Astrocyte cultures derived from human brain tissue express angiotensinogen mRNA

(gene expression/renin-angiotensin system/central nervous system/angiotensin)

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ABSTRACT We have identified human cultured cell lines that are useful for studying angiotensigen gene expression and its regulation in the central nervous system. A model cell system of human central nervous system origin expressing angiotensigen has not previously been available. Expression of angiotensigen mRNA appears to be a basal property of noninduced human astrocytes, since astrocytic cell lines derived from human glioblastomas or nonneoplastic human brain tissue invariably produced angiotensigen mRNA. In situ hybridization histochemistry revealed that angiotensigen mRNA production was not limited to a subpopulation of astrocytes because >99% of cells in these cultures contained angiotensigen mRNA. These cell lines will be useful in studies of the molecular mechanisms controlling angiotensigen synthesis and the role of astrocytes in the human brain by allowing us to examine regulation of expression of the renin-angiotensin system in human astrocyte cultures.

The expression of the renin-angiotensin system (RAS) in mammalian tissue has attracted much interest because angiotensin (Ang) peptides may act as local regulators of tissue function. The brain contains a RAS that may possess both paracrine and autocrine functions (1). Genes expressed in the mammalian brain include angiotensigen (Aogen), renin, and Ang-converting enzyme (ACE) (1–6). Both the precursors and the enzymes that participate in the formation of Ang II are organized in region-specific arrays within the brain (7–12). Furthermore, site-specific cardiovascular responses are produced by intracranial injections of Ang II or administration of inhibitors of ACE (6). However, cellular mechanisms and biochemical pathways that lead to the presence of Ang peptides in elements of the central nervous system remain controversial. Components of the RAS have been ascribed to both neurons and glial cells (13–16). Kumar and coworkers suggest that neuron-enriched cultures derived from neonatal rat brain contained more Aogen mRNA than glial-enriched cultures (14). In contrast, Stornetta and coworkers colocalized glial fibribrillar acidic protein (GFAP) and Aogen mRNA to astrocytes of the rat brain (3), implicating glial cells as the chief cell type producing Aogen. Aogen is the only known precursor from which Ang peptides are formed, and it comprises the major protein of the RAS in cerebrospinal fluid (17). The putative presence of Aogen in astrocytes is of considerable interest because these cells play a key role in brain function, development, and disease and may also constitute a long-range signaling system within the brain (18).

 Cultured cell model systems facilitate analysis of the expression and function of peptide hormones. We used astrocytic cultures derived from human brain tissue to address whether Aogen mRNA is expressed by glial elements of the human brain. Furthermore, we compared Aogen mRNA expression in three separate cell lines derived from neoplastic human brain tissues to three nonneoplastic astrocyte lines to evaluate whether Aogen biosynthesis is an integral property of differentiated astrocytes.

MATERIALS AND METHODS

Cell Culture. Cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units of penicillin G sodium per ml, and 100 μg of streptomycin sulfate per ml. Cells were maintained at 37°C in a humidified atmosphere of 5% CO2/95% air. These cell lines were established over the period 1982–1988 and were subcultured and frozen prior to reconstitution for our studies. While cultures derived from neoplastic brain tissue form continuously dividing cell lines, the nonneoplastic cells have a finite life-span (six to nine passages) in culture.

Biopsy samples from which cultures were originally derived were removed during the course of stereotactic positioning and surgical resection of cerebral tissue. STTG1, CRTG3, and WTTG2 were obtained from neoplastic human brain tissues histopathologically diagnosed as grade IV astrocytoma (glioblastoma multiforme). The CAMS cell line was derived from centro-mesial and was obtained from a patient with multiple sclerosis during stereotactic thalatomy. W3N cells were derived from a specimen of temporal white matter during a surgical procedure for the correction of intractable epilepsy. The CRN line was obtained from non-involved regions of the brain of the patient from whom cell line CRTG3 was derived. The tissue originated from an area of tumor margin histopathologically diagnosed as nonneoplastic in nature.

Isolation and Characterization of Aogen mRNA. Aogen mRNA was assessed by Northern blot hybridization analysis. Isolation of total cellular RNA and poly(A)* RNA, RNA electrophoresis, blot transfer, and hybridization have been described (19, 20). Hybridization probes consisted of 32P-labeled nick-translated human Aogen cDNA insert or intact plasmid pHag3 (21).

For quantitation of relative levels of expression of Aogen mRNA, the probe was stripped from filters, which were reprobed with cDNA standards. Autoradiographic signals representing Aogen mRNA were standardized to signals from unregulated mRNAs (actin or glyceraldehyde-3-phosphate dehydrogenase).

Abbreviations: Aogen, angiotensigen; Ang, angiotensin; RAS, renin-angiotensin system; GFAP, glial fibribrillar acidic protein; NSE, neuron-specific enolase; ACE, Ang-converting enzyme.

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plastic astrocytic cells. Partial characterization of elongated cells bearing GFAP were sometimes preincubated with normal goat serum (1:5). Human skin fibroblasts served as negative cell controls, and human neuroblastoma cells (SK-N-MC) were positive cell controls for NSE staining. Cells were photographed under epifluorescence microscopy.

