Enhanced Sensitivity of Human Colon Tumor Cell Lines in Vitro in Response to Thermochemoimmunotherapy

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ABSTRACT

We have investigated the cytotoxic responses in vitro of three human colon tumor cell lines with epithelial-like morphology, DLD-1, HCT-15, and HT-29, to thermochemoimmunotherapy with hyperthermia (42°C for 2 h), carboplatin, and recombinant human tumor necrosis factor (TNF). Dose ranges of carboplatin and recombinant human TNF were administered essentially simultaneously and were followed 1 h later by hyperthermia. A two-tiered approach was used to evaluate cytotoxicity. In the first tier, a 5-day microtiter assay using vital dye staining was done; the effect on surviving fraction of simultaneously varying carboplatin and recombinant human TNF doses was evaluated by response surface methodology. From this analysis doses were selected for use in the second-tier clonogenic survival assays. A similar treatment protocol was used in clonogenic assays. Both assays revealed significant interline treatment response heterogeneity. Only the HCT-15 cells were sensitive to TNF alone; carboplatin activity against all three tumor cell lines was enhanced by TNF. Hyperthermia had minimal effect as a sole agent but enhanced the effects of carboplatin and TNF in DLD-1 and HCT-15 cells. Triple modality treatment resulted in 3-4-log decreased survival and could reduce cytotoxic resistance expressed against single- or dual-modality treatments by some of these cells.

INTRODUCTION

Colorectal carcinoma and resultant hepatic metastases are largely refractory to current chemotherapeutic regimens and demand improved treatment strategies (1). Recent investigations have explored the use of platinum drugs as well as BRMs, some in conjunction with 5-fluorouracil, currently a drug of choice in the clinic (2–11). Other in vitro and in vivo studies in various tumor models have combined platinum drugs or BRMs such as TNF with hyperthermia (12–20). These studies have reported enhanced tumor cytotoxic interactions with these dual-modality combinations. However, there has been scant attention given to the application of these various dual combinations on human colon tumor cells.

We report here on the use of three dual-modality combinations (heat/TNF, heat/carboplatin, and TNF/carboplatin) on human colon tumor cell lines in vitro; furthermore, triple modality treatment (heat/TNF/carboplatin) has been evaluated as well. Cytotoxic endpoints have been assessed using both vital dye-uptake and clonogenic survival assays.

MATERIALS AND METHODS

Target Cells and Cell Culture. The target cells were human colon adenocarcinoma cell lines with epithelial-like morphology and were purchased from American Type Culture Collection (Rockville, MD). The pseudodiploid DLD-1 line was derived from a male and displays tumorigenicity in nude mice. The pseudodiploid HCT-15 line is also tumorigenic in nude mice. The hypertriploid HT-29 line was established from a female and is also tumorigenic. All lines were adapted to culture and twice-weekly passage in antibiotic-free Dulbecco's Modified Eagle F-12 medium prepared from powder ( Gibco, Grand Island, NY) with pyrogen-free water to which fetal calf serum (GIBCO) was added to a final concentration of 5%. All cell lines were verified Mycoplasma-free by the Gen-Probe Mycoplasma T.C. II detection kit (Gen-Probe, San Diego, CA).

Cytotoxicity Assays: Neutral Red. We used techniques similar to those previously reported (21). Tumor cells were seeded in 180 µl of medium/well of replicate 96-well plates at 2 x 10^3 cells/well on day 1. On day 0, recombinant human TNF (Biogen, Cambridge, MA) dissolved in medium was tiered at a 1:3 ratio over five serial doses. Control wells did not receive TNF. Immediately thereafter, 20 µl of carboplatin dissolved in sterile water were added over four serial doses to both TNF-treated and control wells; additional controls included cells treated with TNF alone and those that received neither TNF nor carboplatin. The final TNF concentrations ranged from 3.3 to 900 ng/ml and the carboplatin concentrations ranged from 2.5 to 20 µg/ml. These manipulations were made as rapidly as possible and then the plates were incubated at 37°C for 1 h.

