[CANCER RESEARCH 37, 3619-3622, October 1977]

Growth Stimulation of Endothelial Cells by Simultaneous Culture with Sarcoma 180 Cells in Diffusion Chambers

Anne Atherton[1]

Department of Cancer Chemotherapy, Imperial Cancer Research Fund, Lincoln's Inn Fields, London W.C.2., England

SUMMARY

Endothelial cells derived from pig aorta were cultured in Petri dishes containing Sarcoma 180 cells growing in Millipore diffusion chambers. The presence of tumor cells consistently increased the colony-forming ability of the endothelial cells, an effect not produced by control-killed cells or 3T3 fibroblasts. This observation suggests that the cultured tumor cell retains the ability to produce diffusible substances that stimulate endothelial cell growth. These products may be similar to the angiogenic factors elaborated by malignant cells *in vivo*. The use of such a method may allow a closer study of the effect of tumor cells on the growth of a variety of tissues.

INTRODUCTION

The ability of solid tumors to elicit the directional growth of blood vessels in order to establish a vascular supply for their expansion is a well documented phenomenon (1, 3, 11). This stimulation of blood vessel growth has been shown *in vivo* to be due to the production of tumor angiogenic factor by the neoplastic cells (8). Tumor angiogenic factor has been extracted from a variety of malignant cells and has been shown to be capable of stimulating blood vessel growth across avascular tissues (8), and also to increase the division rate of endothelial cells *in vivo* (5). However, the detailed chemical characterization of this substance has not yet been achieved.

In vivo studies have shown that tumor cells growing within Millipore chambers are also able to elicit blood vessel proliferation (9), suggesting that the angiogenic factors were readily diffusible. In an attempt to provide an *in vitro* method for the study of this process, Sarcoma 180 cells were grown within Millipore diffusion chambers placed in Petri dishes containing cultured endothelial cells. The CFA[2] of the endothelial cells was scored at various times during the simultaneous growth of Sarcoma 180 and endothelial cells; the 2 cell types being separated only by the Millipore filter, which allowed free passage of substances contained in the culture medium. The CFA was used in preference to a direct count of numbers, since it was considered to be a more suitable index of the ability of any substance diffusing from the Millipore chambers to stimulate quiescent endothelial cells into division. Sarcoma 180 cells grown in this fashion retain the ability to stimulate blood vessel growth when implanted s.c. in the flanks of Swiss Schneider mice (M. Finch, personal communication). The successful culture of endothelial cells derived from a variety of animal tissues has been reported by many workers (10, 12), and a modification of 1 method with collagenase harvesting of pig aortic endothelium was used (6).

The following reports the methods used for the routine culture of both malignant and nonmalignant cells within diffusion chambers, and the effect of their presence on cultured endothelial cells.[3] The use of these methods may allow a more detailed study of the mechanisms by which diffusible substances from neoplastic cells stimulate angiogenesis.

MATERIALS AND METHODS

Endothelial Cell Culture. Endothelial cells were derived from the aortas of freshly slaughtered pigs with a collagenase harvesting technique, with some modifications (6). After transport to the laboratory (2 hr) in phosphate-buffered saline (containing 10 g of NaCl, 0.25 g of KCl, 1.437 g of Na_2HPO_4, and 0.25 g of KH_2PO_4 in 1 liter of distilled water), the aortas were washed thoroughly in heparinized 0.9% NaCl solution (5 units/ml) until the vessel lumens were free from residual blood. After the intercostal vessels were clamped off, the lumens were occluded at one end while 10 ml of 0.2% collagenase (Worthington Biochemical Corp., Freehold, N. J.) in phosphate-buffered saline was passed into the other end. After incubation for 7 min at 37°, the contents of the lumens, containing sheets of endothelial cells, were withdrawn. One ml of this suspension was plated out immediately into 100-mm Petri dishes (Sterilin, Teddington, Middlesex, England) containing 10 ml of Eagle's basal medium modified with Hanks' salt solution. Glutamine (0.83%), penicillin (0.1%) and streptomycin (0.1%) (Crystamycin, Greenford, England), and 20% fetal calf serum (20%) were added to the medium. Sterile precautions were observed throughout these operations.