**In Situ Hybridization Histochemistry.** Cells were plated on plastic chamber slides (Nunc) and processed as described (3, 23) with minor modifications. Synthetic oligodeoxynucleotide probes were 3'-tailed with deoxyadenosine 5'-[α-35S]-triphosphate using terminal deoxynucleotidyltransferase. Hybridizations were carried out overnight at 52°C in 0.1× SSPE (1× SSPE = 0.15 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA). Slides were coated with Kodak NTB2 nuclear track emulsion diluted 1:1 with deionized water. After exposures of ~2 weeks, the film emulsion was developed and cells were stained with cresyl violet before being photographed under bright-field microscopy. A grid was placed over photographs, and silver grains per unit area were counted by two individuals.

STTG1, p21, and W3N, p3, were hybridized to a 35S-labeled 30-nucleotide synthetic oligodeoxynucleotide probe complementary to the nucleotides encoding human Ang I. Nonspecific hybridization was assessed by hybridizing parallel cultures with a radiolabeled oligodeoxynucleotide not complementary to human mRNAs [5'-AGAGTGCCCA-GAGAGGCAT-3'], complementary to a region of rat renin cDNA (24). All oligodeoxynucleotides were synthesized in the Department of Molecular Biology, Cleveland Clinic Foundation.

**RESULTS AND DISCUSSION**

Partial characterization of morphology and growth properties of some of the cell lines used in this study has been reported (25, 26). With one exception (CRN), all lines demonstrated cells bearing the astrocyte marker GFAP. STTG1, the astrocytoma line that was examined most extensively, was noted to exhibit diffuse GFAP staining in the cytoplasm and in elongated processes in 80% of early passage (two to five) cells. GFAP staining in comparable cell passages was observed in 20% of WITG2 and in 60% of CRTG3 astrocytoma cells (B.P.B., unpublished data). Cells in STTG1 and CRTG3 were predominantly labeled with antisera to the astrocyte-specific intermediate filament component GFAP, confirming their astrocytic lineage (27), while less than one-third of cells in culture WITG2 were labeled. Immunostaining of nonneoplastic cell lines at passages one to three showed that CRN was the only cell line that did not contain GFAP-positive cells. In contrast, cell lines W3N and CAMS showed staining by amounts that ranged between 70% (28) and 80%, respectively, indicating that the majority of cells are astrocytes. Further verification was obtained by the finding that none of the astrocytic cell cultures that showed positive immunostaining for GFAP stained for NSE. The absence of NSE-stained cells confirms that these cultures were not contaminated with neurons or neuroendocrine cells. In the present study, immunocytochemistry demonstrated GFAP-containing cells (Fig. 1) but no NSE-containing cells in W3N cultures.

We detected two mRNA species approximately 2 and 2.2 kilobases long in RNA isolated from STTG1 and CRTG3 cells (Fig. 2). The mRNA sizes agree with those reported for the two human Aogen mRNA transcripts found in liver (21, 29). Aogen mRNA was observed in each of seven experiments done in STTG1 cells. We estimated that Aogen mRNA levels in the STTG1 astrocyte cultures were at least 2-fold less than levels of Aogen mRNA in (dog) liver (A.M., unpublished data). The possible significance of this observation in terms of tissue-specific expression and regulation of Aogen mRNA levels remains to be determined. Variations in culture conditions, including growth in serum-free medium and passage number (passages 16–27), did not affect levels of Aogen mRNA. Similar results were found in four different experiments with CRTG3 cells. However, in CRTG3 cells the level of Aogen mRNA was consistently lower than in STTG1 cells. In WITG2 cells, where levels of Aogen mRNA are very low, we detected only the larger Aogen mRNA species. Expression of Aogen mRNA in WITG2 was at or just above the limits of detection with our current methods of analysis.

![Fig. 1. Immunocytochemistry. A well-spread cell in a culture of W3N cells containing GFAP immunoreactive fibers. (×600.)](image)

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![Fig. 2. Northern blot of Aogen mRNA in total cellular RNA prepared from glioblastoma (A) or nonneoplastic (B) cell lines. (A) Lanes (left to right): WITG2 (10 μg), CRTG3 [10 μg of poly(A) + RNA], and STTG1 (20 μg). (B) Lanes (left to right): CRN, W3N, and CAMS cell cultures. Gels contained 0.8% agarose (A) or 1.2% agarose (B). Exposure times were 4 days. Migration of HindIII-digested DNA markers is indicated.](image)
Table 1. Expression of Aogen mRNA

<table>
<thead>
<tr>
<th>Culture</th>
<th>Relative level</th>
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</thead>
<tbody>
<tr>
<td>WITG2</td>
<td>ND</td>
</tr>
<tr>
<td>CRTG3</td>
<td>0.63</td>
</tr>
<tr>
<td>CRN</td>
<td>0.74</td>
</tr>
<tr>
<td>STTG1</td>
<td>1.00</td>
</tr>
<tr>
<td>W3N</td>
<td>3.08</td>
</tr>
<tr>
<td>CAMS</td>
<td>3.51</td>
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ND, at or below the level of detection. Relative level is compared to STTG1 (standardized to actin mRNA).

