Expression of androgen and progesterone receptors in primary human meningiomas

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Meningiomas are common brain tumors that show a predilection for females and become more aggressive during pregnancy and menses. The existence of gender-specific hormone receptors in meningiomas has long been a matter of controversy; the recent cloning of androgen, estrogen, and progesterone receptors has facilitated their direct evaluation. The authors have demonstrated the expression of androgen and progesterone receptor messenger ribonucleic acid and protein product in nine primary human meningiomas by Northern blot analysis. Cellular localization was achieved by in situ hybridization analysis. Estrogen receptor expression was not detected. Normal adult meninges were shown to express very low levels of both androgen and progesterone receptors.

Key Words: hormone receptor · meningioma · gene expression · proto-oncogene · epidermal growth factor

MENINGIOMAS are common human intracranial tumors that arise from meningothelial cells found in the arachnoid villi of the meninges, the membranes covering the brain and spinal cord. Meningiomas are primarily benign tumors that may be cured by excision, although some are inoperable or invasive and are not amenable to surgical removal.

The association of gender-specific hormone receptors with these tumors is indicated by a wealth of clinical and epidemiological data, including the greater predilection for females, the documented association between meningiomas and breast carcinomas in the same patients, and the promotion of symptomatic aggressive growth of previously asymptomatic tumors during pregnancy and the luteal phase of the menstrual cycle. However, the existence of gender-specific hormone receptors in meningiomas has long been a controversial issue. This may be due to a wide variation both in binding assay techniques and in the assessment of binding specificity by competitive studies. Despite the frequent discrepancies, the following picture of sex hormone receptor expression has emerged from binding assays: meningiomas have been shown to contain high levels of progesterone receptor and moderate concentrations of androgen receptor, while the level of estrogen receptor is still equivocal. The recent cloning of complementary deoxyribonucleic acid (cDNA) encoding the human estrogen, progesterone, and androgen receptors has facilitated the direct investigation of hormone receptor gene expression, uncomplicated by variations in binding assay interpretation.

In this study, we have demonstrated the coexpression of androgen and progesterone receptor messenger ribonucleic acid (mRNA) and protein product in nine primary meningiomas. Estrogen receptor in mRNA expression was not detected. Cellular localization of both mRNA and protein product has been achieved through the use of in situ hybridization and immunohistochemistry.

Materials and Methods

Tissue Collection

For Northern blot analysis, tissues were collected intraoperatively and were immediately snap-frozen in liquid nitrogen before being stored at -80°C. For in situ hybridization, tissue specimens were immersed in ice-cold 4% paraformaldehyde and processed as described below. All tumor samples were derived from patients with meningioma. Normal pachymeninges were harvested intraoperatively from trauma patients and served as normal control tissue.
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Northern Blot Analysis

Fragments of tissue were immediately placed in 4 M ice-cold guanidinium isothiocyanate,* then homogenized by a polytron (setting 6 for 45 seconds). After the tissue was centrifuged for 2 minutes at 1000 rpm, the supernatant fluid was carefully layered on a cesium chloride cushion and centrifuged in a rotor† at 35,000 rpm at 20°C for 18 hours. Total RNA was then extracted by standard ethanol precipitation after phenol extraction. Aliquots of 25 mg RNA were heated at 95°C for 2 minutes in a solution containing 50% formamide, 6% formaldehyde, and running buffer (20 mM morpholino-propanesulfonic acid, pH 7.0, containing 5 mM sodium acetate and 1 mM ethylenediamine tetra-acetic acid (EDTA). The samples were electrophoresed at 35 V overnight through a 1% agarose gel containing 6% formaldehyde and running buffer. The RNA was transferred to nylon membranes using 10 x standard saline citrate (SSC) transfer buffer, baked at 80°C for 1 hour, and hybridized with 1 x 10^6 cpm/ml of 32-DCTP (deoxy-cytosine triphosphate) random primer-labeled cDNA probe in a solution containing 50% formamide, 0.1% sodium dodecyl sulfate (SDS), 5 x standard sodium phosphate EDTA, 5 x Denhardt’s mixture, and salmon sperm DNA (200 mg/ml).‡ After washing at 65°C with 0.1 x SSC and 1% SDS, the membranes were subjected to autoradiography at −70°C using intensifier screens. The cDNA probes for the present studies included androgen receptor,† progestosterone receptor,‡ estrogen receptor,§ and β-actin.¶

