Anti-Angiogenesis:
New Concept for Therapy of Solid Tumors

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SOLID tumor growth in animals and in man is accompanied by neovascularization. New capillary growth is elicited by a diffusible factor generated by malignant tumor cells. In the absence of neovascularization most tumors might become dormant at a tiny diameter, perhaps 2–3 mm.[9, 10]

Although evidence for these statements is still indirect and fragmentary, it seems appropriate to speculate that the inhibition of angiogenesis, i.e., *anti-angiogenesis,* may provide a form of cancer therapy worthy of serious exploration. The evidence for such a contention, based on recent findings in our own laboratory and others, will be examined in this report.

Evidence that Tumors Induce New Blood Vessels

Algire [1] attributed to tumor cells the ability to elicit continuously the growth of new capillary endothelium *in vivo.* Feigin [8] observed vigorous neovascularization in the neighborhood of malignant brain tumors, that seemed to be excited or induced by the tumor itself. Many other authors, observing tumor growth in the hamster cheek pouch or in the rabbit ear chamber, have described the intense neovascularization around a growing tumor.[13, 18, 21]

Chalkley [4] and Warren [22] were the first to hypothesize humoral induction of tumor blood vessels.

Evidence for the Humoral Induction of Tumor Angiogenesis

Greenblatt and Shubik [14] demonstrated that vasoproliferative activity was consistently seen in hamster cheek pouch stroma adjacent to tumor implants despite separation of the tumor and its stroma by a millipore filter which prevented the passage of cells. Ehrmann [7] saw the same effect with choriocarcinoma in the hamster cheek pouch. We have demonstrated a similar effect using millipore chambers implanted on the fascial expanse of the dorsal air-sac of the rat. Empty millipore chambers or those containing non-neoplastic cells or fluids, produced no reaction.

More recently we have shown [9] that a diffusible factor which is mitogenic for capillary endothelium, can be isolated from animal and human tumor cells. This factor, called tumor-angiogenesis-factor (TAF) is isolated after disrupting tumor cells by nitrogen cavitation. Its important components are ribonucleic acid (25%) and protein. The activity of TAF is destroyed by ribonuclease and subtilisin but not by trypsin. TAF has a molecular weight of approx-

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imately 100,000. When TAF is injected continuously into the dorsal air-sac of a rat, dense areas of new capillaries proliferate in 48 hours. The regenerating endothelial cells within these capillaries incorporate tritiated thymidine and contain many mitotic figures. Upon cessation of the infusion of TAF, the endothelial mitotic activity subsides and the number of regenerating capillaries diminishes. TAF has not been found in normal tissues with the single exception of human placenta.

Evidence that Solid Tumors are Dependent upon Neovascularization for Sustained Growth

a) Absence of Neovascularization in Perfused Organs. Tumors implanted into isolated perfused organs will grow as solid spheres within the organ. For example, mouse melanoma will grow in perfused rabbit thyroid gland.[11] Time-lapse studies show that expansion of the colony ceases when it reaches a diameter of approximately 2–3 mm. These colonies never become vascularized in the isolated organ. The tumor cells, however, are viable and maintain their ability to elicit new blood supply if they are reimplanted into the original animal. The isolated organ appears unable to generate new blood vessels due either to inadequacy of the organ or a technical artifact of the perfusion.[12] In more than 100 combinations of perfused organs and implanted tumor, the absence of neovascularization has been the most common feature and has always been associated with cessation of tumor growth.

No matter how successfully an organ is perfused in isolation and no matter how long, no one has even been able to demonstrate new vessel formation, even if a wound was made.

b) Anterior Chamber of the Eye. Greene[15] observed that tiny tumors implanted for more than 1 year in the anterior chamber of the eye of a guinea pig could not enlarge beyond 2–3 mm. It is apparent in retrospect that these tumors could not have become vascularized. However, when these tumors were removed from the eye after a year of dormant existence and implanted in the muscle of a rabbit, rapid neovascularization was accompanied by rapid growth.

c) Population Kinetics in the Vascularized Tumor. Tannock[19, 20] has studied the population kinetics of vascular endothelial cells and parenchymal tumor cells within solid neoplasms, with a double labeling technic. His studies show that the probability of a tumor cell entering mitosis decreases with increasing distance of the cell from its nearest capillary. Thus, even after vascularization has been established, the efficiency of diffusion of nutrients and wastes diminishes with increasing distance from each capillary. Using the mouse mammary tumor, Tannock showed that the turnover time of the endothelial cell population (50 hours) lagged behind the turnover time of the tumor cells (22 hours). Thus as the tumor grew, the tardy proliferation of capillary endothelial cells led to an increase in the intercapillary distance. Proliferation of tumor cells furthest from a capillary diminished, so that a decreased rate in growth of the entire tumor resulted. Tannock suggested that the rate of proliferation of endothelial cells may limit indirectly the rate of tumor growth.

