagillin originally derived from a fungus.[11] In vitro AGM-1470 inhibits capillary endothelial cell proliferation,[11] capillary endothelial cell migration,[12] and capillary tube formation.[13]

AGM-1470 is also a potent angiogenesis inhibitor in vivo. It inhibits angiogenesis when administered topically in both the chick chorioallantoic membrane[11] and the rat cornea,[13] and when administered systemically in the mouse, rat,[13] and rabbit.[14]

Certain interferons also have antiangiogenic activity.[15-17] Interferon was found to inhibit lymphocyte-induced[16] and tumor-induced[17] angiogenesis. Based on these studies, human recombinant α-interferon was used to treat life-threatening hemangiomas in children.[18] This was the first successful treatment of an angiogenic disease by antiangiogenic therapy.[19] α-Interferon has dramatically reduced the morbidity and mortality of life-threatening hemangiomas in infants and children.[20]

In the present study we tested the hypothesis that coadministration of two angiogenesis inhibitors, AGM-1470 and interferon α/β, could have an additive or synergistic antitumor effect in mice.

MATERIALS AND METHODS

Seventy-two male C57Bl-6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). The animals were anesthetized by methoxyflurane prior to shaving and at the time of tumor implantation.

Lewis lung carcinoma L1 (LLC-L1) is a highly metastatic variant that was obtained as previously described.[21] Tumors were individually passaged in C57Bl/6 mice subcutaneously by cell injection. Solid tumors grew to 1 cm^3 and were excised from anesthetized mice under sterile conditions in a laminar flow hood. Single cell suspensions were obtained by passing the tumor by syringe through needles of decreasing diameter from 22-gauge to 30-gauge. The number of tumor cells was determined by counting the tumor cells on a hemocytometer under a microscope. Subsequently, 10^6 tumor cells were implanted subcutaneously in the dorsum of each mouse in a volume of 0.1 mL of 0.9% saline. All mice were housed one per cage and were fed ad libitum with Purina chow and tap water. Treatment began after the tumors were visible and palpable (≥ 100 mm^3).

Mouse interferon α/β was prepared and partially purified from suspension cultures of mouse sarcoma C243 cells inoculated with Newcastle disease virus (NDV), as previously described.[22] Control preparations consisted of the supernatant from cultures of C243 cells in which the interferon inducer, NDV, was omitted. Interferon and control preparations were then concentrated 50-fold.

From the Department of Surgery, Children's Hospital, and the Departments of Surgery and Anatomy and Cellular Biology, Harvard Medical School, Boston, MA; and the Department of Viral Oncology, Groupe de Laboratoires de l'Institut de Recherches Scientifiques sur le Cancer, Villejuif, France.

Presented at the 1992 Annual Meeting of the Section on Surgery of the American Academy of Pediatrics, San Francisco, California, October 9-11, 1992.

Supported in part by a grant from the National Institutes of Health (CA-37395-08) (J.F.), the Association for Academic Surgery Award (H.B.), the Leon Hirsch Research Fellowship (H.B.), a grant from the Association pour La Recherche sur le Cancer (I.G.), and a grant to Harvard University from Takeda Chemical Industries, Ltd.

Address reprint requests to Judah Folkman, MD, Children's Hospital, Hunnewell 103, 300 Longwood Ave, Boston, MA 02115.

0022-3468/93/2810-0007$03.00/0

The specific activity of the interferon α/β preparation was 2×10^7 units for each milligram of protein. Interferon was assayed by inhibition of cytopathic effect of vesicular stomatitis virus (VSV) on L cells in monolayer cultures (0.2 mL/well) in Falcon microplates. Units are expressed in mouse reference units.[23] Concentrated α/β-interferon was stored at −20°C. Prior to use it was thawed and then diluted 1:3 with phosphate-buffered saline. In the first experiment each 20-g mouse received 200,000 units per day in the peritoneum in a volume of 0.2 mL. In the second experiment each mouse received 800,000 units of interferon in a volume of 0.2 mL in the peritonem. In addition 800,000 units of α/β-interferon was injected directly into the tumor in one group of mice that received no other treatment and in one group of mice that received subcutaneous AGM-1470. AGM-1470 was synthesized by Takeda Chemical Industries, Ltd. It was administered in a vehicle of 2% ethanol + 0.9% saline,[21] at a dose of 30 mg/kg by subcutaneous injection every other day at a site remote from the tumor, beginning on day 4 after tumor implantation. Control mice received approximately 0.3 mL of a 2% ethanol solution in 0.9% saline subcutaneously every other day and 0.2 mL of the interferon control preparation into the peritoneal cavity every day.