In Situ Hybridization

Fresh tumor tissue was fixed immediately after excision in ice-cold 4% paraformaldehyde for 2 hours and then allowed to sink in 30% sucrose phosphate-buffered saline (PBS) overnight at 4°C to decrease freezing artifacts. In situ hybridization utilizing 32P-labeled cRNA probes for androgen, progesterone, and estrogen receptors was performed on 8-μm frozen sections of both meningiomas and control normal meningiuses according to the method of Hoefler, et al.∥

The specificity of the probes for in situ hybridization was controlled by Northern blot analysis and by hybridization of serial sections with noncomplementary "sense" RNA probes.

Immunocytochemistry

Tissues were prepared as described for in situ hybridization. The tissue sections were hydrated in PBS, quenched with 0.3% H2O2 in methanol, and reacted with the appropriate antibody by means of a staining kit.§ The tissues were counterstained with hematoxylin, then dehydrated, cleared, and mounted. The specificity of the immunostaining was tested by replacing the primary antibody with preimmune sera, which should lead to negative results. The following specific antisera were used in these studies: AR52 anti-androgen receptor,∥ anti-progesterone receptor, and anti-estrogen receptor.

Results

Expression of the Androgen Receptor Gene and Protein

The androgen receptor gene encodes multiple transcripts, two of which (3.0 kb and 4.5 kb) were clearly visible in five (55%) of the nine meningiomas (Fig. 1 and Table 1). Androgen receptor mRNA transcripts were detected in an additional case (Fig. 1, Lane 1) by laser densitometric analysis. In situ hybridization of the meningioma tissue revealed great overexpression of androgen receptor mRNA (Fig. 2A). This was substantiated by immunocytochemical demonstration of androgen receptor protein, which showed both nuclear and cytoplasmic localization (Fig. 2B). In situ hybridization with a "sense" cRNA control resulted in background signal intensity (Fig. 2C), and immunocytochemistry with preimmune sera showed no staining (Fig. 2D). Normal adult pachymeninges expressed low

* Guanidium isothiocyanate supplied by Fluka Chemicals, Buchs, Switzerland.
† Centrifuge, Model SW 50.1, manufactured by Beckman Instruments, Inc., Fullerton, California.
‡ Nylon membrane supplied by Schleicher & Schuell, Inc., Keene, New Hampshire; cDNA probe supplied by Amersham Corp., Arlington Heights, Illinois; formamide provided by Kodak, Rochester, New York; and salmon sperm DNA supplied by Sigma Chemical Co., St. Louis, Missouri.
§ Vectastain avidin-biotin complex kit manufactured by Vector Laboratories, Inc., Burlingame, California.

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TABLE 1
Histopathological classification of nine meningioma tumors and correlation with receptor expression*

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<tr>
<th>Lane</th>
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<th>Sex</th>
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<th>Progesterone</th>
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<td>M</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>F</td>
<td>++</td>
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<td>-</td>
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<td>syncytial</td>
<td>F</td>
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<td>F</td>
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* Graded according to the World Health Organization standards.††
See Fig. 1 for Northern blot analysis data.
† † and ++ = relative expression as determined through laser densitometry; − = no detectable expression.

Levels of androgen receptor transcripts by in situ hybridization (Fig. 3A).