d) Population Dependence *In vivo*. When tumor cells are transplanted from one animal to another, either as packed cells or as a tiny graft, there is a minimal cell number for any given experimental tumor system, critical to further growth of the tumor. Donahoe,[6] working in our laboratory, has shown that this threshold mass can be markedly reduced by implanting the cells into a bed which has been previously vascularized. When 100 Walker-256 carcino-sarcoma cells are inoculated into the subfascial expanse of the dorsal air sac in a rat, no tumors are present at 6 days, even upon microscopic sectioning. How-

ever, when the fascial expanse had previously been exposed to a millipore chamber containing Walker cells, an area of neovascularization developed beneath the filter. When the millipore chamber was removed and 100 tumor cells were inoculated into this vascular bed, large, grossly visible tumors were present at 6 days. Thus the growth of fewer tumor cells in an inoculum is facilitated by pre-vascularization. In the controls, a millipore chamber containing no cells or non-malignant cells did not produce neovascularization and consequently facilitation of tumor growth was not achieved. One hundred Walker tumor cells implanted into this area did not produce any tumors at 6 days. Even if the vascular bed was produced by a millipore chamber containing B-16 melanoma cells, grossly visible tumors appeared 6 days after Walker tumor cells were inoculated into this area. The B-16 melanoma grows only in mice and does not take in rats. This experiment overcomes the objection that tumor cells passing through a leak in the millipore chamber might contribute to the 100 cells implanted subsequently. These experiments imply that when the implanted tumor population is very small, a few viable cells may take a very long time to generate a population necessary to elicit new blood supply. However, if new capillaries are already present, then a small population of tumor cells can become vascularized in an accelerated fashion.

Analogues to Unvascularized Tumors in Which Clonal Growth May Be Restricted by Phenomena of Simple Diffusion

a) **Spheroidal Tumor Colonies in Culture.** Folkman and Hochberg [17] have recently shown that tumor cells growing in three dimensional spheroids in soft agar grow rapidly up to a diameter of 1.75 mm. ± 0.2. No further expansion occurs beyond this diameter even in the face of unlimited exchange of fresh media. An outer layer of cells remains viable. These cells continue to undergo mitosis indefinitely. Cells in the center of the sphere slowly degenerate.

A spheroid thus reaches a steady state when birth rate and death rate are equal and no further expansion takes place. At this critical diameter the surface area of the sphere becomes insufficient to allow escape of catabolites generated; they accumulate and become a major factor in preventing further expansion of the spheroid. At the same time the limited diffusion of oxygen keeps the thickness of the outer shell constant at approximately 200 microns. It is because of these limitations of diffusion that a spheroidal colony of cells self-regulates its growth.

Without capillaries to expand the diffusion capacity such a nodule remains dormant although a major portion of its cells are still viable.

b) **Embryogenesis.** That growing cells, crowded together are affected by diffusion phenomena, may also extend to embryogenesis. This has been pointed out by Crick [5] who stated "A simple order of magnitude calculation suggests that diffusion may be the underlying mechanism in establishing morphogenetic gradients in embryonic development."

Discussion and Clinical Implications

It seems clear that within most solid malignancies, the interplay between tumor cells and endothelial cells constitutes a highly integrated ecosystem.

It is probable that most tumors whether they originate from a cell transformed by virus or carcinogen, or whether they begin as a metastatic implant, must exist early as a small population of cells dependent upon nutrients which diffuse from the extravascular space (Fig. 1). Such a pinpoint colony eventually expands to a size where simple diffusion of nutrients (and wastes)

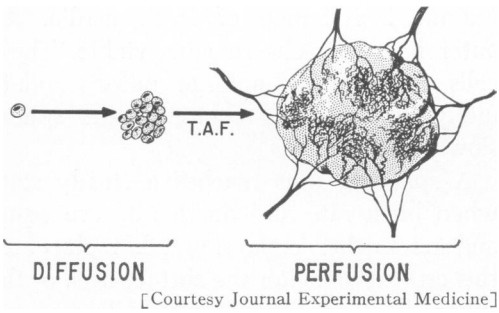

FIG. 1. Illustration of concept that most solid tumors may exist early as tiny cell populations living by simple *diffusion* in the extravascular space. Further growth requires vascularization and the tumor mass then maintains itself by *perfusion*. Tumor-angiogenesis-factor (TAF), may be the mediator of neovascularization.

is insufficient. New capillaries are elicited and the tumor then enters a phase in which nutrients arrive and wastes are removed by perfusion.