These results provided initial evidence that human cells of astrocytic lineage expressed Aogen mRNA in culture (Table 1).

It was important to learn whether the expression of Aogen mRNA by STTG1 and CRTG3 was a consequence of the neoplastic phenotype or a reflection of astrocytic differentiation. To address this question, we utilized three additional lines of human nonneoplastic glial cells. By blot hybridization analysis (Fig. 2), CAMS and W3N expressed high levels of Aogen mRNA, while CRN showed much lower levels of the mRNA species. The correlation between GFAP-staining and Aogen mRNA expression in the three cultures of nonneoplastic human brain suggested that Aogen mRNA biosynthesis is a property of differentiated astrocytes. We observed Aogen mRNA production in each astrocyte cell line, suggesting that the mRNA encoding Aogen may be invariably present in astroglia. Since astrocytes constitute about one-half of the cerebral matter, this finding suggests a possible source of the large quantities of Aogen that have been reported in cerebral tissue and cerebrospinal fluid (17). In our studies, cultured astrocytes were not under the co-regulatory influences that may be exerted by other neuronal elements or humoral factors circulating in the brain fluid microenvironment. Therefore, isolation from these regulatory influences may be one factor that accounts for the finding that cultured astrocytes produce Aogen mRNA in the noninduced state.

The possibility existed that production of Aogen mRNA was accounted for by a minority population of the cells within each astrocytic line. Indeed, Stornetta et al. (3) had provided some evidence for this possibility. Therefore, we performed in situ hybridization histochemistry to evaluate whether production of the Ang precursor is confined to a selected subpopulation of cells.

The data shown in Fig. 3 confirmed the presence of Aogen mRNA in the cell lines. Every cell expressed Aogen mRNA, and some cells expressed much higher levels than other cells, as reflected by the different numbers of silver grains present over individual cells (Fig. 3). Analysis of micrographs revealed that cultures expressing Aogen mRNA (as determined by Northern blots) contained cells that hybridized to human Aogen probe (Fig. 3). Silver grains over cells were counted and compared to silver grains over regions not containing cells (grains per unit area over cells compared to grains per unit area over background regions of slide). Analysis revealed an average ratio of 8.2:1 for STTG1 and 11.5:1 for

**Fig. 3.** In situ hybridization histochemistry shows expression of Aogen mRNA in every cell in STTG1 and W3N cultures. (Upper Left) STTG1, Aogen probe. (Upper Right) STTG1, nonspecific probe. (Lower Left) W3N, Aogen probe. (Lower Right) W3N, nonspecific probe. (×1000.)
W3N on slides hybridized with Aogen mRNA-specific probe. This compares to an average ratio of 1.5:1 for STTG1 and 2.1:1 for W3N (grains per unit area over cells compared to grains per unit area over background) on slides hybridized with a nonspecific control oligodeoxynucleotide probe. Results of analyzing STTG1, W3N, CRTG3, and CRN cell cultures in this manner showed that each culture expressed Aogen mRNA, consistent with the results after Northern blot analysis of isolated total cellular RNA. Thus, we verified with in situ hybridization histochemistry that Aogen mRNA expression is a basal property of these noninduced cultured human astrocytes.

To determine whether the cells that produced Aogen mRNA also exhibited other components of the RAS, we hybridized W3N and CRTG3 cells with a synthetic oligodeoxynucleotide probe complementary to ACE mRNA. In these experiments, we found no specific signal above background. During the course of these analyses, we subsequently probed the same Northern blotted RNA samples with cDNAs encoding other components of the RAS. At no time were we able to detect mRNAs hybridizing to either human renin or human ACE probes in any of the cultures. However, all cultures expressed high levels of glyceraldehyde-3-phosphate dehydrogenase and actin, mRNAs that are normally abundant in mammalian cells.

Although relationships between the original brain tissue placed in cell culture and the cell lines in their present condition are expected to be tenuous at best, we reviewed the medical records of the five patients from whom tissue biopsy samples were obtained to evaluate the possibility that we had inadvertently cultured cells from an atypical patient population. No obvious patterns were found.

The significance of this study lies in our identification of human cell lines derived from central nervous system tissues where Aogen synthesis and the molecular mechanisms regulating it can be elucidated. Aogen, as the sole precursor of biologically active Ang peptides, is a critical component of the RAS. Moreover, regulation of some components of the RAS may be different in the brain than in the periphery. That these glioblastoma and nonneoplastic astrocytes maintain expression of Aogen mRNA after removal from brain and loss of interconnections with neurons and other brain cells indicates that all factors required for expression of Aogen mRNA transcription are contained within the cultured astrocytes. The availability of a central nervous system cell culture system will allow us to address directly how Aogen mRNA transcription and mRNA levels are regulated in astrocytes, the chief cellular source of this critical component of the central RAS.

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