Induction of Thymidine Phosphorylase in Both Irradiated and Shielded, Contralateral Human U87MG Glioma Xenografts: Implications for a Dual Modality Treatment Using Capecitabine and Irradiation[1]

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Abstract

In the United States, tumors of the central nervous system remain the third leading cancer-related cause of death in young adults with a median survival time of <1 year. A recent case study suggested that Capecitabine (a novel, fluoropyrimidine prodrug) may be effective in the treatment of brain metastases. Pharmacogenomic studies have correlated the antitumor response to Capecitabine with the expression of the drug metabolizing enzymes thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD). In the current study, we examined TP and DPD expression in normal human brain tissues and in glioblastoma multiforme, the most common and malignant type of brain tumor. Because previous reports suggest a tumor necrosis factor (TNF)-α-mediated increase in TP expression after irradiation (a current standard of care for glioblastoma multiforme), we also examined the effect of irradiation on the expression of TP, DPD, and TNF-α in both irradiated and lead-shielded contralateral U87MG glioma xenografts within the same animal. Expression levels were determined using real-time quantitative PCR as described previously. Results demonstrate an ~70-fold increase in TP mRNA levels 4 days after irradiation, relative to initial control levels. Interestingly, TP mRNA in the lead-shielded tumors (contralateral to irradiated tumors) increased ~60-fold by day 10 relative to initial control levels. Elevated TP levels were sustained for 20 days in irradiated xenografts but began to decrease after 15 days in the shielded/contralateral tumors, returning to baseline by 20 days. TP mRNA levels in normal mouse liver were unaltered, suggesting a tumor-associated effect. TNF-α mRNA levels did not increase after irradiation; therefore, mRNA expression of 11 additional cytokines [interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p35, IL-12p40, IL-15, and IFN-γ] in both the irradiated and shielded xenografts was quantitated. Results demonstrated increased levels of IFN-γ, IL-10, and IL-1α by 6.3-, 3.7-, and 1.6-fold, respectively, in irradiated tumors only. DPD mRNA levels did not change after irradiation. The tumor-associated induction of TP in irradiated and lead-shielded tumors within the same animal may have significant implications for the combined modality treatment of cancer patients with Capecitabine in conjunction with radiotherapy and may apply to the treatment of distant tumors and or metastatic disease.

Introduction

In the United States, tumors of the central nervous system remain the most prevalent solid neoplasm of childhood and the third leading cancer-related cause of death in adolescents and adults between the ages of 15 and 34 years (1). Unfortunately, despite over 50 years of research (including highly aggressive therapeutic approaches), the median survival of patients with malignant brain tumors remains <1 year (2, 3). GBM[3] remains the most common and malignant type of brain tumor and is characterized by an unusual resistance to treatment with both radiation and chemotherapy. Nitrosourea agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea are frequently used concurrently with radiotherapy (4, 5); unfortunately, severe dose-related toxicity limits the usefulness of these compounds (6). Although chemotherapy agents have not generally demonstrated survival benefit in this disease, a recently published case report suggested that Capecitabine might be an effective treatment for brain metastases (7).

Capecitabine is a recently introduced oral prodrug that is converted into 5-FU by three sequential enzymatic steps. TP is the final and rate-limiting enzyme responsible for Capecitabine activation (8–10). Once converted into 5-FU, metabolism results in either: (*a*) anabolism into cytotoxic nucleotides, which are ultimately responsible for tumor cell death; or (*b*) catabolism into biologically inactive metabolites that are ex-

Received 6/6/02; revised 8/19/02; accepted 8/27/02.
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[3] The abbreviations used are: GBM, glioblastoma multiforme; 5-FU, 5-fluorouracil; IL, interleukin; TNF, tumor necrosis factor; TP, thymidine phosphorylase; DPD, dihydropyrimidine dehydrogenase.