Expression of the Progesterone Receptor Gene and Protein

The progesterone receptor gene encodes at least two mRNA transcripts of approximately 2.5 kb and 3.5 kb, both visible in three of the nine meningiomas (Fig. 1 and Table 1). These transcripts were detectable (Fig. 1, Lanes 1, 2, 3, 7, and 8) via laser densitometry. The 10-day exposure time indicates weak but tangible expression. The degree of expression varied significantly among the samples tested, as determined by laser densitometric analysis. None of the samples showed detectable RNA degradation. Loading errors for RNA were controlled for by subsequent hybridization of the membranes with the cDNA encoding β-actin.

In situ hybridization and immunohistochemical analysis revealed progesterone receptor expression by characteristic fusiform meningioma cells (Fig. 4A and

Fig. 2. Photomicrographs showing the localization of androgen receptor (AR) mRNA (A) and protein (B) in a primary human meningioma by in situ hybridization and immunocytochemistry, respectively. Very high levels of AR mRNA are seen in the meningioma cells (A), with commensurate protein levels in nuclear and cytoplasmic sites (B). Hybridization of a "sense" control results in background signal levels (C), and staining with preimmune sera expressed no AR protein (D). × 466.
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**Fig. 3.** Photomicrographs of *in situ* hybridization of androgen receptor (A) and progesterone receptor (B) in normal adult pachymeninges control tissue. Only background expression is detectable. × 630.

B). *In situ* hybridization with a "sense" cRNA probe (Fig. 4C) and immunohistochemical staining with preimmune sera (Fig. 4D) depicted background signal levels. Control normal adult pachymeninges did not express significant levels of progesterone receptor mRNA (Fig. 3B).

**Fig. 4.** Photomicrographs demonstrating the localization of progesterone receptor (PR) mRNA (A) and protein (B) in a primary human meningioma by *in situ* hybridization and immunocytochemistry, respectively. Clear expression of PR mRNA is apparent in the meningioma cells (A) and of protein in a cytoplasmic localization (B). Background signal is obtained using a "sense" cRNA probe (C) and following staining with preimmune sera (D). × 454.
Expression of the Estrogen Receptor Gene and Protein

The estrogen receptor gene encodes a 6.2-kb mRNA, which was absent from all meningioma samples on both Northern blot analysis (Fig. 1) and in situ hybridization. Immunocytochemistry failed to detect estrogen receptor protein product. Estrogen receptor mRNA was not identified in normal adult meninges by in situ hybridization analysis (data not shown).

Discussion

Analysis of Results

The compelling clinical and epidemiological evidence for the influence of hormones on the growth of meningiomas is attended by much discrepancy in the literature. This may reflect the use in some studies of preoperative glucocorticoids, which may bind to and thereby block part of the gender-specific hormone receptors in the tumors. Alternatively, the conflicting results may reflect the variations in binding assay techniques and in the qualification of binding specificity by competitive studies. For this reason, we employed direct molecular techniques using recently cloned cDNA's of hormone receptors and immunocytochemical assays to determine the presence of hormone receptor mRNA transcripts and their respective protein products in nine primary human meningiomas.

In this study, we have demonstrated the in vivo coexpression of genes encoding androgen and progesterone receptors in a large proportion of primary meningiomas. Despite the considerable discrepancies in gender-specific hormone receptor expression in meningiomas, these findings confirm the most consistent data in the literature.

We were unable to detect estrogen receptor mRNA or protein in any of the nine meningiomas studied, but we found progesterone receptor mRNA and protein in 88% of tumors analyzed. This confirms reports identifying progesterone receptor in the majority of meningiomas examined. Progesterone receptor protein distribution was clearly both nuclear and cytoplasmic. The relationship between progesterone receptor and histological tumor type has been debated. Progesterone receptor was detected at high levels in syncytial, transitional, and fibroblastic tumors. Previous reports indicate that the levels of progesterone receptor were significantly lower in fibroblastic meningiomas and were absent in anaplastic meningiomas. Hayward, et al., found no progesterone receptor in two aggressive hemangiopericytic angiolastic meningiomas. It has also been suggested that high levels of progesterone receptor are to be found in some of the more aggressive tumors.

