References and Notes

1. J. A. Svoboda, J. N. Kaplanis, W. E. Robbins, M. J. Thompson, *Annu. Rev. Entomol.* **20**, 205 (1975).
2. M. J. Thompson, J. N. Kaplanis, W. E. Robbins, S. R. Dutky, H. N. Nigg, *Steroids* **24**, 359 (1974).
3. D. H. S. Horn, in *Naturally Occurring Insecticides*, M. Jacobson and N. G. Crosby, Eds. (Dekker, New York, 1971), chap. 9, p. 333; H. H. Rees, in *Aspects of Terpenoid Chemistry and Biochemistry*, T. W. Goodwin, Ed. (Academic Press, London, 1971), chap. 7, p. 181; M. N. Galbraith, D. H. S. Horn, E. J. Middleton, J. N. Kaplanis, M. J. Thompson, *Experientia (Basel)* **29**, 782 (1973).
4. A. Faux, D. H. S. Horn, E. J. Middleton, H. M. Fales, M. E. Lowe, *Chem. Commun.* (1969), p. 175.
5. S. Imai, M. Hori, S. Fujioka, E. Murata, M. Goto, *Tetrahedron Lett.* (1968), p. 3883; S. Imai, S. Fujioka, E. Murata, Y. Sasakawa, K. Nakanishi, *ibid.*, p. 3887.
6. Extracts from sunflower and milkweed seeds were inactive in the housefly assay when tested at a sensitivity of 10 housefly units per gram-equivalent (wet weight). The housefly unit in our assay system (*8*) is now 3 ng based on α-ecdysone.
7. J. N. Kaplanis, W. E. Robbins, M. J. Thompson, S. R. Dutky, *Science* **180**, 307 (1973).
8. J. N. Kaplanis, L. A. Tabor, M. J. Thompson, W. E. Robbins, T. J. Shortino, *Steroids* **8**, 625 (1966).
9. Because ecdysone conjugates are retained on alumina and conjugates in crude extracts are not readily hydrolyzed by enzymes, the sample was first fractionated on silicic acid and the polar fraction or fractions from the column known to contain conjugates were subjected to enzymic hydrolysis with a mixture of sulfatase and glucosidase. Conditions for hydrolysis, extractions, and fractionations were as described by J. N. Kaplanis, S. R. Dutky, W. E. Robbins, M. J. Thompson in *Invertebrate Endocrinology and Hormonal Heterophylly*, W. J. Burdette, Ed. (Springer-Verlag, New York, 1974), chap. 14, p. 161. No biological activity was detected in the extracts after enzymic hydrolysis, indicating again that, in the embryo (*7*), conjugation does not occur to an appreciable extent in this period of development.
10. Woelm neutral grade I alumina deactivated with 20 percent water. Column, 9.5 cm (inside diameter) by 2.7 cm high; 225 g of adsorbent. The crude extract was eluted with methanol (2 liters) then with 75 percent methanol (1 liter). Only the methanol fraction was biologically active.
11. Unisil, 100 to 200 mesh (Clarkson Chemical Co., Williamsport, Pa.)
12. Two authentic standards of makisterone A were available for these studies. One of the samples was from *Podocarpus macrophyllus* and the other was from *Podocarpus elatus*.
13. J. N. Kaplanis, M. J. Thompson, R. T. Yamamoto, W. E. Robbins, S. J. Louloudes, *Steroids* **8**, 605 (1966).
14. J. N. Kaplanis, unpublished work.
15. R. H. Dadd, *Annu. Rev. Entomol.* **18**, 381 (1973).

3 July 1975

In vitro Demonstration of an Endothelial Proliferative Factor Produced by Neural Cell Lines

Abstract. *Cultured endothelial cells exhibit a six- to tenfold increase in thymidine labeling index in response to a soluble factor elaborated by clonal cell lines of neural origin. This factor, endothelial proliferation factor, appears to be a unique property of tumor cells and may mediate the vascularization of these neoplasms.*

Neovascularization has long been recognized as necessary for tumor growth and maintenance. Algire (*1*) suggested that an attribute of tumor cells is their capacity to elicit the continual production of capillary endothelium in vivo. This concept was strengthened and extended by the work of Tannock *et al.* (*2*) who showed that the rate of tumor growth could be directly related to vascular supply, and of Gimbrone *et al.* (*3*) who showed that in the absence of neovascularization a tumor becomes restricted in size to a spheroid about 2 to 3 mm in diameter. Folkman and his coworkers (*4*) have suggested that tumor cells elaborate a factor known as tumor angiogenesis factor (TAF), which stimulates the proliferation of endothelial cells and thereby the neovascularization of the tumor. Endothelial cells in the adult have an extremely low proliferation rate (*5*), which suggests the existence of a stimulatory factor associated with neoplasms.

