Comparison of Corticotropin-Releasing Factor, Dexamethasone, and Temozolomide: Treatment Efficacy and Toxicity in U87 and C6 Intracranial Gliomas

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Abstract

Purpose/Experimental Design: Treatment of cerebral tumors and peritumoral brain edema remains a clinical challenge and is associated with high morbidity and mortality. Dexamethasone is an effective drug for treating brain edema, but it is associated with well-documented side effects. Corticorelin acetate (Xerecept) or human corticotrophin-releasing factor (hCRF) is a comparatively new drug and has been evaluated in two orthotopic glioma models (U87 and C6), by a direct comparison with dexamethasone and temozolomide.

Results: In vitro combination therapy and monotherapy showed a variable response in 6 different glioma cell lines. In vivo studies showed a dose-dependent effect of hCRF (0.03 and 0.1 mg/kg q12h) on survival of U87 intracranial xenograft–bearing animals [median survival: control – 41 days (95% CI 25–61); "low-hCRF" 74.5 days (95% CI 41–88); "high-hCRF" >130 days (95% CI not reached)]. Dexamethasone treatment had no effect on survival, but significant toxicity was observed. A survival benefit was observed with temozolomide and temozolomide + hCRF-treated animals but with significant temozolomide toxicity. C6-bearing animals showed no survival benefit, but there were similar treatment toxicities. The difference in hCRF treatment response between U87 and C6 intracranial gliomas can be explained by a difference in receptor expression. RT-PCR identified CRF2r mRNA in U87 xenografts; no CRF receptors were identified in C6 xenografts.

Conclusions: hCRF was more effective than either dexamethasone or temozolomide in the treatment of U87 xenografts, and results included improved prognosis with long-term survivors and only mild toxicity. The therapeutic efficacy of hCRF seems to be dependent on tumor hCRF receptor (CRFr) expression. These results support further clinical assessment of the therapeutic efficacy of hCRF and levels of CRFr expression in different human gliomas.

Introduction

Peritumoral brain edema is a significant cause of morbidity and mortality, and there have been relatively few advances in brain edema treatment since the introduction of dexamethasone in the 1970s. Relatively new treatments that have shown a significant impact on tumor-associated brain edema include human corticotropin-releasing factor (hCRF; ref. 1–4) and antiangiogenic therapy (5, 6), although the latter (bevacizumab) has recently been associated with potentiation of tumor-cell invasion and rapid progression following the cessation of therapy (7–10). This study focuses on hCRF monotherapy in 2 xenograft mouse models and provides a direct comparison between hCRF, dexamethasone, and temozolomide monotherapy.

hCRF is a 41-residue neuropeptide, which was initially isolated from sheep hypothalamic extracts in 1981 by Vale and colleagues (11). It is produced in the hypothalamus and is an important component in regulating the hypothalamic–pituitary–adrenal (HPA) axis. CRF is the predominant regulator of adrenocorticotropic hormone (ACTH) formation and release by the pituitary (12). In addition to its primary location in the hypothalamic paraventricular nucleus, it has been identified in cerebral cortical interneurons, the limbic system, brainstem, and spinal cord (13). Furthermore, it has been shown that hCRF possesses antiedema properties. As an antiedematous agent, hCRF prevents vascular leakage induced by inflammatory mediators that selectively act on postcapillary venules and veins in the skin (14), from alveolar capillaries (15–18),...
Translational Relevance

Tumor-associated edema is a major negative factor in patients with brain tumors and is responsible for high morbidity and mortality in this patient group. Dexamethasone has been the most effective drug, which is commonly used for treating brain edema for decades, but it is associated with well-known side effects. Recently, bevacizumab has shown remarkable transient responses in patients with high-grade gliomas, both on MRI and in clinical performance that is at least partially related to its anti-edematous effect. In this study, we evaluated the efficacy and toxicity of human corticotropin-releasing factor (hCRF) in the treatment of 2 orthotopic intracranial brain tumors (C6, rat; and U87, human) in a nude mouse model. In addition, comparisons were made with dexamethasone and temozolomide treatment regimens.

