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# Tumor Angiogenesis Activity in Cells Grown in Tissue Culture[1]

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## SUMMARY

Normal, viral transformed, and tumor-derived cells grown in tissue culture and representing different species were tested for their ability to produce an extracellular tumor angiogenesis factor (TAF). TAF was assayed by measuring the host-mediated vascular response of the chorioallantoic membrane to TAF preparations. All of the viral transformed and tumor-derived cells tested, including SVT2, SVW126, Walker 256 rat carcinoma, B-16 mouse melanoma, human glioblastoma, and human meningioma cells, produced TAF.

The potency of the TAF preparations, as measured by the number of cells needed to induce a positive vascular response on the chorioallantoic membrane, varied from cell line to cell line. The most potent cells tested were the glioblastoma and meningioma brain tumor cells. Since these brain tumors are found to be the most highly vascularized tumors *in vivo*, it was concluded that a correlation exists between the vascularity of a tumor *in vivo* and the potency of TAF *in vitro*.

There was no detectable angiogenesis activity induced by density-inhibited BALB/c primary mouse embryo or early-passage human skin fibroblasts, even when relatively large numbers of cells were used to make a sample. However, density-inhibited BALB/c 3T3 and W138 human embryonic lung fibroblasts, two cell lines widely regarded as demonstrating "normal" growth behavior in culture, produced TAF. From these and other observations, it was suggested that BALB/c 3T3 and W138 are not fully "normal" cells. Furthermore, it was suggested that the production of TAF is an early event in the cell transformation process that precedes the loss of density inhibition of growth *in vitro*.

## INTRODUCTION

Solid tumors release a cell-free diffusible factor, TAF,[2] which induces neovascularization in the host (7, 14). The host blood vessels vascularize the solid tumor and provide nutrients facilitating tumor growth (4). TAF activity has been detected in solid tumors of diverse origins, including Walker 256 rat carcinoma, mouse melanoma, human choriocarcinoma, and human neuroblastoma, but it has not been detected in normal liver and kidney (6).

We have conducted controlled and quantitative studies of TAF activity in a variety of cells grown in tissue culture. The cells tested include normal cells, which are subject to density inhibition of cell division, as well as viral transformed cells and cells cultured from tumors. All viral transformed and tumor-derived cells tested have detectable tumor angiogenesis activity, while primary or early-passage cells do not.

Unexpectedly, BALB/c 3T3 and diploid human embryonic lung fibroblasts (W138), both of which display density-dependent inhibition of growth (9, 12), have substantial tumor angiogenesis activity.

## MATERIALS AND METHODS

**Cells and Media.** BALB/c 3T3 (clone A31) and SVT2 (SV40-transformed BALB/c 3T3 clone A31) cells (1) were obtained from Dr. C. Scher, and diploid W138 human embryonic lung cells (passage 24) were obtained from Dr. G. Bruns, both of Children's Hospital Medical Research Center. SVW126 (8) cells (SV40-transformed human embryonic lung fibroblasts) were obtained from Dr. N. Rosenberg (Massachusetts Institute of Technology, Cambridge, Mass.), and human skin fibroblasts (passage 11) were obtained from Dr. P. Farrell (NIH). Primary mouse embryo fibroblasts were obtained by trypsinization of decapitated 16-day-old BALB/c mouse embryos. Walker 256 rat carcinoma cells were prepared by mincing solid tumors excised from 21-day-old white cesarean-derived (CD) rats that had been given injections of $8 \times 10^6$ tumor cells 4 to 6 days previously. The cells were plated and passed in tissue culture flasks. B-16 melanoma cells were prepared by mincing melanomas excised from 4- to 6-week-old C57/B16J black mice that had been given injections of $2 \times 10^6$ cells the previous week and plating the cells into tissue culture flasks. Human glioblastoma and meningioma cells were obtained by trypsinization of human brain tumors obtained from Dr. S. Brem (Massachusetts General Hospital, Boston, Mass.).