The primary culture was allowed to incubate at 37° for 7 to 8 days in humidified air containing 5% CO_2, by which time a sufficient number of endothelial cells were present to allow plating for experimental purposes and also for routine serial passage. These were removed from the sufrace of the dish by incubation with a trypsin-Versene disaggregation solu-

[1] To whom requests for reprints should be addressed, at Unit for Metabolic Medicine, Guy's Hospital Medical School, London S.E. 1, United Kingdom.

Received November 24, 1976; accepted July 15, 1977.

[2] The abbreviation used is: CFA, colony-forming ability.

[3] During the preparation of this manuscript a report appeared describing similar work, but using a different method (7).

tion to provide a single-cell suspension. The experimental cells used were of the 2nd or 3rd passage, each passage being of approximately 1-week duration. The 4th passage usually contained too many cells with an atypical appearance for successful experimentation.

Growth of Sarcoma 180 Cells within Millipore Diffusion Chambers. Sarcoma 180 cells, cultured routinely in the laboratory in 100-mm Petri dishes (Sterilin), were removed with a trypsin-Versene disaggregation solution, centrifuged at room temperature and 500 × *g* for 10 min, and resuspended in fresh medium at the required cell number. The viability was estimated using the trypan blue exclusion technique; 0.05 ml of this suspension was used to fill each Millipore chamber.

The diffusion chambers were constructed by cementing a Millipore filter (ultrathin, 14 mm in diameter; pore size 0.30 μm) to each surface of a Millipore diffusion chamber ring. After 24 hr the chambers were sterilized by autoclaving and left to cool before use. By using sterile techniques, 0.05 ml of Sarcoma 180 cell suspension was dispensed into each chamber with a 0.5-ml syringe fitted with a 23-gauge needle. The hole used for filling was blocked with a small perspex rod. Control chambers contained either 0.05 ml of medium or 0.05 ml of medium containing cells that had been killed by heating them at 60° for 10 min. Each chamber was placed in a 100-mm Petri dish containing cells that had been previously plated out, as described below.

Measurement of the Effect of Sarcoma 180 Cells Growing in Millipore Chambers on the CFA of Endothelial Cells. Five hundred viable endothelial cells in 10 ml of medium were added to each Petri dish on Day 0 and left to establish themselves for 24 hr; at which time a Millipore chamber containing a known number of viable Sarcoma 180 cells was added to each dish, an equal number of dishes containing control chambers. At various times after plating out, 3 experimental dishes and 3 control dishes were removed for assay. The colonies were fixed and stained for 15 min with a 0.1% solution of Leishman's stain in methanol, washed carefully, and dried for later scoring. The endothelial cell colonies were scored only if they comprised more than 8 cells, arranged in a tightly pavemented fashion, as described by other authors (10, 12). Colonies of fibroblasts were noted but not counted. The number of viable cells within each Millipore chamber was counted after opening the chamber in 2 ml of trypsin-Versene solution, followed by incubation at 37° for 30 min, and thorough agitation to free the individual cells from the surface of the Millipore filters.

Growth of 3T3 Fibroblasts in Diffusion Chambers and Petri Dishes. To test the effect of some nonmalignant connective tissue cells on the CFA of endothelial cells, 3T3 fibroblasts grown routinely in the laboratory were grown in Millipore chambers after being treated in a manner identical to Sarcoma 180 cells. Cells (10^4) were placed within each chamber and the cell colonies assayed after 6 days. This corresponded to the maximal effect seen with tumor cells in the chambers.