Table 1 Primer and probe sequences and optimal concentrations

Target	Sequence	Optimal conc. (nM)
TP (human)		
Fwd	GGAGAAGGGTGACCGACTCA	100
Probe	**FAM**-CGCTGAGATCAATCGGCCCGACTAT-**TAMRA**	200
Rev	TGCCCAGACTCGGCAAAG	200
TP (mouse)		
Fwd	CGGCCAGAGTGCAAAGCT	200
Probe	**FAM**-CAGCATACAGGATCCCATCAGCAGGAA-**TAMRA**	200
Rev	TCCACAGTGGCTGTCACATCTC	300
TNF-α		
Fwd	GGAGAAGGGTGACCGACTCA	300
Probe	**FAM**-CGCTGAGATCAATCGGCCCGACTAT-**TAMRA**	250
Rev	TGCCCAGACTCGGCAAAG	200
S9		
Fwd	ATCCGCCAGCGCCATA	100
Probe	**FAM**-AGCAGGTGGTGGTGAACATCCCGTCCTT-**TAMRA**	300
Rev	TCAATGTGCTTCTGGGAATCC	100
DPD		
Fwd	CCAAAGGCAGTAAAGCAGGAA	300
Probe	**FAM**-TGCGCCTGTCACTCTCCATTGCC-**TAMRA**	25
Rev	TCACGACTCCCCGTATCGA	100

creted in the urine and bile (11). Pharmacokinetic studies have shown that the amount of 5-FU available for anabolism is determined by the extent of its catabolism (11, 12). Thus, a delicate balance exists between the enzymatic activation of Capecitabine into 5-FU (catalyzed by TP) and its catabolic elimination (catalyzed by DPD). Several studies evaluating Capecitabine have demonstrated that intratumoral levels of TP and DPD (expressed as a TP/DPD ratio) are the best indicators of tumor response with increased efficacy characterized by high TP expression (increased conversion of Capecitabine into 5-FU) and low DPD expression (decreased inactivation of 5-FU; Refs. 13–15). In addition, a recent clinical study suggests that high TP expression is a good indicator of disease-free survival for those patients taking 5-FU prodrug-based chemotherapy, with patients expressing high TP and low DPD demonstrating the best disease-free survival (16)

Induction of TP has been suggested as a potential method of increasing efficacy of Capecitabine. Several cytokines (IL1-α, TNF-α, IFN-γ) and anticancer drugs (paclitaxel, docetaxel, cyclophosphamide) have been shown to increase TP expression (17–19). Of particular importance, irradiation has been shown to result in increased TP expression with a concurrent increase in Capecitabine efficacy (20). Because irradiation remains a current standard of care for the treatment of GBM, the addition of Capecitabine represents a rational and potentially synergistic combination.

In this study, we quantitated TP and DPD mRNA expression levels in GBM and normal human brain tissue. The effect of irradiation on the expression of TP, DPD, and several cytokines in contralateral irradiated and lead-shielded U87MG glioma xenograft tumors was also examined. The combined modality treatment of Capecitabine and irradiation may have significant implications for tumor response, which could extend to distant or metastatic tumors and, ultimately, result in prolonged patient survival.

Materials and Methods

Tissue Preparation. After an Institutional Review Board-approved protocol (X980409003), GBM and normal brain tissue samples were obtained from cancer patients undergoing surgical resection. Tissues used for RNA extraction had been snap frozen and stored at −70°C. A representative sample of the tissue obtained was fixed, paraffin-embedded, sectioned, and stained with H&E so that it could be examined by a neuropathologist to establish a diagnosis.

RNA Extraction. Total RNA was isolated as described previously (21, 22). All sample concentrations were calculated spectrophotometrically at A^{260} and diluted to a final concentration of 20 ng/μl in RNase-free water containing 12.5 ng/μl of total yeast RNA (Ambion, Austin, TX) as a carrier.