All cell lines were grown in Dulbecco's modified Eagle's media (11) (Flow Laboratories, Rockville, Md.) containing 4.5 g glucose per liter and 10% calf serum (Colorado Serum Co., Denver, Colo.) and supplemented with glutamine, penicillin, and streptomycin. The cells were grown in T-75 tissue culture flasks or in roller bottles.

**Preparation of TAF.** When exponentially growing cells were about 50% confluent, the culture media was removed and the cells were washed twice with lactated Ringer's medium. Cells in T-75 flasks were rocked gently at 4° for 3 hr in 6 ml of lactated Ringer's. Cells in roller bottles were rolled slowly for 3 hr at 4° in 15 ml of lactated Ringer's. The lactated Ringer's solution was removed and spun for 10 min at 5000 × *g*. The supernatant solution was then filtered through a 5.0-$\mu$m filter (Millipore Corp., Bedford, Mass.)

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[2] The abbreviation used is: TAF, tumor angiogenesis factor.

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and dialyzed for 48 hr against distilled water with 2 changes. The dialysate was lyophilized and stored at −20°. This cell-free crude fraction contains TAF activity. These preparations were free of bacterial contamination. Little if any cell lysis occurred in any of the cell lines during the extraction procedure.

**Bioassay of TAF on the Chorioallantoic Membrane of the Chick Embryo.** TAF fractions were assayed on the chorioallantoic membrane as previously described by Folkman (5) with some modifications. Lyophilized samples, containing TAF produced by a known number of cells, were dissolved in 20 μl of phosphate-buffered saline and applied to sterile discs of Whatman GF/B glass fiber filters (6 mm in diameter; Reeve-Angel, Clifton, N. J.). Mild inflammation occurs occasionally at the edges of, but not underneath, the filter. This response might be caused by the jaggedness of the cut edge. However, this mild response when it does occur does not interfere with the assay since it can be easily differentiated visually from angiogenesis. Angiogenesis is characterized by an increased density of vessels beneath the filter as well as toward the filter. The different characteristics of inflammation and angiogenesis have been discussed previously (4). Prior to implantation of the impregnated filter, 3 holes were made in the chlorioallantoic membrane with a 30-gauge needle at the site of implantation (5). This facilitated the absorption of the TAF through the ectodermal layer of the chorioallantoic membrane. Samples were usually applied on the 10th day of the embryo's development. The response of the chorioallantoic membrane to the test fractions was scored qualitatively as strong, weak, or negative, depending on the number and density of vessels in the chorioallantoic membrane directed towards the implantation site. (Fig. 1, *a* and *b*, depicts a negative and a strong vascular response, respectively.) The chorioallantoic membrane was observed every 24 hr and scored 5 days after implantation. Positive vascular responses were usually seen within 48 hr.

**Photography of Chorioallantoic Membrane.** Embryos were killed by injection of 10% buffered formalin phosphate (Fisher Scientific, Medford, Mass.). The chorioallantoic membrane was excised, fixed in formalin, inverted, and photographed with a Polaroid MP-3 camera.

## RESULTS

**TAF Activity in Cultured Cells.** A number of cell types, displaying either density inhibition of cell division or unrestrained cell division in tissue culture, were grown exponentially and assayed for TAF activity as described in "Materials and Methods." The vascular responses of the chorioallantoic membrane to TAF prepared from human skin fibroblasts and SVT2 cells are shown in Fig. 1, *a* and *b*, respectively. Human skin fibroblasts (Fig. 1*a*) show no tumor angiogenesis activity since there are no more vessels directed toward the filter than is the case for a control glass fiber filter containing lactated Ringer's alone. The vessel architecture in the chorioallantoic membrane shown in Fig. 1*a* resembles that of a normal chorioallantoic membrane of the same age. SVT2 cells (Fig. 1*b*) display strong tumor angiogenesis activity as evidenced by the presence of many vessels directed toward the SVT2 TAF-impregnated disc. Especially significant is the density of vessels seen beneath the filter.