Three important observations were made: (i) hCRF was more effective than either dexamethasone or temozolomide in the treatment of U87 intracranial xenografts; (ii) hCRF treatment was associated with significantly less toxicity than that associated with either dexamethasone or temozolomide treatment; (iii) hCRF efficacy was dependent on hCRF receptor expression in the tumor (e.g., C6 gliomas showed no response to hCRF and had no measurable levels of CRF mRNA on RT-PCR). These results support the development of hCRF-releasing formulations to optimize the therapeutic antitumor effect in human subjects with brain tumors because several clinical trials have shown that hCRF is safe and enables reductions of steroid dosing.

and from muscle capillaries (19). The antiedematous effects of hCRF on systemic vessels seem to be mediated, in part, through activation of CRF2 receptors, whereas in the brain and in brain tumors, this effect is mediated through both CRF1 and CRF2 receptors (20). These observations suggest that hCRF acts throughout the microcirculation to preserve endothelial cell integrity. Several possible mechanisms of hCRF action have been reviewed in the literature (21), but the exact mechanisms are not fully established as yet.

hCRF has been proposed as a new treatment option for peritumoral brain edema. Strong preclinical data supported hCRF as an effective anti-edematous agent for the brain that has substantially less toxicity than dexamethasone (22). The first study of exogenous CRF administration and toxicity assessment in patients was reported by Chrousos and colleagues (23). More recently, Xerecept (corticotelin acetate or hCRF) has been shown to be a well-tolerated drug, based on data from ongoing clinical trials involving nearly 200 patients. The side effects of clinical treatment with hCRF in i.v. single doses (1–5 µg/kg) and continuous i.v. infusions of up to 2,000 µg/24 h per patient were not associated with any significant side effects. Only at much higher i.v. single doses (up to 30 µg/kg) did significant symptoms, including hypotension, tachycardia, arrhythmias, and mental “absences,” develop.

Results from ongoing clinical trials involving nearly 200 patients who have received hCRF indicate that s.c. administration of the drug, often for extended periods, is well tolerated (24–26). The evolving clinical efficacy and safety data support the use of hCRF as a dexamethasone-sparing treatment (if not an alternative to dexamethasone) for the management of symptomatic peritumoral brain edema (4). In contrast to the well-known systemic side effects associated with chronic Dexamethasone administration, which can be more debilitating than the primary disease process, hCRF has shown much less toxicity (27, 28), with the primary effect being transient local reactions at or near the injection site.

The evolving clinical efficacy and safety data indicate that Xerecept might provide a dexamethasone-sparing treatment (if not an alternative to dexamethasone) for the management of symptomatic peritumoral brain edema (24, 25). In addition, our previous preclinical studies indicated that hCRF may have a direct antitumor effect (1, 29) and a well-documented antiedematous effect (1–4). In the present study, we assessed the efficacy and toxicity of hCRF (Xerecept; Celtic Pharma; ref. 30) treatment, both in vitro and in 2 orthotopic glioma animal models. First, we assessed hCRF and dexamethasone for additive effects in cell-culture viability assays involving 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and temozolomide treatment of 6 different glioma cell lines. Then, we focused on a comparison of hCRF and dexamethasone monotherapy and a comparison between temozolomide monotherapy and combination therapy with temozolomide + hCRF.

Materials and Methods

Cell lines, transduction, and fluorescence-activated cell sorting

RG2 and C6 rat glioma and U87 and LN229 human glioma cells were obtained from the American Type Culture Collection. The cell lines were maintained in 75-cm² flasks with minimum essential medium (MEM; RG2 and U87) or Dulbecco’s modified Eagle’s medium (DMEM; C6 and LN229) containing FBS (10%), penicillin (100 U/mL), and streptomycin (100 µg/mL). All cells were maintained in a humidified atmosphere (5% CO2/95% air) at 37 °C.

To monitor intracranial glioma growth by bioluminescence imaging (BLI), we transduced U87 and C6 cells with a retroviral reporter vector containing a firefly luciferase–IREs–green fluorescent protein (GFP) cassette previously developed in our laboratory (31). Both the C6 and U87 cell
lines were stably transduced as previously described (32). After transduction, the cells were expanded in culture for several days and prepared for subsequent fluorescence-activated cell-sorting (FACS) analysis and cell sorting as previously described (33).