Real-Time Quantitative PCR. Expression levels were determined using an ABI 7700 Sequence Detection System as previously described by our laboratory (21, 22). Before sample analyses, housekeeping gene variation was determined for GBM and normal brain tissue as previously described by our laboratory (21). The primers and probes for human TP, DPD, TNF-α, S9 ribosomal RNA (GenBank accession no. NM001953, U20938, M10988 and NM001013 respectively) and mouse TP (GenBank accession no. AW744006) were designed using the Primer Express software (Applied Biosystems, Foster City, CA). The sequence and optimum primer/probe concentrations are shown in Table 1. Expression levels were calculated using the relative standard curve method as described previously (21, 22). All reactions were run in triplicate, and standard curves with correlation coefficients falling <0.98 were repeated. Control reactions confirmed that no amplification occurred when yeast total RNA was used as a template or when no-template control reactions were performed.

Cell Culture. U87MG glioma cells (purchased from the American Type Culture Collection, Manassas, VA) were

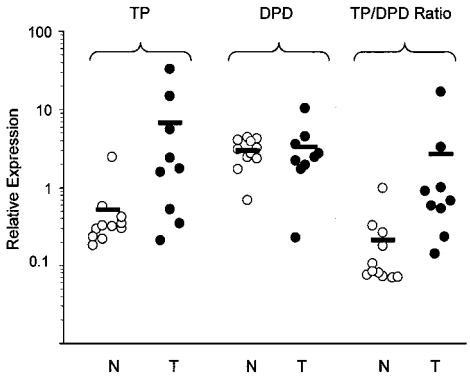

Fig. 1. Expression of TP and DPD in GBM (T) and normal brain (N) tissue. As shown above, TP expression is higher in GBM ($n = 9$) as compared with normal brain ($n = 11$) with a mean difference of 6.2 (SD = 7.2; $P = 0.13$). There was no significant difference in DPD expression levels in the same tissue samples (mean difference of 0.32; SD = 2.13; $P = 0.76$). The average TP/DPD ratio demonstrated in GBM (2.69) is ~16-fold higher than that of normal brain tissue (0.17) and is primarily because of TP overexpression in GBM. This profile should result in selective conversion of Capecitabine in tumor compared with normal brain tissues (16-fold higher TP/DPD ratio in GBM relative to normal brain).

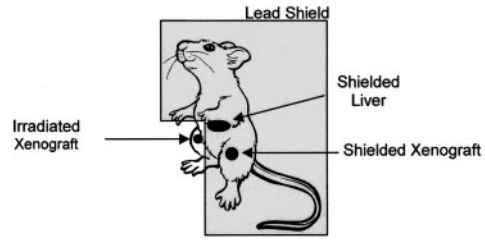

Fig. 2. Schematic of mouse xenograft location(s) and irradiation. One of the two flanks containing a U87MG glioma xenograft was irradiated, whereas the contralateral flank (containing the other U87MG xenograft) as well as the body of the mouse (including the mouse liver) was lead shielded.

maintained in stationary monolayer cultures at 37°C and 5% CO_2 in a humidified atmosphere using a 50:50 mixture of DMEM and Ham's Nutrient Mixture F12 supplemented with 7% heat-inactivated fetal bovine serum and 2.6 mM L-glutamine. All cell cultures were maintained in antibiotic-free conditions and regularly checked for *Mycoplasma* contamination using a PCR-based kit (ATCC). Near confluent (75%) monolayers of cells were harvested by brief exposure to 0.05% trypsin/0.53 mM EDTA (Life Technologies, Inc. Gaithersburg, MD). Harvested cells were pelleted (200 × *g*, 8 min at ambient temperature) in complete medium and resuspended in serum-free medium. Viable cells were counted using a Neubauer hemacytometer using trypan blue (0.4%) exclusion. To determine whether IFN-γ induced TP transcription, 0.5 ng/μl IFN-γ were added to 1×10^6 U87MG glioma cells and omitted in the control population. Cells were incubated for 24 h and collected as described above for RNA isolation.

