Hormonal effects on glioblastoma multiforme in the nude rat model

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Object. The authors studied the effect of gender and hormonal status on survival in nude rats implanted with human glioblastoma multiforme (GBM) cell lines.

Methods. Nude rats received intracerebral implants of either wild-type U87MG cells or U87MG cells transfected with the gene for endothelin-1 (U87/ET-1). In the initial study, survival was compared in males and females for each of the two cell lines. The six second-phase study groups were composed of: 1) males; 2) females; 3) ovariectomized females; 4) sham ovariectomized females; 5) ovariectomized rats given 10 μg/day estradiol benzoate for 21 days; and 6) ovariectomized rats given 20 mg/kg/day progesterone for 21 days. All rats in the second phase were implanted with U87/ET-1 cells. Animals were killed when they exhibited initial signs of neurological deterioration. Female nude rats survived longer than male rats implanted with either U87 or U87/ET-1 cells. In the second phase, ovariectomized, male, and progesterone-treated rats died at approximately 19 days, whereas the female, sham-treated, and estrogen-treated animals died 23 to 25 days after tumor cell implantation.

Conclusions. The authors demonstrate that female nude rats implanted with human GBM cells have a survival advantage over male rats and that estrogen provides the advantage.

KEY WORDS • glioma • gender • estrogen • survival • rat
Hormonal effects on GBM

Transfection With Endothelin-1

The U87 cells were grown in log phase, trypsinized, suspended, and washed twice in phosphate-buffered saline and then resuspended in a final concentration of 10^7 cells per 200 μl. Transfection was accomplished by electrophoration using 10 μg purified, intact pMEXneo/ET-1 plasmid. Thereafter, cells were grown for 48 hours in RPMI-1640 medium with 15% fetal calf serum. The G418 (NEO) selection was performed in media supplemented with 200 μg/ml G418. Neo-expressing clones were grown in 96-well plates by using a 1:3 cell per well limiting dilution method. Individual clones were transferred to T flasks and grown to confluence. Production of ET-1 cytokine was confirmed by enzyme-linked immunosorbent assay. The clone that produced the largest amounts of ET-1 was selected, expanded, and designated U87/ET-1.

Animal Care

The research protocol was reviewed and approved by the Animal Care Subcommittee, Veterans Administration Medical Center, Buffalo, New York. Animal care conformed with National Institutes of Health guidelines. Nude rats (nu/nu), originally obtained from Harlan Sprague-Dawley Co. (Indianapolis, IN), were bred and maintained in specific pathogen-free conditions. All animal procedures were performed in a laminar flow hood.

Surgical Manipulations

All operations were performed after induction of anesthesia consisting of 80 mg/kg ketamine and 4 mg/kg xylazine (1 ml/kg given intraperitoneally).

Ovariectomy. At least 2 weeks before tumor cell implantation, female nude rats were anesthetized, prepared under sterile conditions, and draped. A midline incision was made. A burr hole was drilled at 1.4 mm anterior and 2 mm lateral to the bregma, according to the atlas of Paxinos and Watson. A 1.0-μl Hamilton syringe with a 26-gauge needle was advanced 5.5 mm from the burr hole and then slowly withdrawn; the scalp was closed using No. 3-0 vicryl sutures. Sham ovariectomy was performed as for ovariectomy, including ovarian exposure, but the ovary was replaced and the flanks closed. Success of the ovariectomy was confirmed if five consecutive daily vaginal smears showed females to be in permanent diestrus.

Tumor Cell Implantation. Three-month-old male and female nude rats weighing 270 to 300 mg and 200 to 230 mg, respectively, were anesthetized and placed in a Kopf stereotaxic frame (Kopf Instruments, Tujunga, CA). The scalp was shaved, prepared in a sterile fashion, draped, and a midline incision was made. A burr hole was drilled at 1.4 mm anterior and 2 mm lateral to the bregma, according to the atlas of Paxinos and Watson. A 1.0-μl Hamilton syringe with a 26-gauge needle was advanced 5.5 mm from the dura and 25,000 cells were deposited in 2.5 μl of serum-free RPMI-1640 media over 3 minutes by using a modification of a previously described technique. The needle was left in place for 2 minutes and then slowly withdrawn; the scalp was closed using No. 3-0 vicryl sutures.

Study Groups

Survival. Twenty-eight nude rats (15 female and 13 male) received implants of wild-type U87. In the next experiment, 65 nude rats (38 male and 27 female) were implanted with U87/ET-1. These two sets of animals constituted the simple study of gender-related differences in survival.

