Combination Interferon-α2a and 13-cis-Retinoic Acid Enhances Radiosensitization of Human Malignant Glioma Cells *in Vitro*¹

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**ABSTRACT**

We investigated the individual and combined effects of *cis*-retinoic acid (CRA) and/or IFN-α (IFN) and/or radiation therapy (RT) against a human glioma cell line (American Type Culture Collection; U373MG) to evaluate the possible radiosensitization properties of these agents *in vitro*. Glioma cells were incubated for 24 h in 96-well plates (2 × 10⁵ cells/well) in standard culture medium. Sets of U373 (n = 12) were exposed to CRA (3 × 10⁶ μM), IFN (25 units/ml), CRA plus IFN, or standard culture medium. After an additional 24 h of incubation, the U373 cells were subjected to increasing radiation doses (up to 16 Gy). Glioma cells were harvested 92 h after irradiation, and cell survival curves were determined from [³H]thymidine incorporation data (over the last 24 h). The experiment was repeated for both the untreated control group and the combined CRA/IFN group. To verify the [³H]thymidine assays, a clonogenic assay was also performed. Single cell suspensions of U373 cells were plated out in six-well plates (n = 3). After chemical and RT treatment, colonies of 50 cells or more were counted, and cell survival curves were generated as fractions of nonirradiated controls. The amount of RT (in Gy) that would cause a 50% survival fraction (lethal dose 50 or LD₅₀) was calculated from the survival curves by regression analysis. The following LD₅₀s were obtained:

<table>
<thead>
<tr>
<th>Gloma experiment</th>
<th>RT only</th>
<th>IFN/CRA</th>
<th>CRA</th>
<th>IFN</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H]Thymidine assay</td>
<td>6.8 Gy</td>
<td>3.2 Gy</td>
<td>6.6 Gy</td>
<td>5.6 Gy</td>
</tr>
<tr>
<td>Clonogenic assay</td>
<td>3.9 Gy</td>
<td>2.7 Gy</td>
<td>4.8 Gy</td>
<td>3.3 Gy</td>
</tr>
</tbody>
</table>

The results showed that for both the [³H]thymidine incorporation assay and the clonogenic assay, the combination of IFN/CRA rendered U373 cells more susceptible to ionizing radiation than the untreated control or either single agent alone.

**INTRODUCTION**

Glioblastomas and anaplastic astrocytomas constitute 40–60% of all primary adult central nervous system tumors. Historically, after surgery alone, the median survival for these tumors was only 35 weeks, with a 1-year survival of 33% and 2-year survival of 0% (1). Many patients present with gliomas that are unresectable or that can be only partially resected because of their anatomical location (2). The morbidity of surgery is also a concern, particularly in patients who have recurred after initial therapy.

Radiation therapy is typically used in the management of gliomas, either after surgical resection or as primary therapy in patients who are not surgical candidates (3). The addition of radiotherapy to surgery has been demonstrated to increase life expectancy (4). However, even delivery of doses to 6000 cGy delivered over 5–6 weeks and the use of hyperfractionation radiation therapy have had little effect on survival. Stereotactic radiosurgery (5) and “gamma knife” radiosurgery (6) have enabled more specific delivery of even higher doses of radiation and have clearly decreased the morbidity associated with treating gliomas, but their impact on survival has been limited. Because of the radiation-resistant nature of glioblastomas and the sensitivity and importance of the surrounding brain tissue, there is a critical need for treatments that can increase radiosensitivity of these tumors. Additionally, due to the invasive and aggressive nature of this tumor type, new approaches are needed to stop the rapid spread and growth of the tumor on a cellular level.