The tumor angiogenesis activity of a number of different cell types is summarized in Table 1. The results are scored as strong, weak, or negative when compared with lactated Ringer's impregnated discs. The viral transformed and tumor-derived cell lines tested (SVT2, SVW126, mouse melanoma, Walker carcinoma, human glioblastoma, and human meningioma) all produced substantial TAF activity (Table 1A). In these cell lines strong vascular responses were found in over 50% of the eggs assayed and weak or no vascular responses in the remainder.

The potency of the TAF preparations, as measured by the number of cells required to induce a strong vascular response, varied considerably from cell line to cell line. The human brain tumor cells derived from a meningioma were the most potent with strong TAF activity found in samples prepared from as few as $10^5$ cells. The Walker carcinoma cells were the least potent tested, requiring at least $6 \times 10^6$ cells/sample to obtain strong TAF activity. In addition, TAF activity in a sample could be gradually eliminated by dilution with buffer. A sample made from $4 \times 10^6$ SVT2 cells induced a vascular response, while a 1:10 dilution of this sample, representing $4 \times 10^5$ cells, did not.

Two cell types displaying density inhibition of growth in tissue culture were found to contain no detectable TAF activity, even when large numbers of cells were used to prepare a sample (Table 1B). Human skin fibroblasts were negative even when as many as $2 \times 10^7$ cells were used to prepare a sample. BALB/c primary mouse embryo fibroblasts showed no TAF activity with as many as $1 \times 10^7$ cells/sample. Larger number of cells were not tested.

Two other cell types, BALB/c 3T3 and W138 human embryonic lung, which are subject to density inhibition of cell division in tissue culture (9, 12) were also tested (Table 1A). Unexpectedly, both cell types induced vascular responses. Density-inhibited BALB/c 3T3 cells, which showed less than 1% of the cells synthesizing DNA at confluence, displayed strong TAF activity in at least one-half of the eggs assayed. W138 cells also showed at least weak TAF activity in more than one-half of the eggs assayed.

## DISCUSSION

Solid tumors produce a factor mitogenic for endothelial cells (4, 7, 14). This TAF induces capillary proliferation in the host tissue, which leads to tumor vascularization and subsequent growth. We have now extended our studies of TAF to cells grown in tissue culture, since these experimental growth conditions allow for controlled and quantitative studies.

Six cell types that lack density inhibition of cell division were tested for TAF activity. All 6, including mouse and human cells transformed by SV40, melanoma cells, carcinoma cells, and cells derived from human brain tumors, were found to produce substantial amounts of TAF activity. This was observed in a bioassay measuring the vascular response of the chorioallantoic membrane to samples of TAF.

Table 1

*TAF activity in tissue-cultured cells as measured by a vascular response on the chorioallantoic membrane*

| A. Cells with TAF activity | Species | No. of eggs assayed | No. of eggs with following vascular response | | | Minimum no. of cells[a] needed for a positive vascular response |
|---|---|---|---|---|---|---|
| | | | Strongly positive | Weakly positive[b] | Negative | |
| SVT2 (BALB/c 3T3 transformed by SV40) | Mouse | 42 | 24 | 16 | 2 | $2–4 \times 10^6$ |
| BALB/c 3T3 | Mouse | 14 | 7 | 5 | 2 | $2–4 \times 10^6$ |
| B-16 melanoma | Mouse | 10 | 5 | 3 | 2 | $2–4 \times 10^6$ |
| Walker 256 carcinoma | Rat | 13 | 8 | 2 | 3 | $4–6 \times 10^6$ |
| W138 embryonic lung | Human | 9 | 2 | 5 | 2 | $4–6 \times 10^6$ |
| SVW126 (W126 embryonic lung transformed by SV40) | Human | 20 | 9 | 7 | 4 | $4–6 \times 10^6$ |
| Glioblastoma (brain) | Human | 5 | 5 | 0 | 0 | $0.5–1 \times 10^6$ |
| Meningioma (brain) | Human | 10 | 6 | 2 | 2 | $1–2 \times 10^5$ |

| B. Cells with no detectable TAF activity | Species | No. of eggs assayed | No. of eggs with following vascular response | | | Highest no. of cells tested yielding a negative vascular response[c] |
|---|---|---|---|---|---|---|
| | | | Strongly positive | Weakly positive | Negative | |
| BALB/c primary embryo | Mouse | 14 | 0 | 1 | 13 | $0.6–1 \times 10^7$ |
| Skin fibroblasts (passage 11) | Human | 12 | 0 | 3 | 9 | $1–2 \times 10^7$ |

[a] These are the number of cells in the culture flask at the time of extraction.