It is possible that tumor cells may stimulate the growth of cells other than endothelial cells. In order to study the effect of Sarcoma 180 cells on a population of non-endothelial cells, chambers containing Sarcoma 180 cells were added to Petri dishes containing 200 viable 3T3 cells plated out on the previous day. The number of 3T3 cell colonies was scored on Day 6 after being stained with Leishman's stain as described above.

RESULTS

The endothelial cells grew from a single-cell suspension to form colonies, often comprising as many as 100 cells by Day 10. The colonies consisted of polygonal cells of approximately 50-μm diameter growing in a tightly pavemented fashion, but with characteristically indistinct cell borders. In each dish a very few fibroblast colonies were readily identified by the overlapping, whorled nature of their growth. In control dishes, colonies of more than 8 cells had appeared in small numbers by the 4th day, and subsequently increased rapidly in number. By 10 to 12 days the number of colonies arising from 500 viable cells had reached 100 to 200, representing a plating efficiency of approximately 40%, and scoring was less accurate due to confluency. For this reason, experiments were rarely continued beyond 12 days.

Sarcoma 180 cells grew successfully within the chambers and the cell doubling time was approximately 48 hr during the exponential phase of cell growth, with a lag period of approximately 24 hr before division commenced. Microscopically, the cells appeared to be very similar to those freshly harvested from Petri dishes, and when plated out, grew with the same characteristics as routinely cultured cells. The maximal number of cells that could be contained within a chamber before a stationary phase of growth was reached varied with each experiment, but was generally in the order of 2 to 4 × 10^5 cells. This number did not seem to be dependent on the initial number of cells placed in the chamber. Occasionally, cells escaped from the chambers and formed colonies on the Petri dish. Dishes in which this had occurred were easily recognizable and were discarded.

3T3 fibroblasts grew within the chambers if a very small number of cells (1 × 10^4) were placed in the chamber; the doubling time was 12 to 16 hr, and the cell growth rapidly reached a stationary phase, with cell death occurring by Day 8. Fibroblast growth in Petri dishes was rapid and confluency from 200 viable cells was achieved in 10 days.

The presence of Sarcoma 180 cells growing actively within the diffusion chambers increased the CFA of the cultured endothelial cells. The results of 1 such experiment, in which the number of colonies in both control and tumor cell-containing Petri dishes, were counted on various days after plating out are shown in Chart 1. Similar results were obtained when the experiment was repeated.

The increased number of endothelial cell colonies in Petri dishes bearing tumor cells was not apparent until the tumor cells entered the exponential phase of division, but this difference remained after the number of viable tumor cells had begun to decrease. In the majority of experiments, the maximal increase (varying from approximately 70 to 300%) was experienced about 6 to 7 days after plating out. To check that this increase was not due to a larger number of colonies containing fewer cells, 20 random colonies from control and experimental dishes were counted. In many cases the experimental dishes contained up to 30% more