Thus far, chemotherapy has added little to the benefit achieved by surgery and radiation alone (7). Even for patients who were eligible for investigational protocols, combination therapy with surgery, radiation therapy, and chemotherapy resulted in 2-year survival rates that were no better than 15–25% (8). A meta-analysis of randomized trials of adjunctive chemotherapy determined that the 1-year survival among 884 patients treated with radiation therapy alone was 41% compared with 54% among 1538 patients who received radiation and chemotherapy (9). Two-year survival was only 16% for radiation compared with 23% for radiation plus chemotherapy. Thus, despite recent advances in conventional surgical, radiotherapeutic, and chemotherapeutic treatments, the outcomes of patients with high-grade malignant gliomas still remain poor. Glioblastoma patients continue to have median survival outcomes of <18 months (2, 8).

IFN² has direct growth-inhibitory effects on tumor cells, modulatory effects on the immune system, and up-regulates...
tumor-associated surface antigens (10). Preliminary experiments for our study showed that even low concentrations (10 units/ml) of IFN could inhibit growth of different glioma cell lines as measured by \[^{3}H\]thymidine assay over 48 h (data not shown). IFN also increased potentiation of radiation cytotoxicity in several cell types including small cell lung carcinoma, renal cell carcinoma, head and neck squamous cell carcinoma, and cervical cancer cell lines (11, 12). IFN is also being clinically investigated in combination with radiation and additionally with other chemotherapeutic agents plus radiotherapy to manage high-grade gliomas in patients (13, 14).

CRA is a member of the retinoid family of compounds. Retinoids have been implicated as potent regulators of differentiation in a wide range of cell types and are integral to tissue and organ morphological development (15). Additionally, retinoids and CRA in particular have a direct antiproliferative effect on a spectrum of tumor cell types. In a clinical trial involving 43 glioma patients, who received at least 4 weeks of CRA treatment, some degree of tumor regression was noted in 23% of patients (16). CRA may increase the cytotoxicity of radiation therapy (17).

The combination of IFN and CRA has demonstrated significant antitumor effects against squamous cell carcinomas of the skin and cervix (18, 19). Both agents alone radiosensitize tumor cells in vitro (11, 12, 17, 20). The combination has also exhibited increased antiproliferation and radiosensitization in vitro in squamous cell carcinomas of the head and neck (21). The combination of IFN and CRA plus radiation therapy appears synergistic against cervical carcinoma cells (22), and is being explored clinically against cancer of the cervix (23, 24).

Our preliminary studies in vitro suggested that the combination IFN and CRA plus radiation therapy may have similar benefits for malignant gliomas (25). Our present clinical investigations suggest that this combination may be beneficial in the clinical setting for patients with high-grade malignant gliomas (26). In this study, the combination of CRA and IFN enhanced radiosensitization of human glioma cells in vitro at concentrations that are achievable in humans using standard clinical doses of CRA and IFN. These radiosensitization effects were confirmed both in \[^{3}H\]thymidine uptake and in clonogenic survival experiments.

### MATERIALS AND METHODS

A series of experiments were conducted to test the cytotoxicity of IFN, CRA, radiation therapy, IFN + radiation, CRA + radiation, and IFN + CRA + radiation (27–31).

#### Tumor Cell Line.

U-373 MG (American Type Culture Collection, Rockville, MD; 17-HTB line) is a late-passage glioblastoma derived from a human glioma tumor. This line exhibits features consistent with high-grade astrocytoma including spindle shape morphology and grade III astrocytoma formation in nude mice. Tumor cells were maintained in growth phase using standard methods (31). The cells were maintained at 37°C in an atmosphere of 5% CO₂ in tissue culture medium consisting of RPMI 1640 (Life Technologies, Inc., Grand Lake, NY) supplemented with 5% fetal bovine serum and 5% enriched calf serum (Life Technologies, Inc.). 10 mM HEPES buffer, and 1 mM sodium pyruvate (Gemini Bio-Products, Inc., Calabasas, CA).

