Endothelial growth factor present in tissue culture of CNS tumors

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Human endothelial cells obtained from postpartum umbilical veins and placed in primary tissue cultures were treated with media from cultures of human and experimental central nervous system tumors. Endothelial proliferation was determined by the uptake of \(^{3}\)H thymidine with autoradiography and represented as the thymidine labeling index (TI), which is the proportion of \(^{3}\)H thymidine-labeled endothelial cells to total number of cells counted. There was a marked increase in the TI when tumor-conditioned medium was added to endothelial cultures (range 28.7% to 98.3%) when compared to controls (2.1%) and endothelium with conditioned media from fibroblasts (4.5%). This study demonstrates the presence of a chemical substance produced by tumor cells which results in endothelial proliferation. The system described provides a useful assay technique for the further characterization of this endothelial growth factor.

**Key Words** • CNS tumors • endothelium • angiogenesis • tissue culture • autoradiography

The endothelial proliferation and neovascularization associated with CNS neoplasms is well recognized.\(^{6,14,17-19}\) A specific chemical factor produced by tumor cells which induces neovascularization and more specifically endothelial mitotic activity has been suggested by experiments in which hamster cheek pouch vessel proliferation was induced by a tumor contained in a millipore filter.\(^{8,16}\)

Folkman\(^{8}\) evaluated angiogenic activity of various experimental and human tumors upon vessels in rat tissue and found that extracts of those tumors resulted in neovascularization.\(^{9,10}\) This was also shown by injecting tumor extracts intraocularly and evaluating neovascularization in the iris.\(^{13}\)

Endothelial uptake of \(^{3}\)H thymidine and increase in mitotic activity \textit{in vivo} induced by tumor angiogenesis factor (TAF) has been studied in other experiments.\(^{3}\)

Although these assay systems for TAF are workable, they are also cumbersome and subject to other variables peculiar to \textit{in vivo} bioassay techniques. The purpose of our study was to continue this work in a tissue culture assay program in which tumor specimens and endothelium would be grown in separate cultures; the effect of media in which tumors had been growing (conditioned media) or fractions thereof on the endothelial cultures could be evaluated by the incorporation of \(^{3}\)H
thymidine.\textsuperscript{3,11,20} We are reporting our results with the first \textit{in vitro} assay system for the study of tumor trophic factors on endothelial proliferation.

**Methods and Materials**

**Cell Culture**

\textit{Endothelial Cells}. Endothelial cells were obtained from the umbilical vein of human umbilical cords by collagenase digestion employing the method described by Jaffe, \textit{et al.}\textsuperscript{18} These were then cultured on glass cover slips in six well plates\textsuperscript{*} in F\textsubscript{16} plus 20\% fetal calf serum and 1.5\% CO\textsubscript{2}. Handled as primary cultures the endothelial cells were the vehicle for our assay system; for each endothelial sample we prepared one control and five experimental cultures.

**Human Tumors.** Surgical specimens were diced into 1-mm cubes and placed aseptically in F\textsubscript{16} medium and 20\% fetal calf serum and transferred to Falcon plastic tissue culture flasks as lying drop cultures. Twenty-four hours later fresh F\textsubscript{16} medium was added to the flask and the tumor cells were allowed to grow for 1 week. The tumor type was determined by sections of the original surgical specimen stained with hematoxylin and eosin.

**Experimental Clonal Tumors.** Three different clonal lines of C\textsubscript{6} rat astrocytoma cells\textsuperscript{2} were grown in F\textsubscript{16} medium containing 10\% fetal calf serum.\textsuperscript{†} Fibroblasts were obtained from a biopsy specimen of a human scar and maintained in primary culture with F\textsubscript{16} and 10\% fetal calf serum. The fibroblasts used in these studies had a doubling time of 29 hours, as compared to 20 hours for the C\textsubscript{6} astrocytoma.

**Conditioned Media.** One ml of medium from a growing cell line (conditioned medium) filtered through a 0.22 \textmu m millipore filter was added to 2 ml of F\textsubscript{16} and 20\% fetal calf serum already in the culture well. One ml of fresh medium was added to one of the six wells as a control. Thus, all control and experimental cultures contained a total of 2 ml of medium for the initial 24 hours following plating. These were then supplemented with either 1 ml of conditioned medium or 1 ml of fresh F\textsubscript{16} containing 20\% fetal calf serum.

The fibroblasts were larger than the astrocytoma cells, and were therefore plated at 80\% of the cell density used for the astrocytoma (1.0 \times 10\textsuperscript{6} astrocytoma cells/10 ml of media). Each unit volume of medium was conditioned by approximately the same number of cells.

** Autoradiography**

Twenty-four hours after the addition of the conditioned media, 0.5 \mu Ci of \textsuperscript{3}H thymidine was added to each well. Seventy-two hours later, the cover slips were rinsed with Hanks balanced salt solution, and then fixed in 3:1 fixative (ethanol:acetic acid) for 10 minutes. Subsequently the slides were coated with Kodak NTB-2 nuclear tracking emulsion and exposed for 5 days. After development the cover slips were stained with either toluidine blue or Giemsa.