**Therapeutic drugs**

hCRF (corticorelin acetate, Xerecept) was generously provided by Celtic Pharmaceutical Development Services America, Inc. Dexamethasone and BCNU were obtained from the Memorial Sloan-Kettering Cancer Center (MSKCC) pharmacy. Temozolomide was generously provided by the Developmental Therapeutics Program, National Cancer Institute (Rockville, MD).

**In vitro cytotoxicity assays**

Cytotoxicity assays were conducted using wild-type (WT) and firefly luciferase (FLuc)-transduced cell lines, as previously described (34). Briefly, cells (5,000 per well) were seeded onto 96-well microplates and incubated for 4, 24, and 72 hours under control (nontreatment) condition and with different doses of BCNU and temozolomide, both alone and in combination with low and high doses of dexamethasone (0.01 and 1 μmol/L) and hCRF (0.01 and 1 nmol/L), treated simultaneously. The *in vitro* exposure times for hCRF were selected on the basis of unpublished data obtained from Celtic Pharma. The exposure time for BCNU was selected on the basis of previously published work (35, 36). The total number of experiments was at least 6 for each cell line. The WST-1 reagent (Roche) was used to measure cell viability after drug incubation. Measurement of the absorbance of the samples against a background control used as the blank was done using the Packard ELISA reader (Packard Bioscience Company). EC50 was assessed using Excel and Sigma Plot programs.

**Semiquantitative reverse transcriptase PCR**

Expression levels of CRF receptors (*CRF1* and *CRF2*) were determined by semiquantitative reverse transcriptase PCR (RT-PCR). Briefly, total RNAs from cultured cells and tumor xenografts were isolated using TRIzol reagent (Invitrogen) and then treated with RNase-free DNase 1 (Ambion) according to the manufacturer’s instructions. The first strand of cDNA was synthesized by GoScript Reverse Transcriptase (Promega). PCR was done using the following primer sets, which are located in the conserved domains of *CRF1*, *CRF2*, and β-actin gene from the human, mouse, and rat species: *CRF1*: (F) 5’-ccttgctgtcctgtcctc-3’; (R) 5’-tgttgcctctgtgatgta-3’; *CRF2*: (F) 5’-cctactgcaacagctct-3’; (R) 5’tagcagctctccaaaca-3’; and β-actin: (F) 5’-ggcttcgcgggactg-3’; (R) 5’t-tactctgtcgtgat-3’.

**In vivo studies**

Male nu/nu mice were obtained from the National Cancer Institute [(NCI), Bethesda, MD], and all studies were conducted in accordance with a Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee–approved protocol. Xenografts were established in 3-month-old mice by intracranial injection of 5 × 10^6^ cells of either U87-FLuc or C6-FLuc reporter cells, using a stereotactic device as previously described (29).

Eight treatment groups of 10 athymic nu/nu mice per group, each bearing a U87-FLuc or a C6-FLuc xenograft, were developed as outlined in Table 1. Treatment with hCRF, dexamethasone, temozolomide, or a combination of temozolomide + hCRF was initiated 3 weeks after tumor-cell implantation and lasted for 7 weeks.

For animal groups 1 and 2, the stock clinical grade solution of hCRF (Xerecept; 1 mg/mL) was diluted with 0.9% sodium chloride solution to the required “low” and “high” concentrations prior to each injection (Table 1). For animal groups 3 and 4, clinical grade i.v.-injectable dexamethasone (1 mg/mL) was diluted with 0.9% sodium chloride solution to the required concentrations. Treatment with either hCRF or dexamethasone was done by s.c. injections every 12 hours for 7 weeks, starting in the second week after implantation. For animal group 5, animals were treated with a single daily dose of temozolomide. The temozolomide solution was freshly prepared