Hormone Replacement. We chose the U87/ET-1 line over U87 for the in-depth hormone replacement studies because it led to death more rapidly than U87 and is histologically closer to GBM. Twenty-seven male and 91 female nude rats received intracerebral implants of U87/ET-1. The females were subdivided into five groups: normal females (13 rats), ovariectomized females (32 rats), sham-ovariectomized females (15 rats), ovariectomized rats receiving 10 μg/day estradiol benzoate (13 animals), and ovariectomized female rats receiving 20 mg/kg/day progesterone (18 animals). The hormones were mixed with sesame oil and administered daily by subcutaneous injection for 21 days, beginning on the day of tumor cell implantation. The animals were observed daily, and when they showed signs of neurological impairment (lethargy, head tilt, inability to regain upright posture) they were killed in a CO2 chamber. The brain was removed and in some animals was fixed in 4% paraformaldehyde for histological sections. In others, the brain and tumors were placed in an oven at 60°C, under vacuum, for 1 week or until 2 consecutive dry weight values were identical.

Statistical Analysis

The survival data were plotted on Kaplan–Meier curves and the differences between groups were compared using the Mantel–Cox log-rank test.

Results

Survival Duration

Female rats implanted with wild-type U87 cells survived a mean of 32.7 ± 1.1 days after implantation, compared with 28 ± 1.2 days for male rats. Median survival times were 30 days for females and 26 days for males. This difference was significant at a probability level of 0.02 (Fig. 1). For animals implanted with the U87/ET-1 cells, females survived 27.3 ± 0.7 days compared with 22.6 ± 0.6 days for male rats (median 27 days and 22 days for female and male rats, respectively). This was a significant difference (p < 0.0001, Fig. 2). Thus, the ET-1 transfected cell line was more aggressive, with shorter survival times in both sexes than observed for the wild-type U87 line (p < 0.0001).

Hormone Replacement

Results and statistical analyses for the six study groups are shown in Fig. 3 and in Table 1. Ovariectomy, but not sham ovariectomy, negated the survival advantage of female rats. Estrogen replacement reversed the effect of ovariectomy and restored the female advantage. Progesterone replacement at high physiological levels had no effect on survival. After statistical analysis, the six distinct study groups exhibited the following pattern: male, ovariectomized, and progesterone-treated animals died early,
whereas female, sham ovariectomized, and estrogen-treated animals died in a later cluster.

**Histological Studies**

The tumors that grew from wild-type U87 cells were hypercellular, yet uniform, with little neovascularity and no necrosis, and their appearance was consistent with anaplastic astrocytoma. The U87/ET-1 tumors demonstrated neovascularity, dense cellularity, pleomorphism, and necrosis and were more similar to GBM histologically. However, the appearance of U87/ET-1 tumors did not differ between male and female rats. No significant difference in tumor or brain weight at death was noted between male and female rats (data not shown).

**Discussion**

Questions about possible hormone involvement in human nervous system neoplasms have been broached repeatedly over the years. Male predominance in central nervous system (CNS) tumor incidence in virtually all population subgroups studied and by occasional reports of gender-related survival differences. Male predominance is observed in all but a few CNS tumor subtypes, the most notable exception being meningiomas, which are much more prevalent in females. There is a striking male preponderance in the most common and most deadly CNS tumors: GBM and astrocytoma. The generally accepted observation is that “the more malignant tumors of neuroepithelial origin have a preponderance in the male sex whereas the benign tumors of the coverings of the brain and spinal cord occur more frequently in female hosts.” Most studies of survival in malignant CNS tumors have failed to identify gender as an important predictive variable; age and histological grade stand out. Except for meningiomas, few CNS tumors have shown traditional female hormone receptors. The lack of receptors, coupled with the paucity of reports of gender-related survival differences, have caused the neurooncology community to disregard gender as a significant factor in glial tumors. The results of experiments described here indicate that this body of accumulated evidence, and the conclusions drawn on it, warrant close re-evaluation.

The three animal experiments that we have conducted to assess the influence of hormones on glial tumors all involve the implantation of human GBM cell lines into nude rats. These experiments in human GBM tumors are the first to link a significant positive survival advantage definitively and reproducibly to the direct effect of sex steroids. Tumor progression was slowed in the presence of endogenous or exogenous estrogen, resulting in a 20% prolongation of life, and the positive effect was observed in two separate GBM tumor lines. The female survival advantage was lost with ovariectomy and restored by estrogen replacement, but not by progesterone replacement. Progesterone is known to inhibit the formation of edema in contused rat brain, an effect that might also be important in tumor-induced edema. No difference in peritumoral edema was evident in comparisons between groups in this study. This evidence of a protective effect of female sex steroids in human GBM cell lines in nude rat brain, coupled with the observed lower incidence of these lesions in females, and a few scattered reports of a female survival advantage, is very intriguing. The lower incidence in females and better prognosis, if verified, might point to a very basic hormonal suppression of these tumors. The survival effect observed here is surprisingly large considering that we are dealing with the most aggressive type of brain tumor (almost 80% of patients are dead within 1 year of diagnosis) and considering the failure of experimental treatment modalities, even aggressive cytotoxic regimens.