Tumor dimensions were measured every other day with calipers and volumes were calculated by width^2 × length × 0.52. Tumor volume was also expressed as T/C (ie, mean tumor volume of treated/mean tumor volume of control).

After 21 to 22 days of treatment all mice were killed by continuous inhalation of methoxyflurane. Autopsies were performed on all animals, at which time the lungs were weighed and surface metastases were counted. A surface metastasis was counted if it formed a discrete lesion on the lung as visualized under a stereomicroscope (×12). Tumor and lung weights were measured on a Mettler balance. Animal weights were calculated at the time of necropsy, by subtracting the weight of the resected tumor from the weight of the animal on the last day of treatment. The average animal weight in each experimental group was not statistically different ($P > .05$) on the first day of treatment. Student's t test was used for statistical analysis, and each experimental group was composed of 6 to 8 mice.

Immediately after the mice were sacrificed, the lungs were weighed and stored in 10% formalin. Specimens were fixed in paraffin blocks and sections 6 μm thick were cut. Hematoxylin and eosin were used to stain three paraffin-prepared sections from each set of lungs. Three sets of lungs (9 sections) were analyzed for each experimental group for each experiment. Diameter of metastases was measured under a ×200 magnification grid on a Nikon Projectorscope.

RESULTS

Both doses of α/β-interferon, ie, 200,000 or 800,000 units, gave similarly effective results. There was no statistical difference between each experimental group when compared to controls regardless of the dose ($P > .05$). Results are listed as the average of these two experiments.

After 21 days of treatment, control tumors weighed 5.31 ± 0.30 g. Interferon treatment alone did not significantly reduce tumor weight ($P > .05$). AGM-1470 significantly reduced tumor weight ($P < .05$). AGM-1470 plus α/β-interferon therapy had an additive antitumor effect ($P < .05$) (Fig 1A); right); after 21 days of treatment T/C was 0.21 ± 0.03 ($P < .05$) (Fig 2).

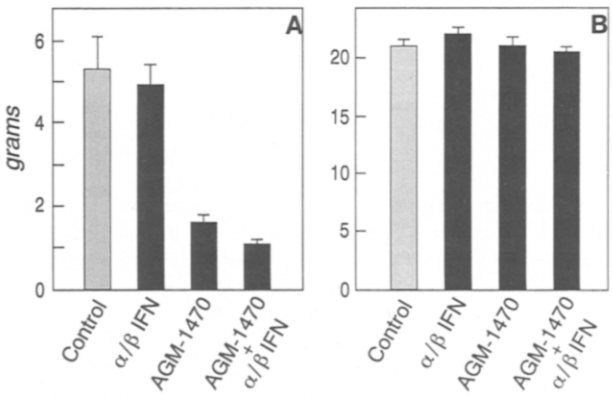

Fig 1. (A) Tumor weights in mice inoculated with Lewis lung carcinoma after 21 days of treatment. α/β-Interferon did not significantly reduce tumor weight. AGM-1470 administered as a single agent significantly reduces tumor weight ($P < .05$). AGM-1470 and α/β-interferon administered in combination significantly reduced tumor weight when compared with either agent alone ($P < .05$). (B) Body weights after 21 days of treatment. No treatment group resulted in a significant ($P > .05$) weight loss of the mice.

After 21 days of treatment, average lung weight of the control mice was 0.38 ± 0.04 g, a 158% increase in the lung weight as compared with age-matched untreated mice without tumors (Fig 3). The average number of metastases in these mice was 32 ± 4. AGM-1470 resulted in only a small reduction in the number of surface metastases, n = 26 ± 3 ($P > .05$), but a large reduction in the size and average weight of each pulmonary metastasis ($P < .05$). α/β-Interferon therapy alone resulted in a significant reduction in the number of pulmonary metastases, n = 13 ± 2 ($P < .05$). However, the actual size of each metastasis was not significantly affected. The combination of AGM-1470 and interferon resulted in an average of 11 ± 2 surface metastases and a sevenfold decrease in the tumor burden of the lungs as compared with controls ($P < .05$). Furthermore, the combination of AGM-1470 and α/β-interferon more potently inhib-