<table>
<thead>
<tr>
<th>Table 1. Treatment Groups</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Volume (mL)</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCRF (high dose)</td>
<td>0.1</td>
<td>s.c.</td>
<td>0.05</td>
<td>Twice daily</td>
</tr>
<tr>
<td>hCRF (low dose)</td>
<td>0.03</td>
<td>s.c.</td>
<td>0.05</td>
<td>Twice daily</td>
</tr>
<tr>
<td>DEX (high dose)</td>
<td>1</td>
<td>s.c.</td>
<td>0.1</td>
<td>Twice daily</td>
</tr>
<tr>
<td>DEX (low dose)</td>
<td>0.3</td>
<td>s.c.</td>
<td>0.1</td>
<td>Twice daily</td>
</tr>
<tr>
<td>TMZ</td>
<td>40</td>
<td>i.p.</td>
<td>0.2</td>
<td>Daily, 5 d/wk</td>
</tr>
<tr>
<td>TMZ + hCRF combination (high dose)</td>
<td>40 + 0.1</td>
<td>i.p. + s.c.</td>
<td>Daily, 5 d/wk + twice daily</td>
<td></td>
</tr>
<tr>
<td>TMZ + hCRF combination (low dose)</td>
<td>40 + 0.03</td>
<td>i.p. + s.c.</td>
<td>Daily, 5 d/wk + twice daily</td>
<td></td>
</tr>
<tr>
<td>Control (vehicle)</td>
<td></td>
<td></td>
<td></td>
<td>Daily</td>
</tr>
</tbody>
</table>

Abbreviations: DEX, dexamethasone; TMZ, temozolomide.
daily and administered by i.p. injection on 5 of 7 days each week, for a total of 7 weeks. Temozolomide powder was dissolved in pure dimethyl sulfoxide (DMSO) and further diluted at a ratio of 1:3 with 0.9% sodium chloride solution to the desired concentration. For animal groups 6 and 7, the effects of combined temozolomide and ‘low’ or ‘high’ hCRF were studied (Table 1). The preparation and administration of the drugs were the same as described above, respectively. For animal group 8 (control), animals were treated with a single daily dose of drug vehicle. All surviving animals were euthanized on day 150.

In vivo bioluminescence imaging

In vivo BLI was done 7 days after implantation of the xenografts and then repeated weekly for the duration of the experiment, using an IVIS-200 Imaging System. Imaging was carried out 10 minutes after i.p. injection of r-luciferin (2 mg per animal; Xenogen), with mice lying in the prone position. Five mice were imaged at the same time with a field of view of 25 cm. An imaging time of 3 minutes with medium binning and an f-stop of 1 were used initially; this was sequentially reduced as the xenografts grew and saturation levels of BLI signal intensity were approached. Measurements of signal intensity were obtained from region-of-interest analysis using Living Image software (Xenogen). The images displayed in each data set were normalized to the appropriate color intensity scale. BLI intensity was expressed as total photons per region of interest per second of imaging.

Statistical analyses

Results are reported as mean ± SD. We applied ANOVA to the EC\textsubscript{50} results for each drug (BCNU and temozolomide) and each cell line (C6, LN229, RG2, and U87) separately and included the date of experiment as an effect in the model for quality control. We compared each of 4 treatments—low- and high-dose dexamethasone and low- and high-dose hCRF against BCNU or temozolomide alone—and adjusted for multiple comparisons within each experiment (combination of drug and cell line) using Dunnett’s method. We considered \( P < 0.05 \), after adjustment for multiple comparisons, to be statistically significant.

To account for possible differences in global levels or calibration of BLI measurement between animals, we used the change from the average of week 1 and week 2 to the average of week 5 and week 6 as a summary value. Survival was measured from time of administration until death, sacrifice, or end of study. Animals that were moribund at the time of sacrifice were considered equivalent as animals that died, and other animals were considered as censored observations in survival analysis. Using parallel factorial designs, we applied ANOVA to change in BLI measurements and the Cox proportional hazard model to survival data. Furthermore, we included each animal’s change in BLI as a potential predictor of survival in the Cox model. Analysis showed that there was no significantly different effect on survival between high versus low doses of hCRF, dexamethasone, or temozolomide + hCRF. We combined high and low doses of each treatment in further analyses.

Results

Effects of hCRF and dexamethasone on BCNU and temozolomide cytotoxicity in cell culture

Cytotoxicity studies were conducted in 6 glioma cell lines (U87, U87-FLuc, C6, C6-FLuc, Ln229, and RG2) with increasing doses of hCRF (0.0001–1.000 nmol/L) and dexamethasone (0.001–10 \( \mu \text{mol/L} \)). Minimal loss of cell viability (<20%) was observed following a 3-day exposure to 1 nmol/L hCRF and 1 \( \mu \text{mol/L} \) dexamethasone, and less than 10% loss of cell viability was noted following a 3-day exposure to 0.01 nmol/L hCRF and 0.01 \( \mu \text{mol/L} \) dexamethasone. On the basis of these results, these concentrations of “high” and “low” hCRF and dexamethasone, respectively, were selected for the in vitro drug-combination cytotoxicity studies.