#### Reagents.

IFN (Roche Laboratories, Nutley, NJ) was diluted from an initial concentration of 3 × 10⁶ IU/ml to 25 IU/ml in tissue culture medium. CRA (Sigma Chemical, St. Louis, MO) was solubilized in 100% ethanol (Quantum Chemical Co., Tuscola, IL) and diluted to 3 μM final concentration in tissue culture medium. Ethanol was added to each group not containing CRA to bring a final ethanol uniform concentration to all groups of 0.03% ethanol. Experiments that compared standard tissue culture medium to culture medium with ethanol showed no radiosensitization or cytotoxic effects on glioma cells (data not shown). These concentrations of IFN and CRA were chosen based on the estimated peak blood levels that might be reached in humans with an estimated 5.5 L blood volume who were receiving IFN at 6 million IU) and CRA at 1 mg/kg, with the assumption that the entire dose would be biologically available. Concentrations were then reduced by about one-half because

### Table 1. \[^{3}H\]Thymidine uptake assay data

<table>
<thead>
<tr>
<th>Radiation dose</th>
<th>Control</th>
<th>CRA</th>
<th>CRA + IFN</th>
<th>IFN</th>
<th>2 Gy set</th>
<th>Control</th>
<th>CRA</th>
<th>CRA + IFN</th>
<th>IFN</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Gy</td>
<td>19417 ± 2948</td>
<td>14108 ± 2389</td>
<td>14934 ± 1428</td>
<td>IFN only</td>
<td>10590 ± 608</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Gy</td>
<td>14445 ± 1420</td>
<td>11138 ± 1529</td>
<td>6493 ± 694</td>
<td>IFN only</td>
<td>7001 ± 805</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Gy</td>
<td>11260 ± 732</td>
<td>7691 ± 1055</td>
<td>3500 ± 282</td>
<td>IFN only</td>
<td>5131 ± 716</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 Gy</td>
<td>6034 ± 504</td>
<td>3826 ± 333</td>
<td>1919 ± 234</td>
<td>IFN only</td>
<td>2110 ± 191</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 Gy</td>
<td>2097 ± 232</td>
<td>1006 ± 186</td>
<td>384 ± 60</td>
<td>IFN only</td>
<td>508 ± 48</td>
<td></td>
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<td></td>
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</tbody>
</table>

* Tritium beta radiation emissions (cpm ± SD).
in vitro tumor cells were in an artificial situation of continuous exposure to the drugs. Although other investigators have used higher concentrations in their in vitro studies, our preliminary experiments suggested that these doses could be radiosensitizing. Those doses were well tolerated and associated with high tumor regression rates in patients with squamous cell of the skin and cervix (18,19), and the combination in these doses is presently being investigated in various squamous cell cancers in trials of the Cancer Biotherapy Research Group (32).

[3H]Thymidine Incorporation Survival Assay. Single-cell suspensions of U-373 MG cells were plated out in flat-bottomed 96-well plates (Corning Costar, Cambridge, MA) at a density of 2 × 10^5 cells/well for 24 h in standard tissue culture medium. Subsequently, the medium was removed and replaced with medium supplemented with either CRA (3 × 10^6 μM) or IFN (25 U/ml), or both CRA/IFN (3 × 10^6 μM and 25 U/ml, respectively). Tissue culture medium without supplementation was used for the untreated control groups. After a 24-h incubation at 37°C, the U-373 MG cells in the 96-well plates were subjected to increasing radiation doses (0, 2, 4, 8, and 16 Gy) at room temperature with a 4-MeV linear accelerator at a dose rate of 2 Gy/min. The plates were then incubated at 37°C for a total of 92 h after irradiation. During the last 24 h of this incubation, 0.5 μCi of [3H]thymidine was added to each well. The cells were harvested from the wells with a cell harvester (Cambridge Technologies, Cambridge, MA). Absolute thymidine counts are presented in Table 1. Survival fractions were calculated by dividing the experimental [3H]thymidine values into [3H]thymidine values for the untreated nonirradiated controls, and these data are shown in Fig. 1 as the subsequent survival curves presented in survival percentages. In Fig. 2, survival fractions were calculated by dividing the experimental [3H]thymidine values into [3H]thymidine values for the respective controls. The survival curves in Fig. 2 were fitted to a linear expression using ProStat (Poly Software Interna-
tional, Salt Lake City, UT), and the lines are shown in Fig. 5. The dose of radiation (in Gy) that would have resulted in a 50% survival fraction (LD_{50}) was calculated from the linear regression line equations and is presented in Table 3.