At that time, parallel sets of plates were removed, bagged in a 5% CO₂/air environment, and then immersed for 2 h in water baths either at 37°C (control) or 42°C. At the end of the 2-h treatment, the bagged plates were removed from the baths, and the plates were removed from the bags and incubated for 5 days at 37°C. The 5-day time period was established in preliminary experiments which indicated that significant cytotoxicity would be manifest by this time; we also chose this time period because it was an effective compromise between retaining the advantages of automated, large-scale, rapid data accumulation and the longer time (14–24 days) needed to reveal clonogens used in the subsequent clonogenic assays. After 5 days the remaining viable cells were stained with neutral red; the incorporated dye was released from the washed cells by treatment with acidified 70% ethanol, and the A540 was determined in an automated plate reader.

The raw absorbance values were normalized to a 100% survival value for control cells that had not been exposed to TNF, carboplatin, or hyperthermia. The normalized values for those cells subjected to varying concentrations of TNF and/or carboplatin were then plotted as a response surface using Delta Graph software (Delta Point, Inc., Monterey, CA). A response surface was generated for both normothermic and hyperthermic treatment and for each cell line. These surfaces allowed effective visualization of multimodality cytotoxic interactions and were used as a guide to select doses of TNF and carboplatin for the clonogenic assays.

Clonogenic Survival. We used approaches similar to those we used previously to evaluate TNF/hyperthermia interactions (22). Initial experiments were conducted to establish control plating efficiencies for these cell lines. We determined that these lines were poorly clonogenic at low seeding densities unless cultured in the presence of feeder layers. Therefore, feeder cells of the same cell line as the test cells were lethally irradiated with 25 gray (U.S. Nuclear Cesium Mouse Irradiator, model E9103) while in suspension and then were inoculated into 25-cm² flasks (Corning Glassworks, Corning, NY) at 1 x 10⁶ cells/flask. After 4 h, serial dilutions of test cells were seeded into triplicate flasks. After...
RESULTS

The response surface analysis of the neutral red data is shown in Figs. 1–3 for the DLD-1, HCT-15, and HT-29 cell lines, respectively. Under normothermic conditions, only the survival of the HCT-15 cell line was significantly reduced by TNF as a single agent (Fig. 2A); this is observed in the surviving fraction versus TNF plane. Carboplatin as a single agent caused a dose-dependent cytotoxic effect above 2.5 μg/ml on all three cell lines (Figs. 1A, 2A, and 3A; surviving fraction versus carboplatin plane). When TNF and carboplatin were combined, greater than additive cytotoxic interactions occurred at intermediate carboplatin doses; this was evident at high TNF doses with the HT-29 (Fig. 3A) and to a lesser extent, the DLD-1 (Fig. 1A) cell lines, both of which normally expressed strong resistance to TNF alone. This interaction was even more striking with the HCT-15 cell line at intermediate TNF and carboplatin doses (Fig. 2A).

Hyperthermia alone had minimal or no effect on any of these cell lines (Figs. 1B, 2B, and 3B, z-axis); however, it increased

overnight culture, the medium was replaced with 2.5–5 ml of control medium or medium containing 10 μg/ml carboplatin (Bristol Myers-Squibb, Evansville, IN) and/or 10–860 ng/ml TNF (Genentech, South San Francisco, CA). One h later the flasks were heated (2 h, 42°C) or sham-heated in water baths as previously described. After returning the flasks to the incubator, the cultures were maintained for 14–24 days without medium change before established colonies were fixed and stained; colonies of >50 cells were scored as surviving clones. The number of colonies in treated cultures was corrected for initial multiplicity and normalized to that of controls, reflecting the difference, if any, in plating efficiency between lines or replicate experiments. The mean and SE for control plating efficiencies were 90 ± 2, 65 ± 4, and 44 ± 5% for DLD-1, HCT-15, and HT-29, respectively.

Statistics. Statistical analyses were done using a two-group, unpaired t test or analysis of variance and the Fisher's PLSD (protected least significance difference) or Scheffe F test at the 95% significance level (StatView II, Abacus Concepts, Inc., Berkeley, CA).

Fig. 1. Response surface analysis of neutral red assays of DLD-1 cells following treatment with carboplatin, TNF, and heat. Medium in 96-well plates was titered with TNF and/or carboplatin as indicated. Carboplatin was added immediately after the TNF. Controls received sham solutions. One h later the plates were sham heated (A, 2 h at 37°C) or heated (B, 2 h at 42°C). At 5 days viable cells were stained with neutral red and surviving fractions were determined and plotted as described in the text. Values are normalized to the sham-treated controls. The means are shown without replication errors to facilitate viewing of these plots. SE (bars) are shown for selected treatments in Fig. 4–6.