Folkman *et al.* (*6*), using the in vivo dorsal air sac and other in vivo assay techniques, have demonstrated an increase in vascularity in response to a soluble factor isolated from animal and human neoplasms. The proliferative response is not, however, restricted to the capillary endothelium but also involves pericytes and surrounding connective tissues. Cavallo *et al.* (*7*) have speculated that this broad mitogenic response reflects an impure TAF fraction containing several mitogenic factors or, alternatively, is a manifestation of the wound-healing process. The wound-healing response is difficult to distinguish from a specific tumor angiogenic response in the in vivo assay. These considerations prompted us to develop an in vitro assay with cultured human endothelial cells as the target of the trophic factor or factors produced by clonal cell lines of tumor origin. We now describe the development of an in vitro tissue culture assay, the use of which clearly demonstrates a stimulation of endothelial proliferation in response to a soluble factor produced by tumor cells. We have called this factor endothelial proliferation factor (EPF).

Table 1. Thymidine labeling index of human endothelial cells cultured with medium conditioned by tumor cell lines. Proliferation response is given as percentage.

Conditioning medium	Response
Controls	
Fresh medium	2.1
Amniotic fluid	1.1
Fibroblast conditioned medium	4.5
Glia tumor cells	
C6 (A)	98.3
C6 (A)	97.6
C6JDV (B)	92.6
C6JDV (B)	83.3
C6-CRL-107 (C)	25.7
C6-CCL-107 (C)	36.7
C6-CCL-107 Co-culture	28.7
Human astrocytoma primary	30.1
Neuroblastoma tumor cells	
NB-41	90.0
IMR-32	78.7

Endothelial cells were obtained from the umbilical veins of human umbilical cords at term by the method of Jaffe *et al.* (*8*) with some modification. We cannulated the umbilical vein using a 16- or 18-gage trochar needle. After removal of blood from the vein by flushing with Hanks solution (Ca and Mg free), collagenase (2 mg/ml) (Sigma) in Hanks solution was introduced into the vein. The endothelial cells were dissociated from the wall of the vein by digestion with collagenase for 20 minutes and flushed free by rinsing with Hanks solution. The Hanks solution containing the endothelial cells and remaining red blood cells was centrifuged for 3 to 5 minutes and resuspended in a small volume of F10 medium plus 20 percent fetal calf serum.

The cells were then plated on glass cover slips in six well culture dishes (Linbro). After 12 to 18 hours the red blood cells were washed free of the cover slips with Hanks solution and then 2.0 ml of growth medium, either medium 199 or F10 supplemented with 20 percent fetal calf serum and containing penicillin (50 unit/ml) and streptomycin (50 μg/ml) (all from Gibco), was added to each well. These primary cultures of human endothelial cells were the vehicle for our in vitro assay. Six uniform cultures from a single umbilical cord allowed each sample to have a control and five experimental cultures.

Tumor-conditioned medium was tested for the presence of EPF as follows. After 1 to 3 days in culture, an additional 1.0 ml of test medium was added to each culture well. Twenty-four hours later, the cultures were labeled with [^{3}H]thymidine (0.5 μc/ml; specific activity > 10 mc/mmole; New England Nuclear) for 72 hours. After fixation in either acetic acid and ethanol (1 : 3) or 10 percent buffered formalin, the cover slips were coated with NTB-2 nuclear tracking emulsion (Kodak) and exposed for 5 days. The exposed cover slips were developed and stained with toulidine blue or Giemsa.

Tumor-conditioned media have been obtained from actively growing cultures of the following cell types:

C6 rat astrocytoma. A clonal cell line derived from a tumor chemically induced with *N*-nitrosomethylurea (*9*). Three subclones of this tumor have been assayed for production of EPF: (A) a subclone obtained from J. de Vellis; (B) a subclone obtained from Stephen Pfieffer; (C) a sub-

clone, C6-CCL-107, obtained from the American Type Culture Collection.

NB-41. A clonal mouse neuroblastoma line derived from the transplantable, mouse C1300 neuroblastoma (Jackson Laboratories) originally cloned by Augusti-Tocco and Sato (*10*), and provided by J. de Vellis.

IMR-32. A human neuroblastoma line originally isolated from a 13-month-old male (*11*) and provided by R. Perez-Polo. This cell line is available from the American Type Culture Collection.