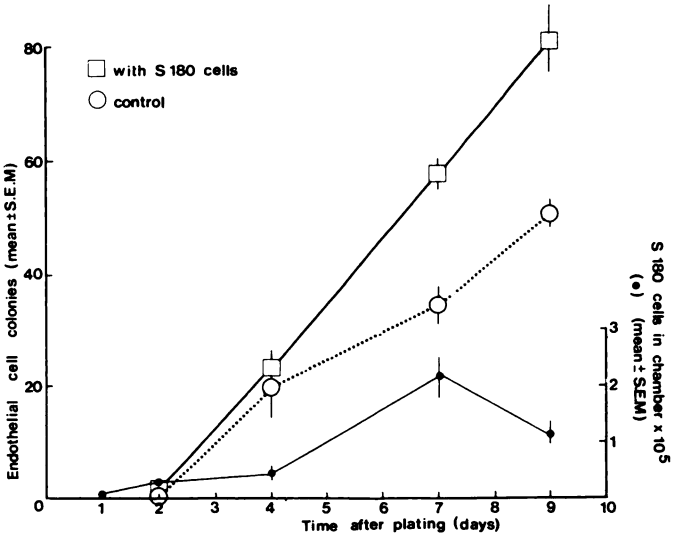

Chart 1. The number of endothelial cell colonies on the surface of 100-mm Petri dishes, containing either viable Sarcoma 180 cells (□) or control-killed cells (○) in Millipore diffusion chambers, were counted on Days 2, 4, 7, and 9 after plating out on Day 0. The number of viable Sarcoma 180 cells present within each diffusion chamber was also scored on the days indicated (●). *Points*, mean ± S.E. of 3 dishes or chambers.

cells in their colonies than the controls did. This would suggest a faster rate of division as well as a greater CFA.

However, 3T3 cells grown in the chambers had no significant effect on the endothelial cell growth when harvested on Day 6. In 1 such experiment, the mean number of endothelial cell colonies in control dishes was 90.2 ± 5.77 (n = 6), and in dishes containing Millipore chambers with 3T3 cells was 86.25 ± 5.96 (total = 0.4711) (not significant by Student's t test). The number of 3T3 cells in each chamber increased from 1×10^4 on Day 1 to $5.4 \pm 1.2 \times 10^5$ on Day 6. Sarcoma 180 cells growing within the chambers did not increase the CFA of 3T3 fibroblasts significantly by Day 6. In 2 typical experiments the mean number of 3T3 cell colonies was 205.8 ± 10.98 (n = 6) and 102.8 ± 8.07 (n = 6) in control dishes, and 224.4 ± 22.84 (n = 6) and 125.80 ± 22.87 (n = 6) in dishes containing Sarcoma 180 cells within chambers (total = 0.7341 and 0.9482, respectively) (not significant by Student's t-test). However, of these dishes, 3 containing tumor cells showed significantly increased numbers of fibroblast colonies. This effect was much less than the effect on endothelial cells, where all of the dishes exhibited an increased CFA.

DISCUSSION

The CFA of cultured pig endothelial cells is increased by the presence of Sarcoma 180 cells growing actively within Millipore diffusion chambers placed in the same Petri dish. This effect is not produced by control-killed Sarcoma 180 cells. During the linear phase of tumor cell growth, the number of endothelial cell colonies increases in proportion with the number of viable tumor cells present. This observed growth increase and the fact that there is a physical separation of the 2 types of cell, suggest that within this system, diffusible substances are produced by the tumor cells that are able to stimulate endothelial cell mitosis. This experimental system failed to demonstrate increased endothelial cell division in response to the presence of growing nonmalignant cells. It is likely, however, that stimulation of blood vessel growth is not specific to the malignant cell. For example, other workers (7) have reported a similar effect produced by homogenates of embryonic tissue. However, the angiogenic factor responsible may not be the same in each instance. 3T3 fibroblasts growing within the Millipore chambers were unable to produce an increase in the endothelial cell CFA. The effect of the tumor cells in the chambers was to increase the CFA of fibroblasts only slightly in a few dishes, and the magnitude of the response was very small compared with that seen when malignant cells were in the chambers. This effect is not unreasonable, since during dissection of many animal tumors an increase in the amount of surrounding fibrous connective tissue is often seen; although Sarcoma 180 *in vivo* has been observed to elicit little of this type of host response.