**Assay Method**

Slides of \textsuperscript{3}H thymidine labeled endothelia were observed under 10\times magnification, and the number of cells labeled with \textsuperscript{3}H thymidine as a proportion of the total number of cells for that field was recorded from 10 randomly selected fields on each slide. The ratio of the cells labeled compared to the total number of cells counted in each field is termed the thymidine labeling index (TI). To eliminate bias, the worker reading the slide was unaware of the group that each particular slide represented. Labeled cells are those which have more than 20 silver grains localized over the nucleus. The labeling index was computed as an average of the 10 low power, random fields for each slide, and these data were tabulated for each of the groups given in Table 1.

**Results**

As shown in Table 1 and Fig. 1, there is a dramatic increase in endothelial incorporation of \textsuperscript{3}H thymidine when conditioned medium from human or experimental tumors from the central nervous system (CNS) is added to primary endothelial cultures. It has been our consistent observation that the addition of conditioned medium to endothelial

\textsuperscript{*}Well plates manufactured by Linbro Plastics, New Haven, Connecticut.

\textsuperscript{†}Rat astrocytoma cells were obtained from S. M. Pfeiffer (University of Connecticut), J. De Veliss (University of California at Los Angeles), and from the American Type Culture Collection (CCL107).
FIG. 1. Graphic display of thymidine-labeling index of endothelial culture with control and conditioned media from human and experimental CNS tumors (see also Table 1).

FIG. 2. Autoradiograph showing endothelial cells after the addition of conditioned media from an angioblastic meningioma. Note high degree of thymidine uptake and mitotic figures.

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Culture also produces morphological alterations as seen by light microscopy. The striking increase of thymidine labeling is shown in Fig. 2 where almost every cell is labeled and mitotic figures may be seen.

A wide range in TI was noted in the endothelium cultures treated with human tumor-conditioned medium, ranging from a Grade III astrocytoma TI of 30.1% to a 98.3% TI resulting from the addition of conditioned medium from an angioblastic meningioma. This tumor had a prominent stain arteriographically and was highly vascular at surgery. It is interesting that the degree of endothelial stimulation does not reflect a tumor's arteriographic staining prominence as demonstrated by the 32.0% and 34.5% TIs that resulted from conditioned media from two arteriographically nonstaining acoustic neurinomas in culture.

The experimental tumors in clonal cultures also have a wide range of endothelial trophic influence. Three varieties of C₆ rat astrocytoma resulted in TIs of 28.7%, 83.3%, and 96.6% respectively. All of these cultures had been growing for some time, and it is interesting that the ability to produce an endothelial trophic factor was not lost after these cells adapted to a tissue culture environment.

There was a slight increase from a control labeling index of 2.1% to a TI of 4.5% when fibroblast conditioned medium was added. Amniotic fluid which contained a number of growth factors did not induce an increase in endothelial uptake of ³H thymidine, neither did a medium conditioned by amniotic cells in culture. The mitotic index of the endothelial cells in control cultures was 0.1%, and increased to 7.0% in the presence of tumor conditioned media. This increase in the mitotic activity indicates actual cell division, rather than mere DNA synthesis or repair.

We also performed an assay in which conditioned medium from a human astrocytoma was treated by heating to 56°C for 10 minutes; we then subjected this to the same experimental protocol. The resultant TI was 4.4%, which suggests that the endothelial trophic factor is inactivated by heat. Filtration of conditioned media through Amicon filters with a 10,000 dalton molecular weight cut-off does not remove the proliferation effects of such media. These findings rendered the possibility extremely unlikely that the
TABLE 1

<table>
<thead>
<tr>
<th>Endothelial uptake of ³H thymidine of control cultures and cultures with conditioned media from human and experimental CNS tumors*</th>
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<tbody>
<tr>
<td>No. Cells Examined</td>
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<tr>
<td><strong>controls</strong></td>
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<tr>
<td>endothelia alone</td>
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<td>endothelia with:</td>
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<tr>
<td>fibroblast conditioned media</td>
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<td><strong>human CNS tumors</strong></td>
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<tr>
<td>endothelia with conditioned media from:</td>
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<td>meningioma (syncytial)</td>
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<tr>
<td>meningioma (angio-elastic)</td>
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<tr>
<td>ependymoma</td>
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<tr>
<td>neuroblastoma</td>
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<tr>
<td>acoustic neurinoma (1)</td>
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<td>acoustic neurinoma (2)</td>
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<tr>
<td><strong>experimental CNS tumors</strong></td>
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<tr>
<td>endothelia with conditioned media from:</td>
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<tr>
<td>C-6 (Pfeiffer)</td>
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<td>C-6 (JDV)</td>
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<td>C-6 (CCL107)</td>
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* Data represent the cumulated averages of multiple cultures and assays.

tumor is deprived of its blood supply it may remain in a dormant state.12 The concept of antiangiogenesis requires definitive characterization of TAF.

In our experiment the fact that human tumors and experimental rodent CNS tumors increased the uptake of ³H thymidine by cultured human endothelium suggested that there is no species specificity in the trophic substance. Because these studies were limited to CNS tumors, we tested the effects of media conditioned by primary fetal brain, spinal cord, and retinal cultures, which were uniformly ineffective in the stimulation of endothelial proliferation. Furthermore, tumor-conditioned medium had no effect in cultured fibroblasts and this fact suggests that the trophic factor must be fairly specific for endothelial cells.

We have described a relatively straightforward in vitro assay system for the further characterization of TAF which we have demonstrated to be present in human or experimental tumor cultures. The relative stability of this as yet unidentified factor in conditioned media should make its future purification and identification possible. Such experiments are presently underway in our laboratory.

References

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