Human astrocytoma. Primary culture of a human astrocytoma was obtained surgically at this institution. To prepare conditioned media, 1×10^6 to 2×10^6 cells were plated into a T-250 Falcon flask (Falcon Plastics) containing 10 ml of F10 growth medium plus 10 percent fetal calf serum. Conditioned medium obtained from such cultures 48 hours after plating (nonconfluent) or from confluent cultures was tested and found to have a similar effect on incorporation of [^{3}H]thymidine.

Control conditioned medium was conditioned by a primary culture of human fibroblasts obtained from a scar biopsy. The conditioned medium was that in contact with any of the aforementioned actively growing cultures for at least 48 hours. The fibroblasts used in our studies had a doubling time of 29 hours, as compared to 20 hours for the C6 rat astrocytoma. All conditioned media were filtered (Millipore, 0.22 μm pore size) to remove any cells before addition to endothelial cultures.

The proliferative response of the endothelial cells is measured as the thymidine labeling index (TI), defined as the percent of labeled cells counted in ten random fields. All slides were coded by one person and counted by another to whom the code was unknown.

Medium conditioned by either of the C6 astrocytoma subclones (A or B) stimulated the proliferation of the endothelial cells about 20-fold when compared to that observed in the presence of fibroblast-conditioned medium (Table 1). This response is seen in Fig. 1 in which nearly all the endothelial cells are labeled and several cells undergoing mitosis are visible. A similar, but not as extensive (sixfold) proliferative response was observed with medium conditioned by astrocytoma subclone C. The difference in the endothelial proliferation response elicited by C6 subclones A, B, and C probably reflects differences in the percent viability of the endothelial cells from cord to cord rather than a quantitative difference in the production of EPF by these subclones. The difference in viability of the endothelia results from each test being done with a separate umbilical cord. Coculture of endothelial cells with the CCL-107 astrocytoma C elicited a proliferation response in the endothelial cells quantitively and qualitively similar to that seen with conditioned media alone. Thus, we have shown that clonal glial cell lines derived from chemically induced tumors are capable of inducing a proliferative response in normal human endothelia.

We then asked whether this property was unique to clonal glial tumor cells and expressed only in long-term tissue culture. Table 1 shows that media conditioned by the human astrocytoma cells induced a sixfold increase in endothelial proliferation over the control. Thus, the production of EPF is a property retained under culture conditions and shared by primary and clonal glial tumor cell cultures. As is shown below, this property is also shared by neuroblastoma cells which are of neuronal origin.

Medium conditioned by neuroblastoma lines NB-41 and IMR-32 elicited a 20-fold increase in endothelial cell proliferation (Table 1). We also have data showing that primary cultures of various human central nervous system tumors, including meningiomas and medulloblastomas, also elicit the endothelial proliferative response (*12*). Thus, it appears that the production of an EPF is a general property of tumors of the nervous system and is expressed in tissue culture.

A general property of all tumor cells is the capacity to undergo rapid proliferation. The endothelial proliferative response observed by us may result from the release of a soluble cell mitogen by rapidly proliferating cells, irrespective of origin or degree of malignancy. However, culture media conditioned by nontransformed fibroblasts do not elicit the endothelial proliferative response. Similarly, no stimulation of endothelial proliferation was seen with media conditioned by peripheral human lymphocytes, or by cells obtained by amniocentesis, neither of which are transformed or malignant. Amniotic fluid is known to contain a number of growth factors and to contain antigens which cross react with surface antigens of tumor cells. In addition, normal human placental tissue has been reported (*6*) to have low levels of TAF activity. The addition of amniotic fluid to the endothelial cultures does not in itself stimulate these cells to proliferate, thus suggesting that the production of EPF is a unique property of tumor cells. Media from primary brain cultures from newborn mice do not stimulate thymidine incorporation by the endothelia, indicating that the response is not the result of a neural mitogenic factor.

Physical characterization of the proliferative factor suggests that a protein moiety may be responsible for the proliferative response. Specifically, the response is abolished by heating to 56°C for 10 minutes (Table 1) or digestion with Pronase for 1 hour at 37°C, but not by ribonuclease digestion.