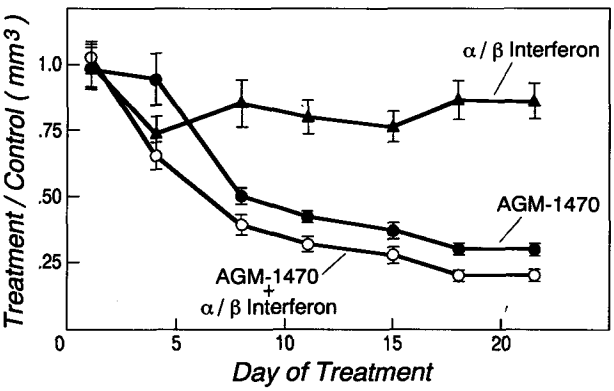

Fig 2. Tumor volume is represented as the average tumor volume of the treated mice (T) compared with the average tumor volume of the control mice (C).

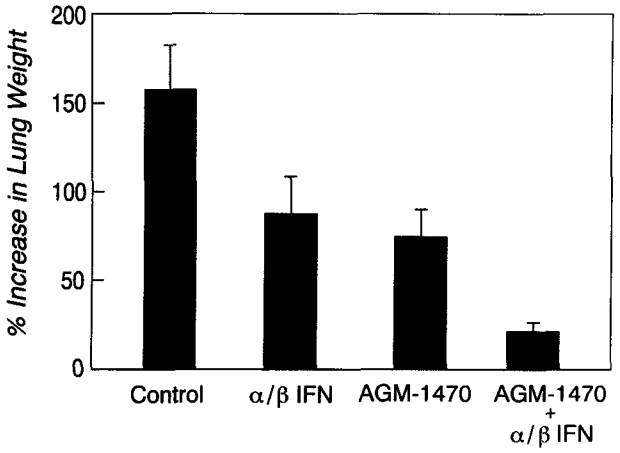

Fig 3. The percentage increase in lung weight after 21 days of treatment. Lung weight correlates tumor burden.

ited the pulmonary metastatic burden than either agent alone ($P < .05$).

In one experiment (n = 12) α/β-interferon was injected directly into the tumor (data not shown). This local therapy did not result in a statistically significant difference as compared to intraperitoneal injections, ie, systemic α/β-interferon therapy.

Histologically, the metastatic tumors in the lungs of the mice that received AGM-1470 and α/β-interferon had tumors that were a maximum of 1 to 2 mm in diameter. By comparison the lungs from the saline treated mice had 40 times more tumor volume when measured microscopically (Fig 4). Furthermore, the tumor metastases in the lungs of the mice that received AGM-1470 and α/β-interferon had a small necrotic rim at the periphery. This necrotic rim in the tumor metastases was not present in mice treated with a single agent, ie, AGM-1470 or α/β-interferon. In contrast the tumor cells in the lungs of saline

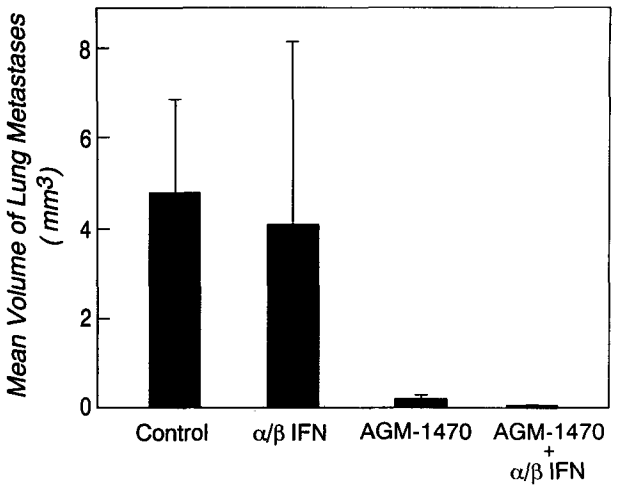

Fig 4. Tumor volume in lungs measured from histological sections.

treated mice were necrotic in the center of the tumors only.