BCNU and temozolomide, both alkylating drugs extensively used for treating high-grade gliomas, were assessed as monotherapy and in drug-combination cytotoxicity studies with either hCRF or dexamethasone. Combining BCNU with either hCRF or dexamethasone had a variable effect; however, there were no significant differences in cytotoxicity between temozolomide treatment alone and combined treatment with hCRF or dexamethasone (data not shown). In a second experimental series, the EC\textsubscript{50} value of BCNU (Table 2, part A) and temozolomide (Table 2, part B) was assessed in 4 different cell lines. Although the EC\textsubscript{50} values varied over an ~3-fold range for each of the 2 drugs, the rank order of sensitivity was quite different for the 4 cell lines. A comparison of the results, accounting for multiple comparisons, showed only a few statistically significant effects. In the temozolomide experiments, the addition of hCRF significantly lowered the EC\textsubscript{50} values in RG2 cells and a non-significant trend toward a lower EC\textsubscript{50} value was observed in U87 cells. There was no significant effect of dexamethasone on temozolomide EC\textsubscript{50} values with any of the cell lines.

The in vitro studies showed little evidence of a consistent additive effect when hCRF or dexamethasone was combined with BCNU or temozolomide treatment. Despite these inconsistent results, we decided to study and compare the effect of hCRF, dexamethasone, and temozolomide monotherapy in 2 orthotopic glioma xenografts in immunodeficient nude/nude rats, and, furthermore, we conducted a combination therapy study involving temozolomide and hCRF. We chose a human (U87) and a rodent (C6) glioma cell line for comparison. Both cell lines were transduced with a reporter gene that constitutively expressed firefly luciferase (U87-FLuc and C6-FLuc). Importantly, there were no significant differences in cytotoxicity observed between U87 and U87-FLuc cells and between C6 and C6-FLuc cells (Table 2). The intracranial xenografts generated from these reporter-transduced cell lines allowed us to monitor tumor growth noninvasively by using BLI.
Table 2. In vitro cell viability studies

<table>
<thead>
<tr>
<th>Cell line</th>
<th>BCNU alone</th>
<th>BCNU + low hCRF (0.01 mmol/L)</th>
<th>BCNU + high hCRF (1.0 mmol/L)</th>
<th>BCNU + low DEX (0.01 mmol/L)</th>
<th>BCNU + high DEX (1.0 mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. hCRF and DEX effects on BCNU EC50 (μmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U87</td>
<td>226 ± 56 (9)</td>
<td>264 ± 91 (9)</td>
<td>193 ± 54 (9)</td>
<td>311 ± 65 (9)*</td>
<td>260 ± 59 (9)</td>
</tr>
<tr>
<td>C6</td>
<td>414 ± 43 (8)</td>
<td>419 ± 33 (8)</td>
<td>388 ± 59 (8)</td>
<td>346 ± 94 (8)</td>
<td>304 ± 102 (9)*</td>
</tr>
<tr>
<td>Ln229</td>
<td>234 ± 43 (7)</td>
<td>261 ± 58 (7)</td>
<td>219 ± 36 (7)</td>
<td>239 ± 47 (7)</td>
<td>201 ± 91 (7)</td>
</tr>
<tr>
<td>RG2</td>
<td>140 ± 22 (6)</td>
<td>259 ± 96 (6)</td>
<td>173 ± 60 (6)</td>
<td>329 ± 165 (6)*</td>
<td>147 ± 72 (6)</td>
</tr>
<tr>
<td>B. hCRF and DEX effects on TMZ EC50 (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U87</td>
<td>4.8 ± 3.5 (5)</td>
<td>3.1 ± 1.8 (5)</td>
<td>3.8 ± 2.8 (5)</td>
<td>4.5 ± 3.3 (5)</td>
<td>3.9 ± 3.2 (5)</td>
</tr>
<tr>
<td>C6</td>
<td>2.9 ± 1.1 (5)</td>
<td>3.6 ± 1.7 (5)</td>
<td>2.9 ± 1.0 (5)</td>
<td>4.5 ± 3.0 (5)</td>
<td>3.8 ± 3.5 (5)</td>
</tr>
<tr>
<td>Ln229</td>
<td>3.1 ± 0.6 (6)</td>
<td>3.2 ± 0.5 (6)</td>
<td>3.3 ± 0.6 (6)</td>
<td>3.4 ± 1.0 (6)</td>
<td>3.5 ± 1.0 (6)</td>
</tr>
<tr>
<td>RG2</td>
<td>9.8 ± 2.8 (5)</td>
<td>6.9 ± 1.4 (5)*</td>
<td>6.2 ± 0.3 (5)*</td>
<td>7.8 ± 2.1 (5)</td>
<td>7.9 ± 1.6 (5)</td>
</tr>
</tbody>
</table>