Human Cancer Xenograft Preparation and Irradiation. Athymic, nude NCr mice (nu/nu) were anesthetized with ketamine/xylozine and s.c. injected bilaterally into hind flanks with a suspension of 5×10^6 U87MG glioma cells. Tumors were allowed to develop between 200 and 400 mm^3 in size. Mice were randomized into control and treatment groups, and one of the glioma tumor-bearing flanks of the treated group was irradiated, whereas the rest of the body (including the tumor in the opposite flank) was lead shielded (Fig. 2). Irradiation was carried out using a ^{60}Co teletherapy X-ray unit (Picker, Cleveland, OH) at a dose of 8.5 Gy. Mice in control groups were anesthetized before the irradiation but were not irradiated. Mice were sacrificed between 0–20 days after irradiation.

Cytokine Expression. The effect of irradiation on the expression of 12 cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p35, IL-12p40, IL-15, IFN-γ, and TNF-α) in the U8MG glioma xenografts was examined using a Taqman Cytokine Gene Expression Plate I (Applied Biosystems) according to manufacturer's instruction. Nonirradiated U87MG glioma xenografts in control mice were used as the calibrator.

Statistical Analyses. Statistical analyses were performed using Student's *t* test where differences were considered to be significant when $P < 0.05$.

Results

Quantitation of TP and DPD Expression in GBM and Normal Brain Tissues. Before quantitation using real-time quantitative PCR, we examined the variability of 12 housekeeping genes in GBM and normal brain tissues as previously described by our laboratory (21). The housekeeping genes examined include: *18S ribosomal RNA*; *β-glucuronidase*; *β2-microglobulin*; *β-actin*; *S9 ribosomal RNA*; *acidic ribosomal protein; TATA binding protein*; *transferrin receptor*; *glyceraldehyde 3-phosphate dehydrogenase*; *cyclophilin*; *phosphoglycerokinase*; and *hypoxanthine ribosyl transferase*. On the basis of these analyses, the ribosomal S9 gene (which demonstrated <1.5-fold variation in expression between normal and tumor tissues) was used to normalize the amount of total RNA in each sample (data not shown).

As shown in Fig. 1, TP expression is higher in GBM (T) as compared with normal brain tissue (N) with a mean difference of 6.2 (SD = 7.2; $P = 0.13$). Furthermore, this data suggests more variability of TP expression among tumor samples (ranging from a low of 0.21 to a high of 33.01) as compared with normal brain tissue (ranging from a low of 0.18 to a high of 2.47). There was no significant difference in DPD levels between normal and tumor tissue samples (mean difference of 0.32; SD = 2.13; $P = 0.76$). The average TP/DPD ratio demonstrated in GBM (2.69) is ~16-fold higher than that of normal brain tissue (0.17). The higher TP/DPD ratio in GBM is primarily because of higher expression of TP in GBM (6.72) compared with normal brain tissue (0.52) because there was no significant difference in DPD levels in the same tissues (3.33 and 3.01, respectively).

TP, DPD, and TNF-α Expression in U87MG Xenografts. As described in the "Materials and Methods" and illustrated in Fig. 2, athymic NCr nude mice were given injections of glioma cells in both hind flanks and allowed to develop tumors. One of the tumor-bearing flanks of the treated group was irradiated, whereas the rest of the mouse was lead shielded (Fig. 2). As shown in Fig. 3*A*, TP mRNA levels did not