Fig. 2. Response surface analysis of neutral red assays of HCT-15 cells following treatment with carboplatin, TNF, and heat. Medium in 96-well plates was titered with TNF and/or carboplatin as indicated. Carboplatin was added immediately after the TNF. Controls received sham solutions. One h later the plates were sham heated (A, 2 h at 37°C) or heated (B, 2 h at 42°C). Other details are as in the legend to Fig. 1.

Fig. 3. Response surface analysis of neutral red assays of HT-29 cells following treatment with carboplatin, TNF, and heat. Medium in 96-well plates was titered with TNF and/or carboplatin as indicated. Carboplatin was added immediately after the TNF. Controls received sham solutions. One h later the plates were sham heated (A, 2 h at 37°C) or heated (B, 2 h at 42°C). Other details are as in the legend to Fig. 1.
COLON TUMOR RESPONSE TO CARBOPLATIN/TNF/HEAT

Fig. 3. Response surface analysis of neutral red assays of HT-29 cells following treatment with carboplatin, TNF, and heat. Medium in 96-well plates was titrated with TNF and/or carboplatin as indicated. One h later the plates were sham heated (A, 2 h at 37°C) or heated (B, 2 h at 42°C). Other details are as in the legend to Fig. 1.

the sensitivity of all the cell lines to carboplatin. This was apparent by comparing the shapes of the response surfaces in the surviving fraction versus carboplatin planes of Figs. 1A, 2A, and 3A with Figs. 1B, 2B, and 3B for each cell line. Under hyperthermic conditions, the loss of surviving fraction became linearly related to the log of the carboplatin dose. In contrast, only the sensitivity of the HCT-15 cell line to TNF was significantly further enhanced by hyperthermia. However, hyperthermia increased the effectiveness of carboplatin/TNF combinations at lower doses which were ineffective during normothermia (compare Figs. 1A, 2A, and 3A with Figs. 1B, 2B, and 3B) for the DLD-1 and HT-29 cell lines (Figs. 1 and 3), and particularly so for the HCT-15 cell line (Fig. 2).

The neutral red cytotoxicity data is shown in Figs. 4–6 for all three cell lines for selected TNF (10–900 ng/ml) and carboplatin (10 μg/ml) doses to allow depiction of statistical significance and comparison to clonogenic survival data over similar dose ranges (Figs. 7–9). For the DLD-1 cell line (Fig. 4) under normothermic conditions, only treatment with carboplatin with or without TNF resulted in significant cytotoxicity (Fisher and F test). Heating enhanced the cytotoxic effect of carboplatin without TNF (Fisher). Apparently heat could not further enhance the effect of carboplatin combined with TNF; however, such a response would not be readily detectable in this case because of the limited dynamic range (1 log) of the neutral red assay and thus it was evaluated in subsequent clonogenic assays where logarithmic ranges can be examined (see below).

The HCT-15 cell line was sensitive to TNF at either dose and to carboplatin under normothermic conditions (Fisher and F test; Fig. 5). When these modalities were combined, significantly increased cytotoxicity was observed (Fisher and F test) which appeared slightly superadditive; but, again, the limits of the range for this assay were met, and further evaluation required the clonogenic assay. Hyperthermia was cytotoxic alone against this cell line and enhanced all the responses of TNF and carboplatin as single agents (Fisher). The triple-modality treatment required evaluation by the clonogenic assay.

The normothermic phenotype of the HT-29 cell line (Fig. 6) contrasted with that of the HCT-15, in that as single agents carboplatin was more effective than TNF. When these two agents were combined, the response was significant for either TNF dose (Fisher and F test). Hyperthermia did not enhance
COLON TUMOR RESPONSE TO CARBOPLATIN/TNF/HEAT

Fig. 5. Neutral red assay of HCT-15 cells following treatment with carboplatin, TNF, and heat. Medium in 96-well plates was titered with TNF and/or carboplatin and heated (h; 2 h at 42°C) or sham heated (37°C). The data shown are for 10 or 300 ng TNF/ml (t10 or t300) and 10 µg carboplatin/ml (p10). Other details are as in the Fig. 4 legend.