Abbreviations: DEX, dexamethasone; TMZ, temozolomide.

hCRF treatment of intracranial gliomas

A dose-dependent effect of hCRF on the survival of animals bearing U87-FLuc intracranial xenografts was observed (Fig. 1A). Both “low” (0.03 mg/kg q12h) and “high” doses (0.1 mg/kg q12h) of hCRF increased survival compared with controls, with median survival ranging from 41 days (95% CI: 25–61) to 74.5 days (95% CI: 41–88; P < 0.05) as well as to more than 130 days (95% CI not reached; P < 0.05), respectively. In contrast, hCRF had no effect on the survival of animals bearing intracranial C6-FLuc xenografts (Fig. 2A).

U87-FLuc intracranial xenografts treated with hCRF usually showed a BLI signal intensity pattern that paralleled survival (Fig. 3). For example, a rapid decrease in BLI signal intensity that remained low for the duration of the experiment was associated with a long survival, whereas, an increasing signal was usually associated with a shorter survival (Fig. 3B). hCRF treatment had no effect on the pattern of increasing BLI signal intensity of C6-FLuc gliomas, consistent with the absence of any survival benefit in these animals (Fig. 2). Further analysis of treatment effects on overall survival, adjusting for experiment and change in BLI effects, showed that hCRF (low or high doses) significantly prolonged overall survival (P < 0.0001).

Very little or no toxicity was observed in animals treated with hCRF alone. The only minor complication was a random nonspecific skin infection that probably reflects the systemic effects of corticosteroids on the immune system. In addition, hCRF-treated animals bearing U87-FLuc gliomas continued to gain weight, similar to control animals (Fig. 1B). No difference in animal weight was noted between control and “low-” or “high-”hCRF-treated animals.

Dexamethasone treatment of intracranial gliomas

Dexamethasone at both low (0.3 mg/kg) and high doses (1 mg/kg) was not effective in prolonging animal survival compared with control nontreated animals bearing either U87-FLuc (Fig. 1A) or C6-FLuc (Fig. 2A) gliomas. Similarly, the patterns of increasing bioluminescence intensity were indistinguishable from that of nontreated control animals (Fig. 3C). Further statistical analysis showed that animals treated with hCRF had significantly longer survival than dexamethasone-treated animals (P = 0.02), whereas no differences between “low” and “high” doses of dexamethasone were noted. Treatment-related toxicity was greater in the dexamethasone-treated animals and included significant weight loss (Figs. 1B and 2B), more severe skin infections, and some necrosis at the injection sites.

Temozolomide treatment of intracranial gliomas

A significant effect of temozolomide alone (P = 0.008) or in combination with different doses of hCRF (P = 0.003) on the survival of animals bearing U87-FLuc xenografts was observed (Fig. 1C), but there was no effect observed on the survival of animals bearing C6-FLuc xenografts (Fig. 2C). The temporal profiles of the bioluminescence images were similar to those of the survival data in both U87-FLuc (Figs. 1B and 4B, respectively) and C6-FLuc (data not shown) xenograft-bearing animals. Many of the animals treated with temozolomide showed both reduction and stabilization of the BLI signal at a low or moderate level for most of the treatment and posttreatment period, followed by a rapid increase in the last 2 weeks of the animal’s life. However, temozolomide monotherapy and temozolomide combined with hCRF was accompanied with high toxicity.
There was a substantial loss of weight (up to 40%), which tended to recover during the posttreatment period if the animal survived. This weight loss is reflected in the photographic images of the animals at week 9, just after completing the course of temozolomide therapy (Fig. 4B and C). A very high incidence (27 of 30) of skin neoplasms was observed in animals treated with temozolomide and there were rare occurrences (2 of 30) of internal abdominal tumors. Spontaneous tumorigenesis was not observed in any of the other treatment groups.