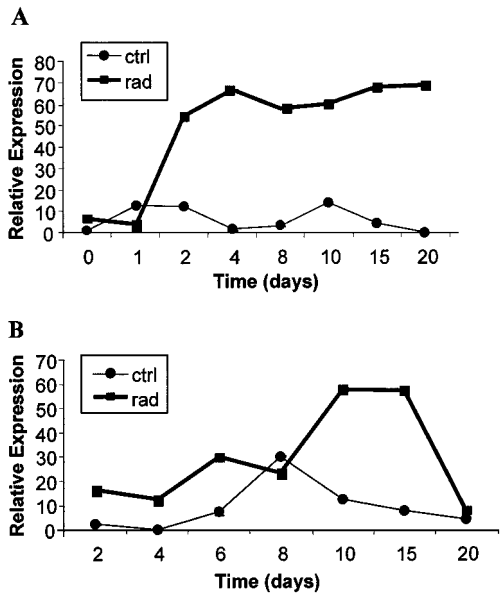

Fig. 3. TP expression in irradiated and shielded U87MG glioma xenografts in mice. *A*, as shown above, TP mRNA levels increased ~70-fold in irradiated tumors relative to nonirradiated tumors in control mice, remaining elevated 20 days after irradiation. *B*, of particular interest, TP mRNA levels in the shielded tumors (in the same animal) increased ~60-fold (relative to initial control levels) 10 and 15 days after irradiation before dropping to control levels at 20 days. *Error bars* have been incorporated into this figure but are so small as to be obscured by the data point.

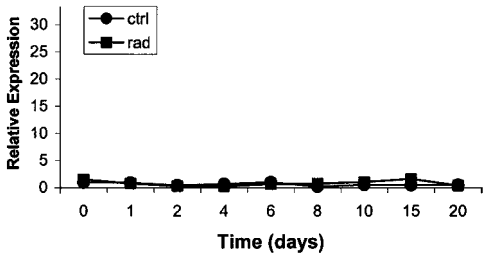

Fig. 4. TP expression in shielded normal mouse liver. Mouse TP mRNA expression in normal liver was evaluated in shielded and nonirradiated (control) mice. As shown above, there is no significant difference between shielded and nonirradiated mouse liver TP mRNA levels. *Error bars* have been incorporated into this figure but are so small as to be obscured by the data point.

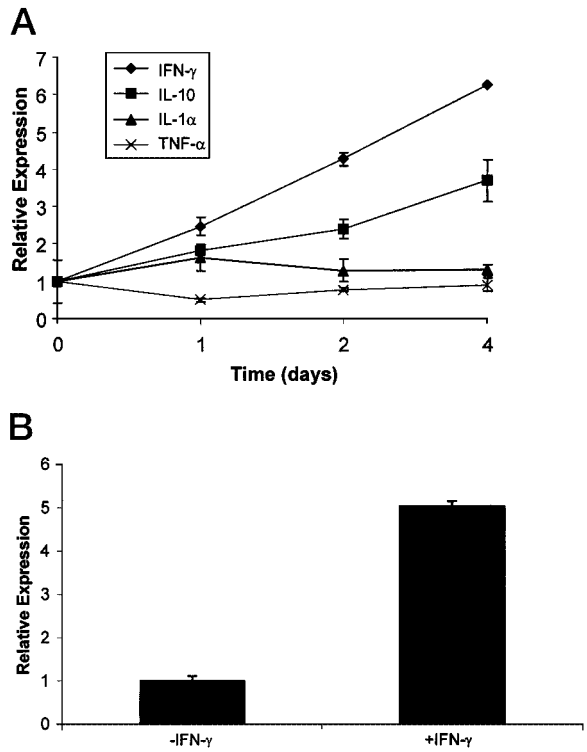

Fig. 5. *A*, effect of irradiation on mRNA expression of 12 cytokines. Cytokine expression in irradiated tumors (days 0–4 after irradiation) was determined for 12 cytokines using a TaqMan Cytokine Gene Expression Plate I as described in "Materials and Methods." As shown above, IFN-γ demonstrated the greatest increase in expression (6.3-fold) followed by IL-10 (3.7-fold) in irradiated xenografts relative to control xenografts at 4 days after irradiation. IL-1α demonstrated a <2-fold increase in expression 24 h after irradiation (1.6-fold), returning to baseline levels 2–4 days after irradiation. TNF-α levels (included in the cytokine plate) did not increase above baseline. IL-2, IL-4, IL-5, and IL-8, which are not shown, did not demonstrate any increases in expression after irradiation. IL-1β, IL-12p35, IL-12p40, and IL-15 mRNA levels were beyond the limits of detection in all of the samples (data not shown). *B*, quantitation of TP in U87MG glioma cells without (−) and with (+) IFN-γ. TP expression (mRNA levels) increased 5-fold in the U87MG glioma cells treated with IFN-γ (+) as compared with untreated cells (−), suggesting a direct effect of IFN-γ on TP transcription.

