Tumor Angiogenesis Factor

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Summary

Recent evidence from our studies indicates that solid tumor growth is not continuous but that it can be separated into two stages, avascular and vascular. In the avascular stage, tumors remain dormant at diameters of 1 to 2 mm. Further growth is possible only after new capillaries have been elicited from the host and have penetrated the tumor. This capillary proliferation is stimulated by a diffusible factor, tumor angiogenesis factor, released by solid tumors, and by neoplastic cells in culture.

The biology and isolation of tumor angiogenesis factor are discussed. Evidence for the mechanism of tumor dormancy in the absence of angiogenesis will be presented. Therapeutic implications of the inhibition of tumor angiogenesis will be mentioned briefly.

Introduction

There is increasing evidence that tumor cells communicate with normal host cells. NGF is an example (14). Certain mouse sarcomas secrete a factor that stimulates growth in neighboring sensory and sympathetic nerve cells. However, the secretion of NGF is limited to a few tumor types. Furthermore, NGF is not necessary for continued tumor growth; in a sense, it is a side product or a luxury molecule. The majority of unusual molecules and inappropriate hormones thus far known to be secreted by tumors are each restricted to a small group of tumors and are of little importance to the successful growth of the tumor. By contrast, the ability of malignant solid tumors to stimulate proliferation of new capillaries is common to a wide variety of neoplasms and appears to be an essential requirement for progressive tumor growth (1, 4). No doubt it will be found that other forms of communication between tumor and host may be important for tumor survival (13).

Therefore, it seems timely to examine our present understanding of the relationship between solid tumor and vascular endothelium and to focus on the concept that information exchange between these 2 tissues operates in 2 directions. New capillaries induced by tumor in turn appear to play a key role in regulating tumor growth.

The Relationship between Tumor and Vascular Endothelium

New information has become available in the past decade concerning tumor vessels and their endothelial cells. For example, it has been shown that from 10 to 40% of a solid tumor may consist of vascular endothelial cells (1). Furthermore, we now realize that the life of a solid tumor can be separated into 2 stages: before vascularization and after vascularization (6). Most solid tumors seem capable of living by simple diffusion until they reach a diameter of a few mm, after which new vessels penetrate them. In animals this conclusion is based on studies of tumor growth in the rabbit ear chamber and the hamster cheek pouch (18–20). In man, this information derives from studies of biopsy and autopsy specimens of tiny metastases that have not yet vascularized (5).

Furthermore, we know that solid tumors cannot make capillaries on their own but must elicit these vessels from the host. This generalization is supported by 3 kinds of experiments. (a) When tumors are implanted into the rabbit ear chamber, the hamster cheek pouch, or the rat dorsal air sac, new vessels always arise from the host and grow toward the tumor; vessels never extend from the tumor into the host (3, 18). (b) Small tumor implants or inoculations of tumor cells from tissue culture inserted into the cornea of the rabbit eye attract new blood vessels from the limbus at the edge of the cornea. These are host vessels; microscopic study of the cornea has never revealed vessels arising from the tumor implant (11). These are host vessels; microscopic study of the cornea has never revealed vessels arising from the tumor implant (11). (c) When a tumor graft is placed within an incision in the chick chorioallantoic membrane, no new vessels penetrate the tumor until approximately 3 days after implantation. The tumor remains pale and unvascularized (D. Knighton, D. Ausprunk, D. Tapper, and J. Folkman, unpublished data). Repeated microscopic observations of such a graft show that vessels previously resident within the tumor graft begin to disintegrate within 24 hr after transplant to the chorioallantoic membrane (D. Ausprunk, unpublished data; Ref. 15). This degeneration occurs despite the fact that the tumor graft is taken from a well-vascularized portion of a rapidly growing animal tumor. In contrast, when grafts of normal embryonic tissues such as muscle or endocrine glands are carried to the chorioallantoic membrane, their own vessels not only survive but also begin to connect with the vessels of the chorioallantoic membrane within 24 hr. Therefore, in a general sense, normal tissues, whether grafted to a syngeneic or to an allogeneic host, usually contain vascular endothelium belonging to the donor tissue; while a tumor graft ulti-

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2 The abbreviations used are: NGF, nerve growth factor; TAF, tumor angiogenesis factor.
It is also becoming clear that vascular endothelium in adult animals is a relatively quiescent tissue, renewing itself very slowly with a labeling index of about 0.5% or less (16). The most rapidly growing vessels in the chorioallantoic membrane of the 7- to 10-day chick embryo may reach a peak labeling index of 27% (2), but in the neighborhood of a tumor implant, whether placed among developing vessels or mature vessels, the labeling index may be 37% or more. Although the precise mechanism by which solid tumors continually elicit new capillaries from the host is not clear, there is evidence that this information is transmitted from the tumor to the vascular endothelial cells by a diffusible factor.