Fig. 6. Neutral red assay of HT-29 cells following treatment with carboplatin, TNF, and heat. Medium in 96-well plates was titered with TNF and/or carboplatin and heated (h; 2 h at 42°C) or sham heated (37°C). The data shown are for 10 or 300 ng TNF/ml (t10 or t300) and 10 µg carboplatin/ml (p10). Other details are as in the Fig. 4 legend.

the effect of carboplatin or TNF alone, except at the highest TNF dose (Fisher); furthermore, no effects of heat on carboplatin with TNF were evident at these doses.

In our previous studies, we had determined that for TNF/hyperthermia, the neutral red assay reflected lower but parallel cytotoxic responses compared to concomitant clonogenic assays (22). Therefore, the 5-day dye assay used here was also compared to the clonogenic assays with these colon tumor cell lines conducted with a single dose (10 µg/ml) of carboplatin and two doses of TNF: (a) 10 or 260 ng/ml for HCT-15 and HT-29, and (b) 33 or 860 ng/ml for DLD-1 cells, because of the greater TNF resistance of the latter. The results are shown in Figs. 7–9 and Table 1. Even the doses of individual TNF or carboplatin treatments which gave mild responses (<50% killing) in the neutral red assay generated significant (up to ~1.5 log) colony inhibition effects. More importantly, greater than additive cytotoxic effects occurred when TNF and carboplatin were combined (Table 1). This cytotoxic interaction was further enhanced by hyperthermia for the HCT-15 and DLD-1 cell lines. The results of the neutral red assay generally foretold but understated the clonogenic response; we have observed a similar pattern comparing neutral red assays and clonogenic assays with murine tumor cells treated with TNF/hyperthermia (22). However, the sensitivity of the HCT-15 cell line to carboplatin, comparable to that of high dose TNF in the neutral red assay (Fig. 5), appeared substantially greater than that to TNF in the clonogenic assay (Fig. 8).

DISCUSSION

Our results demonstrate that strong cytotoxic responses are manifest in human colon tumor cell lines subjected to three distinct modalities: (a) a BRM, TNF; (b) a chemotherapeutic agent, carboplatin; and (c) a physical modality, hyperthermia. These responses were achieved against three cell lines which demonstrated marked heterogeneity, including resistance, to single or even certain dual modalities and suggest that a therapeutic protocol combining these three agents might be effective at overcoming treatment resistance in vivo. Furthermore, the combination of modalities may allow a dose reduction in individual agents to reduce treatment toxicities.

Many previous studies both in vitro and in vivo have well established the ability of heat to interact with BRMs (reviewed in Ref. 12) including TNF (13–15, 21, 22), and certainly also with platinum chemotherapeutic agents, including carboplatin (16–20) in generating a tumor cytotoxic response. Only a few other studies have examined possible interactions between TNF and cisplatin with mixed results (8, 9, 23). In one in vivo study, combined TNF and cisplatin treatment gave a synergistic anti-tumor effect in murine colon adenocarcinoma (MCA 26) or melanoma (B16) tumors implanted s.c. (8). In another study...
using human bladder tumor xenografts in nude mice, this combination was ineffective (9). However, to our knowledge, no prior report exists combining the three agents using human colon tumor cell lines as targets.

Investigating cytotoxic responses to three distinct agents is confounded by the need to examine ranges of doses of each as well as temporal permutations of timing, sequencing, and length of administration of each agent. We have attempted to minimize all but the former by selecting simultaneous administration of TNF and carboplatin, followed 1 h later by a single heat dose and time, and with continuous exposure to the BRM and drug. Nevertheless, the number of different levels of TNF selected (5 + control) combined with the number of different levels of carboplatin (4 + control) and the two temperatures (37°C and 42°C) would pose an extremely unwieldy experimental task if the clonogenic assays were the only option. Primarily for this reason, we have invoked a two-tiered analysis in which the neutral red assay is conducted first. This microcytotoxicity assay is rapid (5 days of treatment), compatible with surveying a wide range of variables including dose levels, and many steps in data accumulation and processing are automated. This assay is well suited to response surface analysis by which the particular dose(s) of TNF and/or carboplatin which induce desired cytotoxic effects can be gleaned. Thus, the combination of the