increase during the first 24 h after irradiation. However, by day 2, TP mRNA levels in irradiated tumors rapidly increased, peaking at day 4 with a ~70-fold increase relative to initial, nonirradiated tumors in control mice. As shown in Fig. 3*A*, the increase in TP mRNA levels was maintained in irradiated tumors for up to 20 days after irradiation (mean difference of 39.31; SD = 18.5; $P < 0.001$) and was an average 10-fold higher than control (nonirradiated) tumors.

TP mRNA in the lead-shielded tumor (from the contralateral flank of the same mouse) did not increase during the first 8 days after irradiation (Fig. 3*B*). However, by days 10 and 15, TP mRNA levels increased ~60-fold relative to initial TP mRNA levels in nonirradiated tumors in control mice (mean difference of 18.07; SD = 16.2; $P = 0.04$). Unlike the irradiated tumors, TP mRNA levels in the shielded tumors decreased to control levels 20 days after irradiation (Fig. 3*B*). No significant change in either DPD (mean difference of 0.13; SD = 0.56; $P = 0.61$) or TNF-α (mean difference of 0.45; SD = 1.2; $P = 0.41$) expression was observed in either the irradiated or shielded tumors relative to control tumors (data not shown).

TP Expression in Shielded Mouse Liver. To determine whether the increase in TP mRNA levels in the shielded xenografts was tumor associated, we examined mouse TP expression in liver tissue from treated (irradiated) and control (nonirradiated) mice. These analyses demonstrated no significant differences in mouse liver TP mRNA levels (mean difference of 0.21; SD = 0.39; $P = 0.24$) between irradiated mice and control mice (Fig. 4) over the time course studied, suggesting that the increase in TP mRNA seen in the xenografts was tumor associated.

Cytokine Expression in U87MG Xenografts. The delay between irradiation and the increase in TP mRNA levels (4 and 10 days in irradiated and shielded xenografts, respectively) suggests a secondary-mediated induction of TP rather than a direct effect. Because several cytokines, which have been shown to increase after irradiation, have also been shown to increase TP, we evaluated the expression of 12 cytokines (including IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p35, IL-12p40, IL-15, IFN-γ, and TNF-α) at several days preceding elevated levels of TP mRNA (days 0, 1, 2, and 4 in irradiated tumors and days 0, 4, 6, and 10 in shielded tumors). As shown in Fig. 5*A*, irradiated xenografts demonstrated the greatest increase in expression of IFN-γ (6.3-fold) followed by IL-10 (3.7-fold) at 4 days after irradiation and relative to nonirradiated xenografts. IL-1α demonstrated a $<$2-fold increase in expression 24 h after irradiation (1.6-fold), returning to baseline levels 2–4 days after irradiation. TNF-α levels (included in the commercially available cytokine plate) did not increase above baseline levels, agreeing with results from our independently designed primers and probes (see above). Expression levels of IL-2, IL-4, IL-5, and IL-8 did not increase after irradiation but were detectable, whereas IL-1β, IL-12p35, IL-12p40, and IL-15 mRNA levels were undetectable in both irradiated and control xenografts.

Cytokine expression was also examined in contralateral, shielded tumors (see Fig. 2) and compared with tumors in nonirradiated mice on days 0, 4, 6, and 10. Of the 12 cytokines examined, TNF-α and IL-10 demonstrated $<$2-fold increases in expression on days 4 and 6. Although IL-8 and IL-1α mRNA levels were detected in the shielded tumors, no increases were observed with these cytokines (data not shown). The remaining cytokines were below the limits of detection (IL-1β, IL-2, IL-4, IL-5, IL-12p35, IL-12p40, IL-15, and IFN-γ).