Because endothelial cell turnover usually lags behind tumor cell turnover, most solid tumors may always be on the verge of necrosis. Interference with their capillary component is likely to disturb this equilibrium and shift it toward more necrosis. If tumor-angiogenesis-factor is indeed the mediator for neovascularization then blockade of TAF might prevent vascularization, i.e., *anti-angiogenesis*.

By *anti-angiogenesis* we do not mean vasoconstriction or infarction of vessels already connected to the tumor. The term *anti-angiogenesis* should serve to indicate the prevention of generation of new vessels by an early tiny tumor implant usually 2–3 mm. in diameter.

Although this diameter is approximate, it is significant because most tumor implants in animals and several which we have seen in man are not yet vascularized at this size or are just beginning to be vascularized (Fig. 2). Further, we think that there is enough evidence to suggest that if neovascularization is prevented many solid tumors might remain fixed at this tiny diameter.

If we could hold a tumor in the nonvascularized, dormant state indefinitely, a number of questions are posed about which we can speculate.

Would such a dormant tumor metastasize? Recent evidence [16] suggests that metastases will diminish when the primary tumor is relatively devascularized. Metastasis from a primary solid tumor may well depend upon the presence of vascularization.

Would nonvascularized, dormant tumors be more susceptible to chemotherapy? Although tiny tumors generally are more vulnerable to chemotherapy, we do not know whether the dormant state resulting from *anti-angiogenesis* would be maintained by a high rate of cell loss or a decline in mitotic rate. Therefore, one cannot predict what kind of drug might be effective with such tumors. It is conceivable that chemotherapeutic killing of unvascularized tumor implants may actually be the mechanism by which Actinomycin, for example, is capable of preventing the recurrence of Wilms' tumor in the unexcised opposite kidney.

Are the cells in a nonvascularized, tiny tumor implant less hypoxic than those cells living in the avascular core of a large solid vascularized tumor? There may be a great difference between these two situations. It is possible that the cells in the former situation are well oxygenated; necrosis is not seen in these tiny implants. However, cells in the necrotic center of a large tumor may be severely hypoxic because they are surrounded by dense layers of cells competing for available oxygen.[8] If there is such a difference, then the susceptibility of the avascular core of a large tumor to chemotherapy or radiotherapy may be far less than the vulnerability of an unvascularized small tumor implant in which simple diffusion of nutrients and wastes is still sufficient for every cell in the clone.

What would be the susceptibility of an unvascularized, dormant tumor implant to immunologic attack? Again, we can only speculate. It is possible that an unvascu-