CRF1 and CRF2 receptor expression

U87-FLuc and C6-FLuc cell lines and xenografts were examined for CRF1 and CRF2 mRNA levels by RT-PCR (Fig. 5). U87 cultured cells and xenografts express measurable levels of CRF2 but not CRF1 mRNA transcript. In contrast, neither CRF1 nor CRF2 transcripts could be detected in the C6 cultured cells and xenografts.

Discussion

We previously reported a dose-dependent decrease in vasogenic peritumoral brain edema following treatment of immunocompetent Fischer 344 rats bearing RG2 intracranial gliomas, with hCRF (Xerecept; 29). Furthermore, we reported a similar result in a single-dose hCRF study involving Sprague Dawley rats bearing W256 intracranial gliomas (1, 17). Both studies showed a significant anti-edematous effect in tumor and peritumoral brain tissue by proton density-weighted and T1-weighted contrast-enhanced MRI and by ex vivo measures of tissue water content. In addition, the initial study reported a significant survival advantage for hCRF-treated animals compared with dexamethasone-treated and control animals (29).

In this study, an immunocompromised animal model harboring either an intracranial human (U87) or a rat (C6) glioma xenograft was studied and therapeutic efficacy was...
evaluated in terms of animal survival. We focused on a comparison of hCRF and dexamethasone monotherapy, although a comparison between temozolomide monotherapy and temozolomide + hCRF combination therapy was also done. Interestingly, a highly significant, dose-dependent effect of hCRF monotherapy on survival was observed in animals bearing U87 gliomas but not C6 gliomas. These results were highly consistent with tumor growth, regression, and regrowth patterns visualized by sequential weekly bioluminescence reporter gene imaging of the xenografts before, during, and following hCRF treatment. Interestingly, dexamethasone treatment had little or no effect on either the survival or BLI profiles of animals bearing either U87 or C6 gliomas, and this may reflect, in part, the toxic side effects of twice-daily s.c. injections of dexamethasone. Temozolomide monotherapy and temozolomide + hCRF combination therapy for U87 gliomas yielded similar survival profiles that were significantly longer than those for control animals. Again, no survival advantage was observed in C6 gliomas.

To further explore the dichotomy in hCRF treatment response between U87 and C6 gliomas, we evaluated the levels of CRF1 and CRF2 receptor mRNAs in the 2 cell lines and xenografts. This investigation was based on our previous study, which showed that significant levels of CRF1 mRNA (0.25 ± 0.01 pg/μg total RNA) were detectable in W256 cells and presumably reflects CRF1-receptor expression on tumor and/or endothelial cell membranes (22). Blockade of CRF receptors with alpha-helical CRF (9–41) analogue abolished the growth inhibitory and differentiation-inducing effects of hCRF. Together, these findings suggested that W256 cells express functional rat CRF receptors in vitro and that these receptors are likely to mediate the effects observed following exposure to hCRF. Our current results suggest that C6 cells and xenografts express neither CRF1 nor CRF2 receptors whereas U87 cells and xenografts express CRF2 but not CRF1 receptors. This difference between the 2 cell lines and xenografts could contribute to the difference in hCRF treatment response that was observed in this study. In addition, these results suggest that CRF1 and/or CRF2 receptor expression is required for the antitumor and antiedematous effects of hCRF. It was previously shown that CRFR1 is expressed in tumor cells and that urocortin (UCN) and CRF, both members of the CRF family, reduce tumor-cell growth via CRF1 receptor (37, 38). Multiple malignancies have
been reported to have high levels of CRFR1 and CRFR2 expression and to be sensitive to the suppressive effects of CRF and its agonists. Graziani and colleagues reported that UCN/CRF inhibited the growth of adenocarcinoma Ishikawa cells in a concentration-dependent manner and that this effect was mediated by CRF1 receptors (38). It was reported that UCN inhibited the proliferation of melanoma cells in vitro and in vivo, also through CRF1 receptor (39) and, in human mammary cancer cells, CRF acted on CRF1 receptor to inhibit the proliferative effects of estrogens on MCF-7 cells in both paracrine and autocrine manners (40). Activation of CRF2 receptor was observed to suppress angiogenesis and rearrange the vasculature. Furthermore, it was reported that CRF2 receptor agonists inhibited hepatocellular carcinoma tumor angiogenesis in vitro and reduced tumor microvessel density in vivo. Our results are consistent with these observations.