The use of this model may allow closer study of the relationship between cells; for example, the inhibition of endothelial cell growth by cartilage (2), or the production of tumor dormancy by the vitreous (4). It would also allow investigation of the mechanisms by which tumor cells are able to stimulate endothelial cell division. It seems that this process, as observed *in vitro*, bears many similarities to the response of endothelial cells to malignant cells *in vivo*. However, the endothelial cells used in culture are derived from large vessel lumens, and the growth of tumors *in vivo* excites growth in small capillaries and venules. The development of satisfactory methods for culturing capillary-derived endothelium may show a greater sensitivity of these cells to angiogenic factors. The response of endothelium derived from aortas is, however, similar enough to warrant further study. With modifications, it may be possible to treat the cell types separately with agents that influence the angiogenic effect. The process may also be observed continuously during the various stages of cell growth and division. Furthermore, collection of the medium, in which the 2 cell types have grown, may help in elucidating the nature of the angiogenic factor by providing a relatively pure source. Preliminary experiments in this laboratory have shown that medium harvested from cultured tumor cells will stimulate *in vitro* endothelial cell growth for a short time only. As the volume of tumor cells increases in culture, it is possible that an increase in the number of cells with an anaerobic metabolism may initiate the production of substances that stimulate endothelial cell division. However, lactic acid (L-lactate) added directly to the medium in varying concentrations does not stimulate endothelial cell growth (G. Murkin, personal communication).

The action of the putative angiogenic substances demonstrated in these studies seems not to be species specific. Mouse Sarcoma 180 cells produced a growth-stimulating effect on pig endothelium, while tumor cell homogenates from rat Walker 256 carcinosarcoma have been shown to increase the growth of bovine endothelial cells in culture (7). It is possible that isologous tumor cells would have an even greater effect on corresponding endothelial cells growing *in vitro*.

REFERENCES

1. Algire, G. H.; and Chalkley, H. W. Vascular Reactions of Normal and Malignant Tissue *in vivo*. 1. Vascular Reactions of Mice to Wounds and to Normal and Neoplastic Transplants. J. Natl. Cancer Inst., *6:* 73–85, 1945.
2. Brem, H., Arensman, R., and Folkman, J. Inhibition of Tumor Angiogenesis by a Diffusible Factor from Cartilage. *In:* H. C. Slavkin and R. C. Greulich (eds.), Extracellular Matrix Influences on Gene Expression, pp. 767–772, New York: Academic Press, Inc., 1975.
3. Brem, S. The Role of Vascular Proliferation in the Growth of Brain Tumours. Clin. Neurosurg., *23:* 440–454. 1976.
4. Brem, S., Brem, H., Folkman, J., Finkelstein, D., and Patz, A. Prolonged Tumor Dormancy by Prevention of Neovascularization in the Vitreous. Cancer Res., *36:* 2807–2810, 1976.
5. Cavallo, T., Sade, R., Folkman, J., and Cotran, B. Tumor Angiogenesis: Rapid Induction of Endothelial Mitoses Demonstrated by Autoradiography. J. Cell. Biol., *54:* 408–420, 1972.
6. de Bono, D. Effects of Cytotoxic Sera on Endothelium *in vitro*. Nature, *252:* 83–84, 1974.
7. Fenselau, A., and Mello, R. J. Growth Stimulation of Cultured Endothelial Cells by Tumor Cell Homogenates. Cancer Res., *36:* 3269–3273, 1976.
8. Folkman, J., Merler, E., Abernathy, C., and Williams, G. Isolation of a Tumor Factor Responsible for Angiogenesis. J. Exptl. Med., *133:* 275–288, 1971.
9. Greenblatt, M., and Shubik, P. Tumour Angiogenesis: Transfilter Diffusion Studies in the Hamster by the Transparent Chamber Technique. J. Natl. Cancer Inst., *41:* 111–124, 1968.
10. Haudenschild, C. C., Cotran, R. S., Gimbrone, M. A., Jr., and Folkman, J. Fine Structure of Vascular Endothelium in Culture. J. Ultrastruct. Res., *50:* 22–32, 1975.
11. Warren, B. A., and Shubik, P. The Growth of the Blood Supply to Melanoma Transplants in the Hamster Cheek Pouch. Lab. Invest., *15:* 464–478, 1966.
12. Wechezak, A. R., and Mansfield, P. B. Isolation and Growth Characteristics of Cell Lines from Bovine Venous Endothelium. In Vitro *9:* 39–45. 1973.