Induction of TP Expression in Cultured U87MG Cells with IFN-γ. To determine whether IFN-γ alone can induce TP transcription, U87MG glioma cells were incubated with IFN-γ for 24 h. As shown in Fig. 5*B*, TP mRNA increased 5-fold compared with control cells.

Discussion

Despite highly aggressive therapeutic approaches, the median survival of 1 year for patients with malignant brain tumors has not appreciably changed in the last 50 years (1–3). Recent advances in the molecular analysis of tumor tissue that allow quantitation of drug-metabolizing enzymes have resulted in the ability to predict response to chemotherapy (22–27). Although previous studies have suggested that this pharmacogenomic approach can be used to increase efficacy through selection of a subpopulation of patients likely to respond to chemotherapy (13–15, 27), a broader approach would be to design novel therapies based on the molecular profile of the tumor type.

A recent study reported the effective use of Capecitabine for brain metastases originating from breast cancer (7). The partial response (shown by decreased lesion size and improved mental performance) was particularly noteworthy because previous hormonal treatment, whole brain irradiation, and systemic chemotherapy, including treatment with 5-FU, proved ineffective. In addition to providing a potentially effective treatment for brain metastases, this study demonstrated that: (*a*) tumor resistance to 5-FU does not preclude effective treatment with Capecitabine; and (*b*) Capecitabine can reach therapeutic concentrations in brain tumor tissues. Because response to Capecitabine has been correlated to intratumoral expression of TP and DPD (expressed as a TP:DPD ratio; Refs. 13–15, 27), we examined the expression of these drug-metabolizing enzymes in GBM and normal human brain tissues. In addition, the effects of irradiation on TP mRNA expression were examined in both irradiated and shielded, contralateral U87MG glioma xenografts.

As shown in Fig. 1, the average TP/DPD ratio in GBM is $\sim$16-fold higher than in normal brain tissue. This increased TP/DPD ratio is primarily because of higher TP expression in tumor compared with normal brain tissue. There was no significant difference in DPD expression levels between normal and tumor tissues. This distribution of TP and DPD should result in selective intratumor activation of Capecitabine (*i.e.,* intratumoral 5-FU levels would be higher than normal tissue), whereas 5-FU clearance from tumor and normal tissues should be similar (equivalent DPD expression). Furthermore, these data suggest that in GBM and normal brain tissue samples, TP and DPD appear to be independent determinants of response with no apparent correlation in expression levels. Interestingly, the variability of TP expression is much higher in GBM as compared with normal brain tissue. However, whether this variability correlates to other factors such as tumor stage, location, or patient survival remains to be determined.

Recent studies have shown that it is possible to increase Capecitabine efficacy by induction of TP (20). Although irradiation has been shown to result in increased TP levels with a concurrent increase in Capecitabine efficacy in colon, cervix, gastric, and breast cancer xenograft models (20), the effects of irradiation in glioma xenograft models have not been examined before this study. As shown in Fig. 3*A*, irradiation increased TP mRNA expression $\sim$70-fold relative to initial control (nonirradiated) levels. Shielded contralateral tumors demonstrated a 60-fold increase in TP expression between 10 and 15 days after irradiation (Fig. 3*B*). Because DPD levels were unaffected, this results in a 70- and 60-fold increase in the TP/DPD ratio for these tumors, respectively.

The current trend in treatment is to reduce the amount of neurotoxicity from whole brain radiation by focusing the irradiation to the area of residual tumor or the site of tumor excision (involved fields or intensity-modulated radiation therapy). However, recurrence is common, generally within a few centimeters of the original tumor site (just beyond the irradiated field; Ref. 28). The contralateral xenograft model used in this study (Fig. 2) was designed to represent invasive, metastatic, and/or micrometastatic tumors in humans. The induction of TP in shielded tumors that were not directly irradiated has potential implications for improving Capecitabine efficacy in patients with invasive tumors that were not directly irradiated during treatment. Furthermore, examination of shielded mouse liver TP (Fig. 4) agrees with previous studies, suggesting that the induction of TP is a tumor-

associated effect (20). Interestingly, TNF-α mRNA levels did not increase after irradiation (Fig. 5).