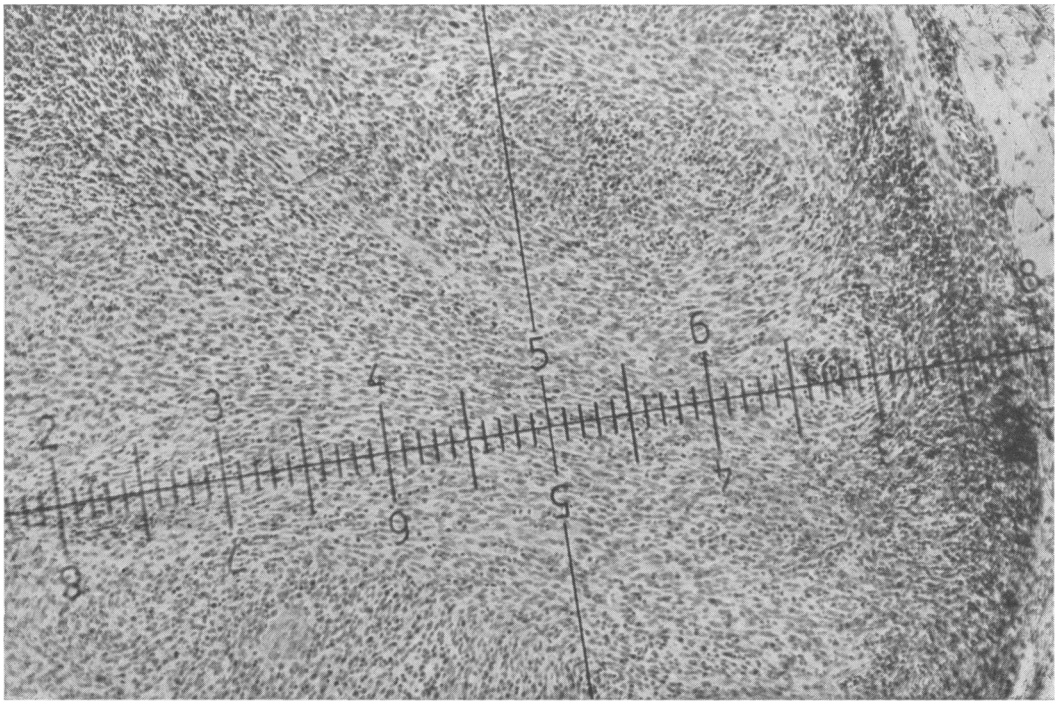

FIG. 2a. Human astrocytoma growing subcutaneously in Wistar rat, (fifth serial transplant). Tumor removed from rat after 22 days, at which time it was 2.0 mm. in diameter. There are no capillaries. Same tumor removed later will reach 1 to 2 cm. diameter and will be completely vascularized. Smallest unit in ocular grid is 0.025 mm. (H&E, 40×). Rats inoculated with this tumor were sent to us by Dr. Paul Kornblith, Neurosurgical Laboratories, Mass. General Hospital.

larized clone of tumor would be vulnerable to attack by immune lymphocytes because these cells can migrate from nearby vessels. It is also possible that circulating antibody would be unable to reach an unvascularized tumor in as high a concentration as it would accumulate in a vascularized tumor because of the diffusion gradient which might exist over such great distances. It is conceivable that unvascularized tumor colonies cannot be enhanced; that they are "out of reach" of humoral antibody and thus are exposed to the full force of mobile, immune lymphocytes. Therefore, *anti-angiogenesis* may synergize immunotherapy.

Assuming that *anti-angiogenesis* could be accomplished, would cessation of growth occur in all solid tumors? We have reviewed the histology of a wide range of human tumors from the point of view of endothelial regeneration within the tumor.[2] We have quantitated the extent of endothelial cell regeneration and capillary density. If one thinks of this not simply as *vascularity* but as endothelial cell dependency of the parenchymal tumor cells then there is a striking stratification of different tumors. Brain tumors appear to be most dependent upon endothelial cell proliferation, carcinomas slightly less, sarcomas less, and at the extreme, chondrosarcomas the least. Certain chondrosarcomas reach enormous dimensions yet capillaries are sparse and endothelial cells in these capillaries are not active. The chondroblasts seem to survive at great distances from capillaries without any necrosis. If this hierarchy of endothelial cell dependency is true, then it is possible that *anti-angiogenesis* might be a powerful therapeutic adjunct for brain tumors and