Animal studies of cerebral neoplasms vary from observational studies of spontaneous tumor incidence, to chemical or transplacental induction, to the direct transplantation of tumor cells into animal hosts. Each of these systems has been used over the years to assess the effects of hormones on CNS tumors, with variable results. Studies of spontaneously occurring glial tumors in rodents show a histological distribution very similar to that of

![Graph of Kaplan-Meier survival plot for nude rats implanted with the transfected GBM cell line U87/ET-1. The mean survival duration is shown in brackets. The difference in survival times between male and female rats was statistically significant according to the Mantel-Cox log-rank test (p = 0.0001).](image1)

![Graph of Kaplan-Meier survival plot for control and experimental groups of nude rats implanted with the U87/ET-1 cell line. The mean survival duration is shown in brackets. The statistical comparisons are shown in Table 1; there is a clear division between the three early death groups and the three longer-lived groups. This division was highly statistically significant. E = estradiol benzoate; Ovx = ovariectomized; P = progesterone.](image2)
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TABLE 1

Comparison of survival times in 118 nude rats implanted with the U87/ET-1 cell line and assessed according to gender and hormone therapy

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>No. of Rats</th>
<th>Median Survival (days)</th>
<th>Mean ± SD (days)</th>
<th>Relative to Males (%)</th>
<th>Ovx + E</th>
<th>Normal Ovx</th>
<th>Early Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>ovx + E</td>
<td>13</td>
<td>24</td>
<td>25.4 ± 3.0</td>
<td>128</td>
<td>—</td>
<td>0.3635</td>
<td>—</td>
</tr>
<tr>
<td>female</td>
<td>13</td>
<td>24</td>
<td>24.1 ± 3.1</td>
<td>122</td>
<td>—</td>
<td>0.2570</td>
<td>—</td>
</tr>
<tr>
<td>sham ovx</td>
<td>15</td>
<td>24</td>
<td>23.9 ± 2.9</td>
<td>121</td>
<td>—</td>
<td>0.7419</td>
<td>—</td>
</tr>
<tr>
<td>ovx + P</td>
<td>18</td>
<td>19</td>
<td>20.4 ± 4.4</td>
<td>103</td>
<td>0.0024</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>male</td>
<td>27</td>
<td>20</td>
<td>19.8 ± 1.8</td>
<td>100</td>
<td>0.0129</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ovx</td>
<td>32</td>
<td>19</td>
<td>18.8 ± 1.9</td>
<td>95</td>
<td>0.0100</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* E = estradiol benzoate; ovx = ovariectomized; P = progesterone; SD = standard deviation.
† Calculated according to Mantel–Cox log-rank statistics.
‡ p < 0.05.
§ p < 0.001.
‖ p < 0.0001.

Garman, et al.,11 reported that male Fischer 344 rats exposed to ethylene oxide vapor developed more astrocytomas than females (ranges 0–6.9% of males and 1.1–3.8% of females, depending on ethylene oxide concentration). Fischer 344 rats exposed to acrylonitrile or to N-methyl-N-nitrosourea showed no gender-related difference in tumor incidence or in survival.1,3,10 Although the number of animals in each of these studies was small, there appears to be a higher propensity to tumor induction in male rats.

In the late 1960s the chance administration of carcinogens to pregnant rats led to the discovery that the fetal nervous system is maximally vulnerable to tumorigenesis at the end of gestation and near birth; it is at least 50 times more sensitive than mature nervous tissue. Because of the high rate of tumor induction and the high organ specificity (92% of the newborn animals showed nervous system tumors, with only 2% of tumors occurring elsewhere), transplacental carcinogenesis soon became a standard model for studying chemically induced CNS neoplasms.7,16,17 Coincidentally, in the 1970s there was intense interest in the synthetic estrogen diethylstilbestrol (DES) because of its adverse effects (that is, carcinoma induction) primarily in the daughters of women who received the drug to prevent miscarriage. This was the first evidence of estrogen’s potential to initiate or promote cancer. Zülch17 reviewed studies in which DES was tried as an additive to boost the systemic carcinogens normally used in the transplacental CNS tumor induction model.Surprisingly, the addition of DES produced fewer tumors than carcinogens alone. A similar pattern was found after the addition of oral contraceptive drugs to methyl nitrosourea in the transplacental model. Researchers at the time were at a loss to explain these results, given the adverse effects of DES in human pregnancies. However, the results may be interpretable in light of the protective effect of estrogen found in our brain tumor transplantation studies.