[b] These are characterized by a smaller number of vessels directed towards the test samples than is the case with strong responses. However, they differ significantly from blank filter controls.

[c] This does not imply that larger numbers of cells gave positive response but rather that larger numbers of cells were not tested.

The potency of a cell line for TAF production, as measured by the minimum number of cells required to produce an extract able to induce a vascular response on the chorioallantoic membrane, differs from cell line to cell line. Cells derived from human brain tumors are the most potent since as few as $10^5$ cells are needed to induce a vascular response. Walker carcinoma cells are the least potent tested and require as many as $6 \times 10^6$ cells to induce a similar response. Cells transformed by SV40 show intermediate values.

The relatively high potency of cells derived from brain tumors for TAF activity is consistent with the results of Brem *et al.* (3), which indicate that brain tumors are the most highly vascularized class of solid tumors found *in vivo*. This suggests that a correlation exists between the vascularization of a tumor *in vivo* and the potency of TAF *in vitro*.

Preliminary diploid mouse and early-passage diploid human skin fibroblasts, which show density inhibition of cell division in tissue culture, display no detectable TAF activity even when more than $10^7$ cells are used to prepare a sample for assay.

BALB/c 3T3 mouse and diploid human embryonic lung fibroblasts (W138) were found to display substantial levels of TAF activity. This was unexpected since these cell types show density inhibition of growth in tissue culture and are generally considered to be prototypes of the "normal" cell (9, 12). There are several possible explanations for these findings. The fact that BALB/c 3T3 is an established and permanent cell line arising from embryo cells implies that transformation, although not necessarily viral transformation, has taken place. Thus, while the BALB/c 3T3 cells exhibit density inhibition of growth *in vitro*, a characteristic associated with normal cells, it is possible that some properties of the transformed state are nevertheless present. For example, BALB/c 3T3 cells have high levels of plasminogen activator (D. Rifkin, personal communication), a characteristic associated with transformation (10). In addition, BALB/c 3T3 cells, when implanted onto the chorioallantoic mem-

brane, become vascularized and grow as a solid mass (unpublished observations). This suggests that the growth pattern of the BALB/c 3T3 cell is partially determined by its environment. The so-called "normal" growth pattern generally attributed to the BALB/c cell may be due to the experimental conditions of the tissue culture flask. Continuous feeding of BALB/c 3T3 cells in tissue culture flasks does lead to piling up and loss of growth control (13). Analogously, the continual feeding of these cells in the chorioallantoic membrane due to the presence of BALB/c 3T3 TAF-induced capillaries leads to 3-dimensional growth on the chorioallantoic membrane.

High plasminogen activator activity has also been found in W138 and other lung cells (D. Rifkin, personal communication). This fact, along with the observation that W138 has TAF activity, suggests that even nonpermanent cell strains have some parameters associated with transformation. These transformed properties may become more evident with increased passage number. Alternatively, it is possible that lung tissue, which is vascularized, might naturally have TAF-like activity.

Recently, it has been demonstrated that density-inhibited BALB/c 3T3 cells are tumorigenic in the mouse when implanted on beads (2). Thus, tumorigenicity *in vivo* and density inhibition of growth *in vitro* are not always mutually exclusive. The fact that density-inhibited cells such as BALB/c 3T3 and W138 produce TAF suggests that cell transformation is a multistep process and that TAF production is an early event in cell transformation that precedes alterations in growth behavior. Since the vascularization of tumors is a necessary step leading to tumor growth, it is suggested here that relative levels of TAF in a cell, as assayed by the vascular response of the chorioallantoic membrane, might be a useful indicator of the transformed state and a predictor of the potential of a cell to be tumorigenic.