We found androgen receptor mRNA and protein in 66% of the meningiomas, confirming the work of others. Androgen receptor protein was localized in both a nuclear and cytoplasmic distribution in meningioma tumor cells.

Steroid Receptors and Oncogenes

Steroid hormone receptors are intracellular proteins that play an important role in regulating gene expression, the synthesis of DNA and protein, and the growth of cells. Cloning of the genes encoding steroid hormone receptors revealed them to be members of a superfamily of potentially oncogenic genes. They share with the erb A, oncogene sequences encoding a DNA-binding domain involved in the specific control of gene expression. Since these hormone receptor gene products control cell growth and differentiation, it is possible that a structurally altered or deregulated receptor could participate in neoplastic transformation.

In support of this hypothesis, alterations of restriction patterns of DNA-binding domains of estrogen receptor genes have been reported in human meningiomas. Alterations to erb A and steroid receptor genes have been reported in hepatocellular carcinomas and small-cell lung carcinomas. In vitro studies have shown that overexpression of some proto-oncogenes can result in neoplastic transformation. Furthermore, aberrant expression of proto-oncogenes encoding growth factors or their receptors has been found in several human tumors, including the proto-oncogenes c-sis/platelet-derived growth factor-B in astrocytomas and meningiomas, and c-erb B (epidermal growth factor (EGF)-R) in meningiomas and astrocytomas. K-ras in primary human meningiomas, and human EGFR-2/neu in breast and ovarian carcinomas, and the nuclear oncogenes N-myc in neuroblastomas and c-myc in lung carcinomas. Androgens have recently been shown to induce the synthesis of EGF-R in human prostatic carcinoma cells, thereby implying a functional link between hormone action and proto-oncogene expression in these neoplasms. Both EGF-R mRNA and protein product have also been demonstrated in these tumors (in preparation). Thus, androgens may promote meningioma cell growth via autocrine or paracrine mechanisms that render the cells more sensitive to growth factor-mediated stimuli. Much data exist to implicate steroid hormones in human cancers. For example, human prostate cancers are often androgen-dependent in the early stages of growth. Detection of estrogen receptor in human breast cancers is predictive of their prognosis. Striking therapeutic success has been achieved through the use of tamoxifen, an anti-estrogen agent, in breast cancer patients. Interactions between EGF and insulin, estrogen, and progestins have been reported in breast carcinoma cells.

Therapeutic Regimens

In vitro studies in cell culture have shown that estrogen, progesterone, and tamoxifen each promote the growth of meningioma cells. Demonstration of the elevation of progesterone receptor in a large proportion of human meningiomas has led to experimentation with the anti-progesterone agent mifepristone (RU-486). Several in vitro studies have lent support to this hypothesis. Mifepristone has been shown to cause a growth inhibition ranging from 18% to 36% in three meningioma cell lines. The same group has also demonstrated a significant reduction in the size of human meningioma tissue implanted into nude mice treated with mifepristone.
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to cause a significant decrease in the thymidine labeling index in 13 human meningioma specimens.\(^7\)

Based on the extensive data from \textit{in vitro} studies, indicating a role for mifepristone in the inhibition of meningioma growth, Grunberg, \textit{et al.}\(^8\) have performed clinical trials. Of 14 meningioma patients who received mifepristone therapy, an objective response of tumor reduction was seen in 35% on computerized tomography or magnetic resonance imaging. An additional three patients (21%) experienced symptomatic improvement. Given the prevalence of androgen receptors in human meningiomas, similar trials using anti-androgen agents are clearly indicated.

Despite considerable variation in the available literature, our data confirm the existing consensus in demonstrating the presence of both androgen and progestosterone receptor (but not estrogen receptor) mRNA and protein by Northern blot analysis, \textit{in situ} hybridization, and immunocytochemistry. We propose that the presence of these receptors may contribute to meningioma tumorogenesis. It is hoped that the therapeutic achievements of hormonal manipulation in the treatment of breast cancer will be equaled in meningioma patients.

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