Clonogenic Survival Assay. For this assay, we used the methodology of DeLaney et al. (21) with some modification. In brief, single-cell suspensions of U-373 MG cells were plated in six-well plates at cell numbers appropriate for colony counting at each radiation dose. After 24 h, the medium was changed to standard tissue culture medium for the control groups or to medium containing either CRA (3 × 10^6 μM), IFN (25 U/ml), or both CRA/IFN (3 × 10^6 μM and 25 U/ml, respectively). After an additional 24 h of incubation at 37°C, the U-373 MG cells in the six-well plates were subjected to increasing radiation doses (0, 2, 4, 8, and 16 Gy) at room temperature with a 4-MeV linear accelerator at a dose rate of 2 Gy/min. After 12 days of incubation at 37°C, cells were fixed with 2% formaldehyde and stained with crystal violet. Colonies of 50 cells or more were counted independently by three different investigators; all three readings yielded similar results. The results of one reading are reported in Table 2. From these data, survival curves were generated by dividing the values of the respective experimental groups by the nonirradiated untreated control values shown in Fig. 2. Fig. 4 presents survival curves generated for each experimental group as a survival percentage of the nonirradiated control for each of the respective four groups. The survival curves in Fig. 4 were fitted to a linear expression, and the lines are shown in Fig. 2. The dose of radiation (in Gy) that would have resulted in a 50% survival fraction (LD_{50}) was calculated from the linear regression line equations and is presented in Table 3.

Statistical Analysis. Thymidine uptake assays were performed in replicates of 12. The clonogenic assay was performed in replicates of three for each group. For the [3H]thymidine uptake assays, the data were first statistically analyzed to insure that data fell between 99% confidence intervals for each data set using the ProStat statistics program and then analyzed for averages, SDs, and statistical significance using a general two-
RESULTS

Table 1 exhibits the [3H]thymidine data incorporation as a function of IFN and/or CRA treatment with and without increasing levels of irradiation and the associated Ps for the various comparisons. In the group receiving no irradiation (0 Gy), it is important to note the effect of the single or combination agent treatment on thymidine incorporation. Treatment with IFN alone caused a highly significant reduction of thymidine incorporation as compared with the control group (no treatment, no irradiation, 54% of the control value). In contrast, CRA alone, although also producing a highly significant reduction as compared with the control value (73%) did not have as great an effect as IFN alone. The combination of IFN/CRA also showed a significant reduction over the control values but at a level similar to CRA alone (77% of the control value with combination treatment). These data show that each of these agents alone has a cytostatic effect in the absence of irradiation, at least against this cell line, and that IFN alone was more potent in this regard than was CRA alone.

With the addition of irradiation, similar effects were demonstrated. For instance, IFN treatment alone with 4 Gy radiation was significantly more effective in inhibiting incorporation of thymidine (46% of the control group at 4 Gy) than the control (no agent treatment, 4 Gy irradiation) group (P < 0.001). The addition of CRA alone was also more potent (68% of the control value at 4 Gy) than the control group at 4 Gy (P < 0.001) but less so than the group treated with only IFN at 4 Gy (P < 0.001). The combination of CRA and IFN at 4 Gy showed the greatest reduction in thymidine incorporation (31% of the control value at 4 Gy) as compared with either single agent or the control group at 4 Gy (P < 0.001 for all groups). As can be observed from Table 1, the combination of CRA and IFN reduced thymidine uptake in comparison with all other experimental groups at each radiation dose. In fact, the only nonsignificant events in Table 1 were observed at: 0 Gy between the CRA alone group and the combination treated group (P = 0.35); at 2 Gy between IFN alone and the combination group (P = 0.21); and at 8 Gy between the IFN alone and the combination treatment (P = 0.09). All other events differ from one another at a statistically significant level. It is important to note that irradiation alone (control group 0–16 Gy) resulted in significant reduction of the incorporation of thymidine. However, the addition of single or combination treatment combined with irradiation significantly decreased the incorporation of thymidine over the irradiation alone. In the presence of irradiation, the combination treatment was the most potent in reducing the incorporation of the thymidine.