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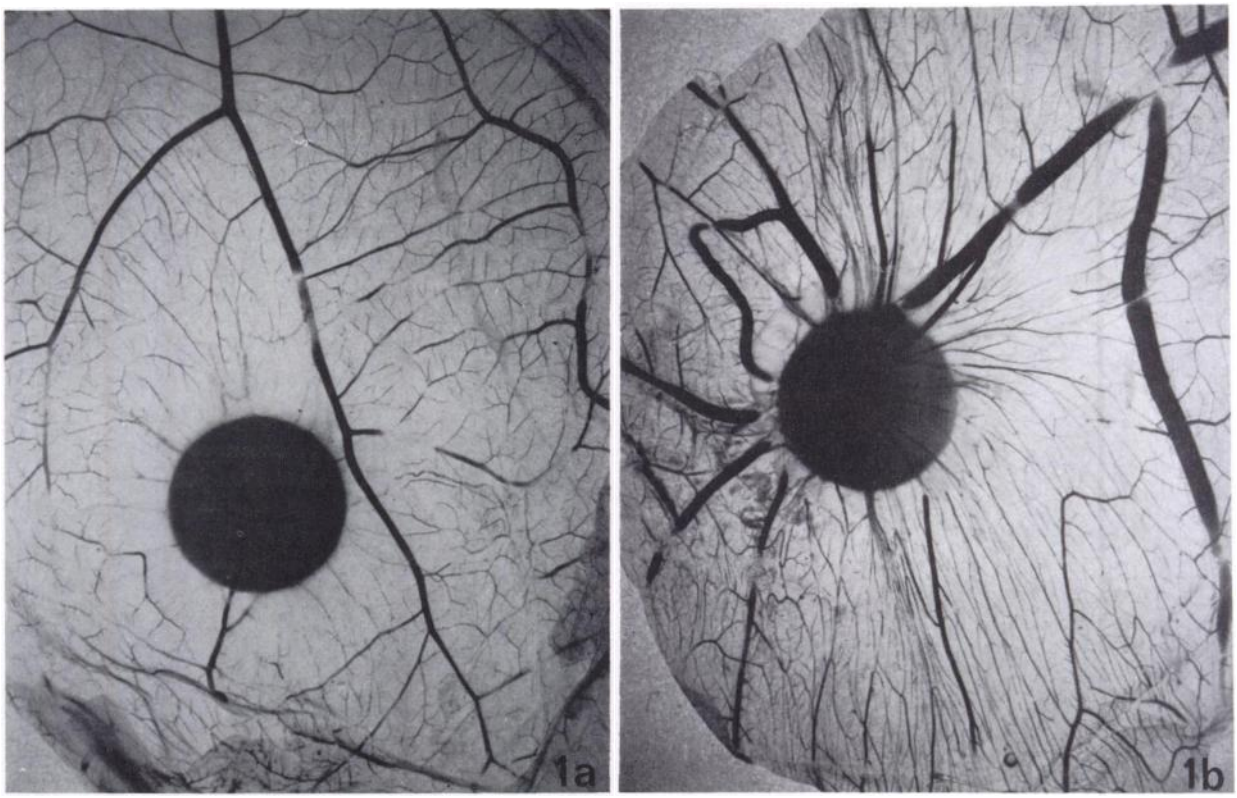


Fig. 1. Vascular responses of the chorioallantoic membrane to samples of TAF. TAF samples were prepared, concentrated, and adsorbed onto GF/B glass fiber discs (6 mm in diameter) as described in "Materials and Methods." The discs were placed on the chorioallantoic membrane of a 10-day-old chick embryo. Five days later, the chorioallantoic membrane was fixed in formalin and cut out of the egg. The chorioallantoic membrane was turned upside down and photographed to best display the blood vessels underneath the filter. *a*, response to TAF preparation from $1 \times 10^7$ human skin fibroblasts (passage 11); *b*, response to TAF preparation from $4 \times 10^6$ SVT2 fibroblasts.