Our data suggest that CNS tumors in primary cultures, as well as clonal cell lines, elaborate a soluble factor, which we call endothelial proliferation factor (EPF). The mitotic index of the endothelium in

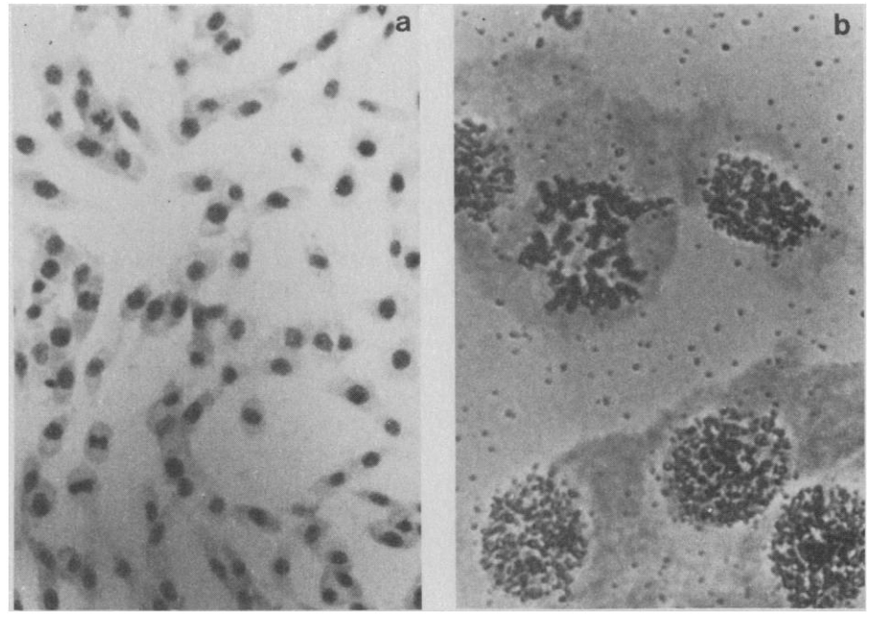

Fig. 1. (a) Endothelial cells cultured in the presence of C6 rat astrocytoma conditioned media. This microscopic field shows the high TI of the endothelia as well as cells in several stages of mitosis. (b) The thymidine labeling localized directly over the early metaphase chromosomes of an endothelial cell.

the presence of EPF is 7 percent as compared to 0.1 percent in the control cultures.

Thus, TI reflects the number of cells incorporating thymidine for cell division, not just DNA repair or movement of the cells into the DNA synthesis period of the cell cycle. Our in vitro assay system has several advantages over other bioassay procedures (*3, 6*) in that the specific response of one cell type is measured in the absence of other cell types, we can screen a large number of tumors and tumor cell lines for the production or content of EPF, and we require less tumor material.

The production of EPF in vitro by clonal cell lines, coupled with the in vitro assay, provides a system for the purification and characterization of EPF. Neither the EPF described by us nor the TAF described by Folkman has been adequately characterized. These factors may prove to be identical; however, we note that our factor differs from Folkman's (*4, 6*) in the following ways: (i) it is produced by tissue culture cells; (ii) it is ribonuclease resistant; (iii) it is sensitive to proteolytic digestion with Pronase; and (iv) it stimulates the proliferation of endothelial cells but not of fibroblasts. Cavallo (*7*) suggests that the nonspecificity of TAF may result from the lack of a purified factor; this may also contribute to the differences we observe between TAF as described in vivo and EPF produced in tissue culture. Neither TAF nor EPF appears to be species specific as demonstrated by the increased proliferative response of human endothelial cells elicited by tumor cells of both rodent and human origin. The in vivo effects of EPF have yet to be determined.

R. L. SUDDITH, P. J. KELLY
H. T. HUTCHISON, E. A. MURRAY
B. HABER

Division of Neurobiology, Marine Biomedical Institute, Division of Neurosurgery, Department of Surgery, and *Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston 77550*

References and Notes

1. G. H. Algire and H. W. Chalkley, *J. Natl. Cancer Inst.* **6**, 73 (1945).
2. I. F. Tannock and S. Hayashi, *Cancer Res.* **32**, 77 (1972).
3. M. A. Gimbrone, Jr., S. B. Leapman, R. S. Contran, J. Folkman, *J. Exp. Med.* **136**, 261 (1972).
4. J. Folkman, *Am. J. Med.* **285**, 1182 (1971).
5. H. P. Wright, *Nature (Lond.)* **220**, 78 (1968).
6. J. Folkman, E. Merler, C. Abernathy, G. Williams, *J. Exp. Med.* **133**, 275 (1971).
7. T. Cavallo, R. Sade, J. Folkman, R. Cotran, *Am. J. Pathol.* **70**, 345 (1973).
8. E. A. Jaffe, R. L. Nachman, C. G. Becker, C. R. Minick, *J. Clin. Invest.* **52**, 2745 (1973).
9. P. Benda, J. Lightbody, G. Sato, L. Levine, W. Sweet, *Science* **161**, 370 (1968).
10. G. Augusti-Tocco and G. Sato, *Proc. Natl. Acad. Sci. U.S.A.* **64**, 311 (1969).
11. J. J. Tumilowicz, W. W. Nichols, J. J. Cholon, A. E. Green, *Cancer Res.* **30**, 2110 (1970).
12. R. L. Suddith, P. J. Kelly, H. T. Hutchison, E. A. Murray, B. Haber, in preparation.
13. Supported by NIH grants NS 11354 and NS 11255, the Lanier Foundation, and Neurosurgery MSRDP research funds. We thank K. Werrbach for technical assistance and the medical and nursing staff of the Department of Obstetrics and Gynecology for providing the umbilical cords from patients at term.