Neither AGM-1470 therapy alone, interferon therapy alone, or AGM-1470 plus α/β-interferon had any significant effect on the weight of these mice after 21 days of treatment (Fig 1B). Furthermore, the mice in all treatment groups did not manifest any gross toxicity, ie, there was no hair loss or seizure activity.

DISCUSSION

We show here that the coadministration of two angiogenesis inhibitors improves the antitumor and anti-metastatic effects of each inhibitor in an additive manner. AGM-1470 by itself inhibited the size of the primary tumor as well as the size and number of its lung metastases. Interferon-α/β by itself inhibited the number of pulmonary metastases as previously reported,[24] but not the size of the primary tumor or its metastases. When both agents were administered together, the size of the primary tumor was inhibited more effectively than with either agent alone, and the same additive inhibitory effect was observed for number and size of pulmonary metastases. Toxicity was not increased when the two inhibitors were given together.

The antiangiogenic activity of interferon is a new property discovered in the past 5 years[16-20] based on an original observation in 1980 that interferon could inhibit locomotion of capillary endothelial cells in vitro.[25] α-Interferon has been successful in the treatment of life-threatening hemangiomas in infants.[18-20] In these patients it causes regression of large hemangiomas over a period of months. The mechanism of the antiangiogenic activity of interferon is unknown, but it is not a very potent angiogenesis inhibitor. While the regression of hemangiomas in the first months of life is dramatic, this neovascular bed may be a special case because of the tendency for hemangiomas to undergo spontaneous regression by 6 to 7 years.

α-Interferon is only effective in about 30% of Kaposi's sarcoma (another highly angiogenic lesion), and it is relatively ineffective against the majority of solid tumors in patients. Nevertheless, in a recent report, when α-interferon was coadministered with 13-cis-retinoic acid, it reduced tumor size in more than 50% of patients with carcinoma of the cervix metastatic to the pelvis.[26]

We previously showed that retinoic acid can inhibit angiogenesis.[27] Thus, the coadministration of α-interferon with retinoic acid in cancer patients is a clinical example of therapy by two angiogenesis inhibitors which parallels our animal study.

Of interest in our study is that histological sections

of lung show a peripheral rim of necrosis around the metastases in animals treated with both α/β-interferon and AGM-1470. The cause of this unusual pattern is unclear, but it could be the result of late regression of newly formed capillaries at the edge of each metastasis. This necrotic ring did not appear when either inhibitor was used alone.

AGM-1470 may soon be studied in clinical trials in patients with Kaposi's sarcoma. If it shows antitumor activity, then our animal studies suggest that coadministration of α-interferon and AGM-1470 may potentiate each other not only during anticancer therapy, but also in the treatment of certain nonneoplastic angiogenic diseases such as diabetic retinopathy, rheumatoid arthritis, retinopathy of prematurity, macular degeneration, and psoriasis.

In this report we combined both AGM-1470 and α/β-interferon because both are inhibitors of angiogenesis when administered as single agents. In the future it may be possible to add multiple antiangiogenesis agents[28-40] to control solid tumor growth and metastases.[12,41]

ACKNOWLEDGMENT

We are grateful to Dr Fumimasa Goto for his expert review of the histological specimens. The assistance with the in vivo experiments provided by Laurence Sanders and Dr Michael O'Reilly is appreciated. We thank Wendy Foss for her editorial assistance.

REFERENCES

1. Folkman J, Brem H: Angiogenesis and inflammation, in Gallin JI, Goldstein IM, Snydderman R (eds): Inflammation: Basic Principles and Clinical Correlates (ed 2). New York, NY, Raven, 1992, pp 821-839

2. Folkman J: Tumor angiogenesis: Therapeutic implications. N Engl J Med 285:1182-1186, 1971

3. Folkman J: What is the evidence that tumors are angiogenesis-dependent? J Natl Cancer Inst 82:4-6, 1990

4. Folkman J: Oncology overview on antiangiogenesis, in Girardi AJ (ed): Oncology Overviews on Angiogenesis. Washington, DC, US Government Printing Office, 1991, pp vii-x

5. Brem H, Tamargo RJ, Guerin C, et al: Brain tumor angiogenesis, in Kornblith PL, Walker MD (eds): Advances in Neurooncology. Mount Kisco, NY, Futura, 1988, pp 89-102