Host hormonal factors can be critical for both growth and differentiation of human brain tumors in rodents. In a study of an estrogen and androgen receptor–negative human GBM transplanted into the flanks of male and fe-
male nude mice, tumor growth was more successful in males. Tumors from the male recipients were subsequently found to be androgen-receptor positive. A second heterotransplant was equally successful in male and female mice, but with different growth patterns; a shorter latency period was seen in the male recipients. Human oligoastrocytomas transplanted into male and female nude mouse flanks were revealed to have more extensive growth in the females, with the mixed tumor differentiating into a pure oligodendroglioma under the influence of estrogen. Transplanted ethyl nitrosourea-induced gliomas into rats followed by injections of adjuvant immunotherapy up to 5 days postimplant. Survival was prolonged and some cures were seen, but only in female rats.

In a very interesting experiment closely presaging our own, Lee, et al. studied the therapeutic effects of radioactive monoclonal antibodies (mAbs) directed against a glioma-associated antigen, tenascin, on the survival of rats with intracranial human glioma xenografts. Male and female rats were used interchangeably in the initial experiments. Different dosage levels of radioactive labeling on the directed mAbs were tested, as well as different control groups, including alternate mAbs. The differences in survival between control animals and those in the dosage treatment groups did not follow a logical pattern, and on closer inspection the authors found that a basic difference in male compared with female survival rates was confounding their experiment. The eight animals that died in the first 3 days postimplant, regardless of the treatment group to which they belonged, were all males. Conversely, the apparent cures and the last groups of animals to die were all females. Male athymic rats with intracranial human glioma xenografts were dying significantly earlier than their female counterparts (p ≤ 0.005), despite their randomization into treatment groups. The authors speculated that body weight may have contributed to what they termed an “apparent” sex difference in survival; however, after attempting to correct for this variable, the effect remained. Another possibility was differences in ages between groups. The latter factor was not discussed and in the end the authors elected to use only female animals in their survival experiments. The expected experimental dosage trends were clearly evidenced in the single-sex studies.

Most glial neoplasms do not express estrogen receptors, or express only trace amounts. Immunohistochemical studies performed using antibody to the estrogen receptor were negative for U87 tumors in nude rat brain (data not shown). Estrogen has not been reported to inhibit GBM cell lines in culture, but the literature is sparse. One possibility is that it is not estrogen per se, but an active metabolite that causes the female survival advantage. One metabolite of estrogen, 2-methoxyestradiol (2ME2), inhibits cell division and angiogenesis in vitro. In cultured human lung cancer cells, 2ME2 induced wild-type p53 expression and led to apoptosis in 30 to 40% of the cells. Lung cancer cell lines that express mutant or no p53 are not affected by 2ME2. Another group of researchers showed that 2ME2 inhibits endothelial cell proliferation and migration in vitro. When given orally to mice, it inhibited tumor neovascularization and tumor growth. In a recent review of 2ME2 it was suggested that this substance may inhibit mammary carcinogenesis by both antiangiogenic and apoptotic mechanisms. Glioblastomas multiforme are highly vascular and our evidence is consistent with an estrogen metabolite such as 2ME2 acting as an inhibitor. Preliminary work in our laboratory has shown that 2ME2 can inhibit growth and induce apoptosis in U87 in vitro.

Glioblastomas occur more commonly in male mice, rats, and humans. Although these tumors are not classically hormone dependent, as is the case with breast cancer, the evidence indicates that sex steroids can play a role in incidence and, in some settings, in survival. Differences in survival related to gender have generally not been reported in patients with glioblastomas. One reason may be that most brain tumor series are relatively small and subtle differences can be missed. Patients who develop glioma in their reproductive years represent a small subset of the total population with glioma. There are several reports in which it is at least suggested that hormonal status may be important in patients. A registry review from Finland showed that women had a better prognosis than men in the 1st year after diagnosis of a glioblastoma. However, the study did not subdivide gliomas or stratify patients by age and gender in the same analysis. Chandler, et al., reported on 449 patients with GBM and characterized the 22 long-term survivors. They did not give the gender breakdown of the total group, but there were 10 male and 12 female long-term survivors. Because almost all series of GBMs have a preponderance of male patients, the larger number of female survivors is intriguing. A Japanese report clearly identified a survival advantage in females, but the number of patients was small. Roth and Elvidge reported more long-term survivors among women with GBM and a “slightly better prognosis” for women. Our animal evidence points to a role for estrogen in the survival of rats with implanted GBMs, and the human data are clear on the gender difference in incidence. It remains to be seen if all or at least a subset of GBMs are responsive to hormone treatment.

Conclusions

Previous experimental work concerning gender and gliomas has suffered from variable histological types of tumors, small numbers, and gender being a peripheral issue. In our experiments, we used human GBM cell lines and focused on the role of gender. We found that female rats have a survival advantage that is due to physiological levels of estrogen; progesterone is not involved. This provides a new biological avenue to explore as a means to understand the growth of gliomas. It may also provide a new therapeutic approach: hormonal manipulation of glial tumors.

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