Reports from other animal and human studies confirm that the antiedematous effects of hCRF- and dexamethasone-treated animals are similar. Patients treated with hCRF and decreasing doses of dexamethasone show clinical improvement in neurologic symptoms as well as have less steroid-associated myopathy and insulin dependence (41). The substitution of a less toxic, but equally effective, drug for dexamethasone to treat tumor-associated cerebral edema has been a long-explored challenge for clinicians.
Our preclinical studies add to the body of data that indicates that hCRF has the potential to meet this challenge. We observed higher therapeutic efficacy with hCRF than with either dexamethasone or temozolomide monotherapy in U87 glioma xenografts. Furthermore, no toxic effects or signs of discomfort were observed in mice that had received hCRF treatment in low or high doses for 2 to 6 months. In comparison, animals that have received dexamethasone or temozolomide therapy exhibited significant toxicity, manifested by a decrease in weight, increased irritability, local skin necrosis, and even spontaneous tumorigenesis.

The current clinical development program for hCRF has focused on the treatment of peritumoral brain edema in patients with metastatic and primary brain tumors requiring dexamethasone to control symptoms. Three major clinical trials (0303, 0501, and 0302) have established 2 important points: (i) hCRF is safe in man, and (ii) hCRF enables significant reductions or elimination of steroid dosing in patients with cerebral tumors, with no apparent impairment of neurocognitive status (26). Blinded, independent review of MRI scans from patients receiving hCRF showed that most of these patients experienced prolonged periods of stable disease and a minority have achieved a measurable level of tumor regression (24). In a phase 1 clinical trial, 10 of the 15 patients who received hCRF had improvement in neurologic symptoms or physical

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**Figure 4.** BLI time course of representative control and temozolomide (TMZ)-treated animals bearing U87-FLuc orthotopic gliomas. The profiles of control (vehicle-treated) (A), temozolomide monotherapy (B), and temozolomide + hCRF combination therapy (C) are shown.
Comparison of hCRF and Dexamethasone Efficacy in Glioma Models

Figure 5. Expression of CRF1 and CRF2 gene transcripts in cultured U87 and C6 parental cells (columns 1 and 5) and reporter-transduced cells (columns 2 and 6) and in s.c. xenografts from U87 and C6 parental cells (columns 3 and 7) and reporter-transduced cells (columns 4 and 8). Normal mouse brain tissue, which expressed both CRF1 and CRF2 mRNAs, was used as a positive PCR control. Expression levels of CRF1 and CRF2 were normalized by the β-actin gene (ACTB).

findings, with little or no toxicity (4). It is notable that there was a measurable decrease in steroid-related side effects, especially myopathy and the appearance of Cushingoid features. One patient, who had lymphoma-related pruritus and a long-standing rash resistant to steroids, noted improvement while receiving hCRF (25). Furthermore, in this preclinical study, we noted differences in toxicity and efficacy of hCRF, dexamethasone, and temozolomide treatment of the glioma-bearing animals.

Overall, we have shown therapeutic efficacy and low toxicity of hCRF in the treatment of a human-derived glioma in an orthotopic nude mouse model. Our results are consistent with the requirement of CRF receptor expression in the tumor cells for hCRF therapeutic efficacy. Notably, hCRF treatment was less toxic than that with dexamethasone or with temozolomide. These results support the development of novel hCRF-releasing formulations or platforms for extended constant administration and studies to optimize the therapeutic antitumoral effect. Further clinical studies will need to evaluate whether long-term hCRF treatment (or the rapid cessation of treatment) is associated with increased glioma cell invasion and rapid tumor progression, as has been described for bevacizumab.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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