6. Folkman J: Tumor angiogenesis, in Holland JF, Frei E, Bast RC, et al (eds): Cancer Medicine (ed 3). Philadelphia, PA, Lea & Febiger, 1993, pp 153-170

7. Liotta LA, Kleinerman J, Saidel GM: Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. Cancer Res 34:997-1004, 1974

8. Liotta L, Kleinerman J, Saidel G: The significance of hematogenous tumor cell clumps in the metastatic process. Cancer Res 36:889-894, 1976

9. Folkman J: What is the role of angiogenesis in metastasis from cutaneous melanoma? Perspectives and Commentaries. Eur J Cancer Clin Oncol 23:361-363, 1987

10. Blood CH, Zetter BR: Tumor interactions with the vasculature. Biochim Biophys Acta 1032:89-118, 1990

11. Ingber D, Fujita T, Kishimoto S, et al: Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. Nature 348:555-557, 1990

12. Brem H, Ingber D, Blood CH, et al: Suppression of tumor metastasis by angiogenesis inhibition. Surg Forum 42:439-441, 1991

13. Kusaka M, Sudo K, Fujita T, et al: Potent anti-angiogenic action of AGM-1470: Comparison to the fumagillin parent. Biochem Biophys Res Commun 174:1070-1076, 1991

14. Gonzalez EM, Adamis AP, Folkman J: Systemic administration of an angiogenesis inhibitor (AGM-1470), inhibits bFGF-induced corneal neovascularization. Invest Ophthalol Vis Sci 33:777, 1992 (abstr 424)

15. D'Amore PA: Mechanisms of endothelial growth control. Am J Respir Cell Mol Biol 6:1-8, 1992

16. Sidky Y, Borden E: Inhibition of angiogenesis by interferons: Effects on tumor and lymphocyte-induced vascular responses. Cancer Res 47:5155-5161, 1987

17. Dvorak HF, Gresser I: Microvascular injury in pathogenesis of interferon-induced necrosis of subcutaneous tumors in mice. J Nat Cancer Inst 81:497-502, 1989

18. White CW, Sondheimer HM, Crouch ED, et al: Therapy of pulmonary hemangiomatosis with recombinant interferon alfa-2a. N Engl J Med 320:1197-1200, 1989

19. Folkman J: Successful treatment of an angiogenic disease. N Engl J Med 320:1211-1212, 1989

20. Ezekowitz RAB, Mulliken JB, Folkman J: Interferon alfa 2a therapy for life-threatening hemangiomas in infancy. N Engl J Med 324:1456-1463, 1992

21. Brem H, Folkman J: Analysis of experimental antiangiogenic therapy. J Pediatr Surg 28:445-451, 1993

22. Tovey MG, Begon-Lours J, Gresser I: A method for the large scale production of potent interferon preparations. Proc Soc Exp Biol Med 146:809-815, 1974

23. Gresser I, Maury C, Belardelli F, et al: Effectiveness of mouse interferon alpha/beta compared to single-agent chemotherapy in increasing survival time of mice after intravenous inoculation of Friend erythroleukemia cells. J Natl Cancer Inst 80:126-131, 1988

24. Gresser I, Bourali-Maury C: Inhibition by interferon preparations of a solid malignant tumour and pulmonary metastases in mice. Nature 236:78-79, 1972

25. Brouty-Boye D, Zetter BR: Inhibition of cell motility by interferon. Science 208:516-518, 1980

26. Lippman SM, Kavanagh JJ, Paredes-Espinoza M, et al: 13-cis-retinoic acid plus interferon alpha-2a: Highly active systemic therapy for squamous cell carcinoma of the cervix. J Natl Cancer Inst 84:218-219, 1992

27. Ingber D, Folkman J: Inhibition of angiogenesis through modulation of collagen metabolism. Lab Invest 59:44-51, 1988

28. Yamashita T, Sakai M, Kawai Y, et al: A new activity of herbimycin A: Inhibition of angiogenesis. J Antibiotics 42:1015-1017, 1989

29. Rastinejad F, Polverini PF, Bouck NP: Regulation of the activity of a new inhibitor of angiogensis by a cancer supressor gene. Cell 56:345-355, 1989

30. Bouck N: Tumor angiogenesis: The role of oncogenes and tumor suppressor genes. Cancer Cells 2:179-185, 1990

31. Klagsbrun M, Folkman J: Angiogenesis, in Sporn MB,

Roberts AB (eds): Peptide Growth Factors and Their Receptors. New York, NY, Springer-Verlag, 1990, pp 549-586