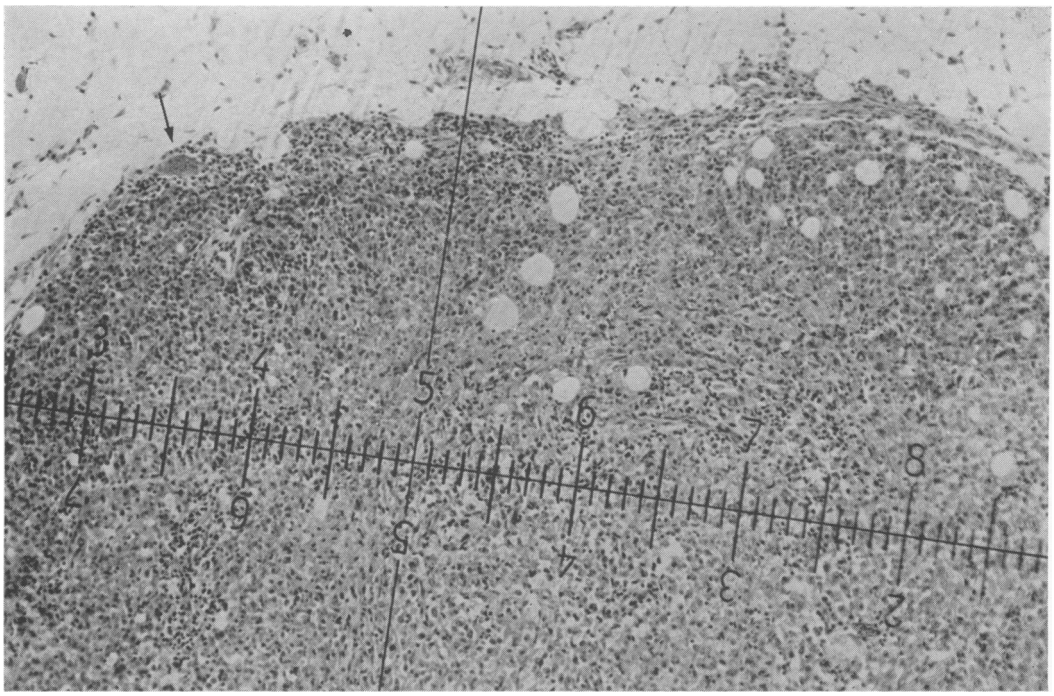

FIG. 2b. Embryonic sarcoma in 2-week-old baby. This nodule was a tiny skin metastasis measuring 2.5 × 3.0 mm. Vessels are not seen throughout the entire nodule except at the outer edge where a few capillaries are just beginning to penetrate the tumor (arrow). Smallest unit in ocular grid is 0.025 mm. (H&E, 40×)

carcinoma but not for sarcoma, and might be ineffective for chondrosarcoma. It is interesting in this regard that after a tumor field has been heavily irradiated, as for Wilms' tumor, the tumor most likely to occur years later in this irradiated field is the chondrosarcoma, as if it had almost no requirement for an endothelial cell population. In regard to nutrients and wastes, it behaves as the "cactus" of solid tumors.

The concept of *anti-angiogenesis* raises two questions which are more fundamental.

How is information transferred between the malignant tumor cell and the benign endothelial cell? Capillary endothelial cells, which normally exist in a non-renewal state, are stimulated by neighboring malignant cells into high mitogenic activity. However, a permanent genetic lesion is not produced in the endothelial cell; the regenerative activity of the endothelial cell is only transient.

Why should a tumor colony stop expanding when it cannot become vascularized? What metabolites are responsible? Do they operate by suppressing mitosis or by increasing the rate of cell loss?

Some of the arguments we have presented have certain weaknesses: the evidence is not yet available which would guarantee their validity. For example, we have shown that TAF extracted from human and animal tumors produces new blood vessels in rats and rabbits. There is a possibility (although an unlikely one) that TAF may not be what tumors use to stimulate new vessels. An alternative hypothesis is that a growing tumor acts as a "sink" for the consumption of some repressor which might exist in endothelial cells to prevent them from entering mitosis under normal conditions. *Anti-angiogenesis* would still be possible but through a different pathway.

Despite these difficulties, it is my belief that *anti-angiogenesis* is not only possible but plausible. The attempt to achieve it may provide a new understanding of the early phase of solid tumor development.

Summary

Evidence is presented to suggest that neovascularization is mediated through a humoral factor called TAF.

The concept of *anti-angiogenesis* is proposed to indicate that blockade of TAF might prevent many solid tumors from growing beyond a diameter of 2–3 mm.

Extension of this concept raises new questions for future study.

a) Is it possible to make antibody against tumor-angiogenesis factor?
b) If vascularization of a new tumor implant could be blocked, would this prevent metastasis from this tumor?
c) Would an unvascularized tumor be more susceptible to chemotherapy?
d) Would an unvascularized tumor be more vulnerable to cell mediated immunological attack than a vascularized, rapidly growing tumor?
e) Is there a hierarchy of endothelial cell dependency in which brain tumors are the most dependent and chondrosarcomas the least?
f) How is this transfer of information from malignant cells to endothelial cells accomplished?
g) If blockade of angiogenesis leaves a tiny dormant tumor, is the dormant state due to the accumulation of inhibitory metabolites?

If *antiangiogenesis* can be achieved it may become a powerful adjunct to present methods of therapy. If it cannot, or even if the concept is wrong, the careful exploration of its consequences may enable us to see something fundamental about the nature of growing cells in packed population *in vivo*.

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Erratum

In Inahara, T.: Acute Superior Mesenteric Venous Thrombosis: Treatment by Thrombectomy, Ann. Surg., 174:960, 1971, line 5 under **Treatment** should read: intervention should *not* be delayed. etc.