Fig. 1 displays the thymidine incorporation data as survival fractions rather than the thymidine raw data, which is exhibited in Table 1. Survival fractions were obtained by dividing the results of the thymidine incorporation of all groups by the value for the control group at 0 Gy, which is the reference point for 100% survival. The statistical results discussed above are the same for this data set and are not repeated for this reason. As can be seen from Fig. 1, combination therapy resulted in the lowest surviving fractions at all points tested with the exception of 0 Gy point. This suggests that the IFN plus CRA is more effective than single-agent treatment only when combined with irradiation.

Fig. 2 exhibits the thymidine incorporation data as a percentage of each group’s nonirradiation control. These data points were calculated by taking the values at each irradiation point (2, 4, 8, and 16 Gy) for each group and dividing them by their respective values at 0 Gy. For this reason, the values for all groups (control, CRA, IFN, and CRA + IFN) start at 100% in Fig. 2. By setting each radiation control and experimental group as a survival percentage of its respective control, the cytotoxic and cytostatic effects of CRA and IFN could be negated. This enabled us to compare their radiosensitization effects independent of their cytotoxic/cytostatic effects. Treatment with single
agent was more effective at all doses of irradiation tested than no treatment with the exception of CRA treatment at 2 Gy. Again, treatment with the combination of IFN and CRA had the greatest effect. With respect to the single-agent treatment, this analysis suggested that IFN was more potent as a radiosensitizer than CRA as a single agent. The combination therapy was the most effective radiosensitizing condition based on inhibiting thymidine incorporation as a function of irradiation.

To verify the results of the thymidine incorporation experiments, the same sets of experiments were performed using clonogenic assays. The results are exhibited in Table 2, along with the statistical analysis of this data. At the 0 Gy irradiation point, the effects on colony formation produced by IFN versus CRA versus IFN + CRA without irradiation are shown. In contrast to the thymidine incorporation, the clonogenic assay suggested that of the single-agent treatments, CRA alone was more effective than IFN (P = 0.048) in reducing colony formation in the absence of irradiation (0 Gy, no irradiation).

The effects of increasing irradiation alone can be observed in the control group (no agent treatment). As was seen in the thymidine experiments, irradiation alone reduced the number of colony-forming units as a function of increasing dose. With the addition of CRA and/or IFN, colony formation was inhibited compared with irradiation alone with the exception of CRA at 4 Gy (Table 2). At 16 Gy, for all three of the experimental groups and the untreated control group, no colony formation was observed. This suggests that the limit of sensitivity for the clonogenic assay was reached at a radiation dose between 8 and 16 Gy. This is probably not the case for the thymidine incorporation experiments as interpretable data are clearly shown at the 16 Gy irradiation point. However, as was the case in the thymidine incorporation experiments, the combination of CRA + IFN combined with radiation therapy significantly reduced colony formation over the control values except at the 8 Gy level (P = 0.07), where statistical significance was not met.

Fig. 3 displays the clonogenic assay data as a survival percentage where the 0 Gy, no treatment group value is the reference value for 100% survival. Combination treatment with IFN and CRA produced the lowest values of the various test conditions. Statistical analysis for this data was the same as that shown in Table 2.

Fig. 4 depicts the clonogenic data as a survival fraction within each group, with the nonirradiation value for each group being used as the 100% survival value. In all instances, the addition of irradiation decreased colony formation. Once again the lowest values in this analysis were seen with the IFN + CRA combination. Consistent with the thymidine data analyzed in the same fashion, the clonogenic results suggested that single-agent IFN was a more effective radiosensitizer than single agent CRA (see Fig. 2).