Tumors separated from the vascular bed by a Millipore filter will induce new capillary growth on the other side of the filter (12). In separate experiments using the dorsal s.c. air pouch in the rat (3) and the anterior chamber of the rabbit eye (11), we were able to show that the distance over which tumor can stimulate endothelial mitosis is greater than the 25-µ thickness of the Millipore filter and can be as great as 3 to 5 mm.

More recently, we have been able to separate a cell-free fraction from tumor cells that will induce the growth of new capillary sprouts. This fraction, called TAF, has not been detected in normal tissues or fibroblasts in primary culture, with the possible exception of trophoblast and mouse salivary gland (7), although the possibility is not precluded that the TAF may eventually be found in other nonneoplastic tissues at very low levels. TAF is mitogenic to endothelium of capillaries and small venules. It is a protein with a molecular weight of approximately 100,000; it contains about 3 parts RNA and is inactivated by RNase. TAF activity has been found in both the cytoplasm and the nucleus of several animal and human tumors, although the majority of our work has utilized the Walker 256 carcinosarcoma. The material can now be isolated both from tumor in the solid or ascites form and from tumor cells in culture. Where tumor nuclei are used as the source of TAF, angiogenesis activity is associated with the nonhistone proteins of chromatin; while the histone proteins lack activity (17). Angiogenesis activity in the nonhistone proteins has been resolved to approximately 20 protein moieties by acrylamide gel electrophoresis. We are presently engaged in purifying TAF obtained both from the nonhistone fraction and from fractions isolated from the cytoplasm of intact cells in culture.

Bioassays for TAF

The bioassays available for TAF are carried out in vivo and are only qualitative. We do not as yet have a quantitative or an in vitro assay. Two assay systems are routinely used as follows.

Chorioallantoic Membrane of the Chick Embryo. A window is made in egg shell at Day 8 of incubation and the chorioallantoic membrane allowed to drop. The test fraction is then added on Day 10. The fraction to be tested, usually an eluate from a Sephadex column, is first steri-

lized by Millipore filtration, dialyzed against distilled water, and lyophilized. Approximately 0.01 ml of 0.9% NaCl solution is added to the few crystals that remain (usually in the range of a 5 to 25 μg of total protein). This solution is soaked up into 3 tiny pads made of glass fiber filter paper previously impregnated with 5% polyacrylamide gel and then washed in Ringer’s lactate solution. Each paper is placed on the chorioallantoic membrane of a separate egg together with a control filter paper containing no protein or inactivated protein. The filter paper is placed over 3 or 4 tiny holes made in the chorioallantoic membrane with a No. 30 hypodermic needle just before positioning. This guarantees that the ectodermal layer of the chorioallantoic membrane will not be a barrier to diffusion of the test fraction. When fractions are positive, this is detectable in 2 to 4 days by new vessels, which grow toward the filter paper, forming a “red spoke wheel” appearance. The density of the vessels in this spoke wheel allows a qualitative grading of 1+ or 5+. No new vessel reaction is excited by the control filter. This assay will respond to approximately 5 μg of total protein (containing angiogenesis activity).

Rabbit Cornea. Fractions of protein to be tested are inserted into pockets made in the cornea as previously described (11). Highly concentrated fractions can be inserted directly into this pocket, which holds a volume of approximately 20 μl. Alternatively, these fractions can be dispersed in 5% polyacrylamide gel and injected into the pocket, allowing a slower release of the protein. The polyacrylamide gels are occasionally inflammatory in the cornea unless they are buffered to 7.4 with N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid and made isosmotic. Approximately 1 μg of total protein can be dispersed in 20 μl of acrylamide gel in such a pocket. The eye is examined daily with a stereoscopic slit lamp. New vessels grow out from the limbus, traverse the cornea, and enter the pocket. Inactive fractions do not attract vessels. In this technique, the rate of vessel growth can be measured. Capillary growth reaches a maximum of 0.3 mm/day. This assay is used as a supplement for the chick embryo assay.

Inhibition of Angiogenesis

If neovascularization were only a side effect of tumor growth, then the effort to isolate, characterize, and inhibit TAF might seem unimportant. However, we are beginning to appreciate that the induction of new capillaries by tumor is critical to progressive tumor growth. In terms of tumor progression, TAF may be one of the most important molecules secreted by a tumor, because without TAF most tumors would remain avascular and dormant at a diameter of a few mm. This idea turns upon 4 pieces of experimental evidence, as summarized in Chart 1.

The 1st information comes from tumor implants grown in isolated perfused organs (7). We have shown that small organs such as rabbit thyroids, when perfused through their arterial tree in isolated chambers, will support tumor growth. However, vascular endothelial cells in these organs
are injured by the perfusion in such a way that neither endothelial mitosis nor capillary proliferation is possible during isolated perfusion. Because of this unique artifact, tumors growing deep within these organs reach diameters of 1 to 2 mm but fail to be vascularized and never grow further. They remain viable, as can be demonstrated by reimplantation into their original host animal, whereupon they vascularize and grow.