27 January 1975; revised 30 June 1975

Production of Globules in Mouse L Cells Penetrated with Hamster Sperms

Abstract. *Globule formation has been observed in long-term cultures of mouse L cells penetrated with hamster spermatozoa. These numerous, uniform, and spherical structures are approximately 10 micrometers in diameter when formed, and are positive to stains specific for proteins and nucleic acids. They may be produced either by sperms within their target cells or by the cells in response to penetration by the spermatozoa.*

Active penetration of spermatozoa into somatic tissues was initially observed by Kohlbrugge (*1*) in the female genital epithelia of domestic fowls and mammals. It was further demonstrated in vitro by Reid (*2*), who incubated (in tissue culture) human sperms with biopsy materials from the postparturient uterus. Both intercellular and intracellular penetrations were evident upon examination of the tissues after short-term culture. Reid also noticed the formation of vacuoles and the alignment of mitochondria around the heads of sperms within their target cells, thus indicating some form of interaction between the host cells and the spermatozoa which penetrated them. Extensive works by Bendich *et al.* (*3*) have shown that, in tissue culture, mouse sperms are capable of penetrating various mammalian cell lines. Within their target cells, the release of labeled DNA from mouse spermatozoa, followed by the production of substances immunologically specific to the sperm donor, have also been demonstrated, implying that protein synthesis might have been initiated by the spermatozoa as a result of their entry.

I now report a cellular activity hitherto not observed, namely, the production of globules by a line of mouse fibroblasts penetrated with spermatozoa from a hamster.

In my experiment, motile spermatozoa, aseptically extracted from the cauda epididymis of a Syrian hamster, were mixed with mouse L cells in tissue culture medium (*4*) supplemented with 0.5 percent fructose to enhance sperm motility (*5*). There were ten sperms to each cell, and the final density in the mixture was approximately 5×10^5 cells per milliliter. Adhesion of sperm heads to cells was observed immediately after mixing, and vigorous vibration was maintained by the tails for 8 to 12 hours while the heads remained tightly bound to their target cells. As sperm motility subsided, the cells settled down and spread out on microscope slides that were incubated under standard tissue culture conditions at 34°C. Most cells were attached with single sperms, but multiple attachment was not infrequent (Fig. 1). Within 12 hours after admixture of sperms and L cells, intracellular location of sperm heads was evident; and in 48 hours most of these intracellular sperm heads had already disappeared in the cytoplasms of their target cells (Fig. 1). In agreement with observations by Bendich *et al.* (*3*), each target cell then began to display more than one nucleus, indicating the arrest of cell division due to sperm penetration. From then on, the cells increased both in size and in nuclear number until, in 8 to 12 days, a population of polynucleates prevailed in which most cells exhibited more than eight nuclei (Fig. 2). Due to the low density of plating and the large initial intercellular distances, it seemed quite unlikely that these giant cells could have arisen from the fusion of adjacent cells. Studies on their chromosomal numbers revealed a widespread polyploidy among these giant cells, suggesting endomitosis might have been the mode of cell growth subsequent to sperm penetration (*6*).

Further incubation of the sperm-penetrated cells gave rise to numerous spherical structures hitherto nonexistent in the culture (Figs. 3 to 5). And, depending on the state of the host cells before admixture with spermatozoa, the appearance of these globules came within 2 to 3 weeks after sperm entry. These uniform globules can be seen inside both giant polynucleates and smaller cells (Fig. 3). They are also visible around those cells from which they seem to have been extruded (Fig. 4), and they appear in aggregates on sites where giant cells may have disintegrated to yield a group of mononucleate cells together with the globules (Fig. 5). The formation of globules seemed to be a fairly synchronous activity, with a peak period of about 4 days during which most cells were associated with a number of these spherical objects.

The globules average about 10 μm in diameter when sparsely attached to glass.