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Treatment</th>
<th>Calculated additive surviving fraction</th>
<th>Actual surviving fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLD-1</td>
<td>t33 + p10</td>
<td>0.085 ± 0.0600</td>
<td>0.008 ± 0.0026(^d)</td>
</tr>
<tr>
<td></td>
<td>t860 + p10</td>
<td>0.072 ± 0.0461</td>
<td>0.005 ± 0.0015(^d)</td>
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<td></td>
<td>h + t33 + p10</td>
<td>0.059 ± 0.0438</td>
<td>0.003 ± 0.0006(^d)</td>
</tr>
<tr>
<td></td>
<td>h + t860 + p10</td>
<td>0.050 ± 0.0337</td>
<td>0.002 ± 0.0007(^d)</td>
</tr>
<tr>
<td>HCT-15</td>
<td>t10 + p10</td>
<td>0.053 ± 0.0141</td>
<td>0.026 ± 0.0080(^d)</td>
</tr>
<tr>
<td></td>
<td>t260 + p10</td>
<td>0.034 ± 0.0077</td>
<td>0.002 ± 0.0001(^d)</td>
</tr>
<tr>
<td></td>
<td>h + t10 + p10</td>
<td>0.032 ± 0.0074</td>
<td>0.010 ± 0.0027(^f)</td>
</tr>
<tr>
<td></td>
<td>h + t260 + p10</td>
<td>0.019 ± 0.0034</td>
<td>0.001 ± 0.0000(^f)</td>
</tr>
<tr>
<td>HT-29</td>
<td>t10 + p10</td>
<td>0.032 ± 0.0130</td>
<td>0.016 ± 0.0083(^d)</td>
</tr>
<tr>
<td></td>
<td>t260 + p10</td>
<td>0.014 ± 0.0032</td>
<td>0.001 ± 0.0006(^d)</td>
</tr>
<tr>
<td></td>
<td>h + t10 + p10</td>
<td>0.027 ± 0.0085</td>
<td>0.025 ± 0.0177</td>
</tr>
<tr>
<td></td>
<td>h + t260 + p10</td>
<td>0.012 ± 0.0015</td>
<td>0.002 ± 0.0008(^f)</td>
</tr>
</tbody>
</table>

\(^a\) Combined treatments with TNF plus carboplatin or heat plus TNF and carboplatin; t10, t260, and t860 are ng TNF-α/ml medium; p10, 10 μg carboplatin/ml; h, 2 h at 42°C.

\(^b\) Mean ± SE of additive surviving fraction calculated from clonogenic surviving fractions when treatments are given separately. Data from 3-4 repeat experiments shown in Figs. 7-9.

\(^c\) Mean ± SE of actual surviving fractions of combined treatments. Data from 3-4 repeat experiments shown in Figs. 7-9.

\(^d\) Mean ± SE of additive surviving fraction calculated from clonogenic surviving fractions when treatments are given separately. Data from 3-4 repeat experiments shown in Figs. 7-9.

\(^e\) Mean ± SE of actual surviving fractions of combined treatments. Data from 3-4 repeat experiments shown in Figs. 7-9.

\(^f\) Significantly different by unpaired, one-tailed t test (P < 0.05).
dye uptake microassay and response surface analysis can be used effectively to greatly reduce the scope of the more laborious and costly clonogenic assay, which also involves a much longer turnaround time and tedious, observer-dependent colony counting. Despite these differences, these two assays were generally congruent, which we believe validates this two-tiered approach.

The mechanistic basis for cytotoxic interactions between these diverse agents is open to speculation; any mechanistic overlap between them would at first appear unlikely. Platinum is believed by most investigators to exert its cytotoxic effects by DNA cross-linking (24), although other possibilities have been proposed (25). Hyperthermia may induce its effects via perturbation of the protooncogene c-myc is influenced by TNF (37–42) and hyperthermia (43, 44) and can influence tumor cell cytotoxic response to platinum chemotherapeutics (45); this suggests another possible common mechanistic element. Our ongoing studies are designed to examine these possible mechanisms using the current treatment schedules which demonstrate strong interactive effects and cytotoxic responses. These will be compared to schedules involving abbreviated drug exposure and fractionated heat doses as well as other altered sequences of agent administration.

ACKNOWLEDGMENTS

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