32. Maione TE, Gray GS, Petro J, et al: Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. Science 247:77-79, 1990

33. Missirlis E, Karakiulakis G, Maragoudakis ME: Antitumor effect of GPA1734 in rat Walker 256 carcinoma. Invest New Drugs 8:145-147, 1990

34. Moses MA, Sudhalter J, Langer R: Identification of an inhibitor of neovascularization from cartilage. Science 248:1408-1410, 1990

35. Klagsbrun M, D'Amore PA: Regulators of angiogenesis. Annu Rev Physiol 53:217-239, 1991

36. Li WW, Casey R, Gonzalez EM, et al: Angiostatic steroids potentiated by sulfated cyuclodextrins inhibit corneal neovascularization. Invest Ophthalmol Vis Sci 32:2898-2905, 1991

37. Folkman J, Hanahan D: The angiogenic phenotype in cancer, in Brugge J, Curran T, Harlow E, et al (eds): Origins of Human Cancer: A Comprehensive Review. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1991, pp 803-812

38. Tamargo RJ, Bok RA, Brem H: Angiogenesis inhibition by minocycline.[1] Cancer Res 51:672-675, 1991

39. Moses MA, Langer R: Inhibitors of angiogenesis. Bio/Technology 9:630-634, 1991

40. Brem H, Klagsbrun M: The role of fibroblast growth factors and related oncogenes in tumor growth, in Benz CC, Liu ET (eds): Oncogenes and Tumor Suppressor Genes in Human Malignancies. Boston, MA, Kluwer, 1993, pp 211-231

41. Sakamoto N, Tanaka NG: Effect of angiostatic steroid with or without glucocorticoid activity on metastasis. Invasion Metastasis 7:208-216, 1987

Discussion

N.S. Adzick (San Francisco, CA): About three decades ago Dr Judah Folkman postulated that "solid tumors are angiogenesis-dependent." This concept now has extensive support and has led to a series of remarkable discoveries by Dr Folkman's group including: (1) the demonstration that human tumors develop through a dormant prevascular phase followed by a vascular phase associated with explosive tumor growth; (2) the definition of the morphological and biological steps of capillary proliferation and neovascularization; (3) the detection of factors that modulate angiogenesis such as heparin and angiostatic steroids; (4) the sequencing and cloning of an entire family of angiogenic molecules such as basic fibroblast growth factor; (5) the characterization of a whole new class of angiogenic diseases such as the hemangiomas that were shown; and now the use of "anti-angiogenesis" as a potentially extremely powerful approach for cancer treatment.

Dr Brem has shown us that the combination of two antiangiogenesis agents, AGM-1470 and α-interferon, have a synergistic effect in limiting primary tumor growth and metastases in a Lewis lung carcinoma mouse model. When either of these agents was used alone, the effects were different. The angioinhibin AGM-1470 dramatically decreased tumor size of either the primary tumor or the metastases. In contrast, alpha interferon limited only the number of lung metastases.

I have two questions. Do you have any information on how these two drugs interfere at different levels of the angiogenesis cascade? Second, what is the status of clinical trials to evaluate these drugs in cancer patients—particularly for the fumagillin analog AGM-1470? Angiogenesis inhibitors may soon serve as powerful adjuncts to present methods of cancer therapy.

H. Brem (response): First, in terms of clinical trial, 13 days ago the first patient at the National Cancer Institute received AGM-1470. This is obviously very early, but yet it represents a milestone and it's now in clinical use. Phase 1 trials will be to determine its safety in patients. In our animal studies it has been very safe. Then the progression will be to test its efficacy.

An exciting area is, what is the mechanism of both of these compounds?

Interferon has been around for 30 years but only in the last 5 years has it been found to be an angiogenesis inhibitor. We don't know the mechanism of interferons. We do know that it is probably by secondary messenger mechanism. For example, interferons bind specific protein receptors and then there is an induction of many other proteins synthesized by the cell, which explains its antitumor effect. One example is it induces induction of tumor necrosis factor receptor. There are many other examples. Tumor necrosis factor by itself inhibits endothelial cell proliferation.

How AGM-1470 works is still a very active area of investigation.