The 2nd piece of evidence originated from a clinical observation and was expanded in laboratory studies. In certain eye tumors, such as the retinoblastoma, cells metastasize into the aqueous humor of the anterior chamber and into the vitreous humor of the posterior chamber. Tumor nodules grow from these and live in suspension (Fig. 1A; Chart 2). Capillaries do not traverse the liquid environment, and they fail to reach the tumors. These nodules remain avascular and rarely exceed a diameter of 1 mm. They do remain viable, as demonstrated by microsections (Fig. 1B). In our laboratory, Gimbrone et al. (10) demonstrated this phenomenon in animals by inoculating the anterior chamber of the rabbit eye with Brown-Pearce tumor. Again, it was observed that floating tumor nodules always remained avascular, but viable, and never exceeded a volume of 0.9 cu mm. They could be transplanted from one anterior chamber to another. The nodules could be vascularized by bringing them in contact with the iris vessels. Once these spheroids were penetrated by vessels, there was rapid exponential growth; and tumor volumes increased as much as 16,000 times within 2 weeks of the onset of vascularization.

The 3rd piece of evidence is derived from a study of tumor spheroids in vitro (8). Tumors were grown in soft agar to simulate conditions in the anterior chamber. Each tumor was suspended in a large volume of medium which was changed frequently. These isolated spheroids eventually reached a size (2 to 4 mm) beyond which no further growth was observed. This was called the "dormant diameter." These spheroids (B-16 mouse melanoma, V-79 Chinese hamster lung, and L-5178Y murine leukemia cells) remained viable for the duration of the experiment, up to 280 days. Cells in the periphery incorporated thymidine-3H. Daughter tumors grew from spheroids which were fragmented or dispersed, but these also stopped growing at the dormant diameter. The total number of viable cells in any spheroid never exceeded \(10^6\). When other spheroids were added to the same culture flask, the mean dormant diameter diminished in proportion to the number of neighboring tumors. This more closely resembled the in vivo situation, where many cell populations are bathed by the same fluid. It would also account for the variance between the somewhat larger dormant diameter of an isolated spheroid compared with the dormant diameter of multiple spheroids growing in the anterior chamber of the eye. We used the term "dormancy" to indicate an equilibrium between proliferating cells at the periphery of the spheroid and dying cells in the necrotic center. In 3-dimensional cell populations, the surface area expands by the 2nd power, while the volume increases by the 3rd power. For this reason we proposed that the onset of dormancy would begin when the volume of cells reached a point at which their aggregate surface area was insufficient to provide for adequate escape of catabolites and absorption of nutrients (including oxygen).
The 4th piece of evidence comes out of experiments with chick embryos by Knighton et al. (unpublished data). Tumors were implanted into the yolk sac as early as Day 3 and into the chorioallantoic membrane by Day 5. These implants, surrounded by vessels, grew to diameters of approximately 1 mm and then became dormant until penetrated by new capillaries at approximately 72 hr. At this point, there was rapid tumor growth. In this experiment, the tumor—during its avascular phase—is surrounded by contiguous capillaries, yet the surface area of the aggregate population still limits the diffusion of wastes and nutrients, just as it does in the tumor immersed in soft agar or aqueous humor. The surface area is suddenly expanded only after new capillaries penetrate the tumor. The use of the chick embryo allows the avascular phase and the vascular phase of tumor growth to be visualized and the dormant period displayed. A similar experiment is not yet possible in an animal-tumor system, because the separation between the avascular and the vascular phase is imperceptible. When we have learned to inhibit TAF, it should be possible to prolong the avascular phase in animals.

Conclusion

In conclusion we can say that: (a) tumors must elicit new capillaries from the host; (b) tumors do this by a chemical signal that diffuses over a distance of at least 3 to 5 mm; (c) vessels must penetrate a tiny tumor spheroid before further growth beyond approximately $10^5$ cells is possible; (d) without penetration by capillaries, surface area becomes limiting to further growth, and tumor nodules enter a dormant phase at a diameter of a few mm; and (e) ves-
sels induced by tumor have a short half-life and quickly regress when the stimulus is turned off.

What is much less clear is the nature of this chemical signal (TAF), its uniqueness to malignant cells, and whether it is related to mitotic rate in tumor cells.

Evidence from the study of avascular spheroids suggests that malignant tissues are no more autonomous with respect to food supply and catabolite elimination than their normal counterparts. Once a means can be found to inhibit TAF, either by the corresponding antibody or by some other pathway, then it might be possible to hold tumors in an avascular or dormant state indefinitely. This potential approach to cancer control, “antiangiogenesis,” could well become an important therapeutic adjunct. For example, it is possible that vascularized tumors are largely beyond the reach of conventional immunotherapy and that immunotherapy preceded by antiangiogenesis would be a powerful synergistic approach.

References
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