Figs. 5 and 6 illustrate the linear regression curves derived from the thymidine incorporation assay (Fig. 5) and from the clonogenic assay (Fig. 6). The LD_{50} shown in Gy were derived from these curves and are shown in Table 3. The thymidine experiments yielded an LD_{50} value for the combination treatment (3.2 Gy) that was much lower than the control value (6.8 Gy) and either of the single-agent treatment values (5.6 and 6.6 Gy for IFN and CRA, respectively). The values for single-agent treatment, although lower, were comparable with the control value. The clonogenic assay showed a decrease in the amount of irradiation needed to reduce tumor colony-forming units for the combination treatment (2.7 Gy) in comparison with the control value (3.9 Gy). The single-agent treatment with CRA showed a higher value (4.8 Gy) than the control with the single-agent IFN treatment showing a lower value (3.3 Gy) than the control. Again, the values for single-agent treatment are comparable
with the control, whereas the combination treatment with IFN and CRA was associated with the lowest value.

DISCUSSION

In this series of experiments, we have shown that in vitro, at concentrations that are achievable (based on serum measurements and estimates of blood volume distribution for the doses of IFN and CRA used in clinical trials) in cancer patients, IFN and CRA produce cytotoxic/cytostatic effects on glioma cells in vitro, and they enhance radiation-induced cytotoxicity when used in combination. The precise basis for this radiosensitization in vitro is unknown and was not explored in the present work. In the absence of radiation, at these concentrations IFN had the greatest cytotoxic effects in the [3 H]thymidine assay, whereas IFN + CRA and CRA alone had greater cytotoxic effects in the clonogenic assay. In the presence of radiation, IFN alone appeared to be more important in producing radiosensitization than CRA alone in terms of reducing the uptake of [3 H]thymidine. In the clonogenic assay, the radiosensitization roles of IFN and CRA as single agents are not as clear. However, it is apparent that when IFN and CRA are used in combination, they act in a synergistic manner to produce an increased radiosensitization and decreased overall survival of glioma cells in both the clonogenic assay and in the [3 H]thymidine uptake assay.

These data support the rationale for combining IFN and CRA with radiation therapy in patients who are undergoing brain RT as part of the management of their gliomas. We showed previously that IFN alone could be safely added to RT in such patients (13) and have recently launched a human clinical trial with IFN and CRA plus RT (26).

A variety of cellular events probably lead to increased radiosensitization of cancer cells via CRA and IFN treatment (33). These may include down-regulation or inhibition of DNA repair enzymes, decreased control of cell cycle regulation, or an enhanced sensitivity to apoptosis. Ideally, these effects would be preferentially exerted on malignant but not normal brain cells. It is likely that the promotion of differentiation by CRA would be less pronounced on the highly differentiated cells of the normal brain. Similarly, IFN, the biotherapeutic effects of which would presumably preferentially target virally transformed or other abnormal cancerous cells, might not be as potent against normal cells in the brain. However, because the radiosensitization mechanism of CRA and IFN is presently unknown, it is possible that the same radiosensitization effects may be exerted on normal brain tissue and not just glioma cells.

The in vitro results from this study support the concept that the IFN and CRA combination treatment renders high-grade glioma cells more susceptible to radiation-induced cytotoxicity. It is possible that the combination of CRA and IFN may mediate additional effects in vivo that we have not tested in this in vitro system. For instance, it is known that these agents exert significant immunological effects that could provide additional antitumor effects in vivo. They may also exert antiangiogenesis or other effects on blood vessels that could also enhance an anti-tumor effect in vivo. Thus, there are rationales beyond these in vitro observations that also support investigation of these agents in the clinical management of gliomas. A clinical trial in patients with gliomas is the best way to determine whether there is a sufficient therapeutic index to safely administer such a combination in vivo. Our initial results in the Cancer Biotherapy Research Group Trial 95-08 suggest that IFN at a dose of 3–6 million IU per day 5 days a week and CRA at a dose of 1 mg/kg/day 5 days a week can be safely administered with partial-brain radiation therapy in patients with gliomas (26). Accrual to that trial was completed recently, and follow-up analysis is in progress.

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REFERENCES


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