The molecular basis for induction of TP after irradiation remains to be elucidated. Although a previous study suggests that increased TNF-α levels after irradiation results in increased TP expression, no increase in TNF-α was observed in these glioma xenografts. Examination of Fig. 3 reveals that elevated TP mRNA levels occurred at 4 and 10 days in irradiated and shielded tumors, respectively. This delayed increase in TP mRNA levels suggests a mediated and potentially complex mechanism with induction possibly involving a currently unidentified, soluble cell factor(s). The sustained increase in TP mRNA levels (particularly in irradiated tumors by up to 20 days) may also be suggestive of other mechanisms involved such as stabilization of the TP mRNA transcript. Because IL-1α and IFN-γ have also been shown to induce TP (17–19), we examined the expression of 12 cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p35, IL-12p40, IL-15, IFN-γ, and TNF-α) in irradiated and shielded glioma xenografts (Fig. 5*A*). Although significant changes were not detected in most of the cytokines examined, IFN-γ, IL-10, and IL-1α mRNA levels increased 6.3-, 3.7-, and 1.6-fold, respectively. In fact, IFN-γ has been shown to induce the highest levels of TP expression relative to TNF-α and IL-1α in human macrophages with gamma-activated sequence elements in the TP promoter being essential for IFN-γ-dependent activation of the TP gene (29). In the current study, we demonstrate that IFN-γ induced TP mRNA levels in U87MG glioma cells (Fig. 5*B*). Taken collectively, these studies suggest that the molecular basis for the induction of TP after irradiation may vary depending on tumor type with at least two mechanisms: (*a*) a TNF-α-independent mechanism (as demonstrated in the glioma xenograft models); or (*b*) a TNF-α-dependent mechanism [as demonstrated in the in colon, cervix, gastric, and breast cancer xenograft models (20)]. In addition, increased IFN-γ levels have been shown to have antitumor activity in recurrent gliomas by inhibition of angiogenesis, apoptosis of endothelial cells, suppression of glioma growth, and decreased cell proliferation (30, 31). These studies suggest the potential use of IFN-γ to induce TP expression in patients treated with Capecitabine (where irradiation is not a treatment option) or as an addition to the combination of Capecitabine and irradiation.

Previous studies have shown induction of IL-10 (a potent anti-inflammatory cytokine) after irradiation (32). In addition, immunohistochemical staining has suggested that elevated IL-10 protein levels correlate with elevated TP expression in a study examining oropharyngeal carcinoma (33). The data presented in this study suggest that increased IL-10 expression precedes increased TP levels. Interestingly, IL-10 has been reported to suppress TNF-α (34). Additional studies will need to examine whether the increased levels of IL-10 observed in this study are related to the lack of increase in TNF-α mRNA levels in irradiated xenografts. The increase in IL-1α after irradiation that was observed in this study is in agreement with studies that have also implicated IL-1α in TP up-regulation (20). However, only a slight increase in IL-1α (<2-fold) during the first 24 h after irradiation was observed. Future *in vitro* studies will examine the role of these cytokines in the molecular basis for TP induction after irradiation.

Although, chemotherapy has not yet emerged as a standard of care for brain tumors, a recent case study reports the successful treatment of brain metastasis with Capecitabine (7). Because ~24% of human cancers are known to metastasize to the brain (35), this may provide a new and potentially effective treatment option in a disease where incidence is increasing and median survival remains poor. However, whether this same approach can be used for primary brain tumors such as GBM remains to be determined. Molecular analysis of patient samples suggest that the high TP:DPD ratio (the determinant of response to Capecitabine) in GBM compared with normal brain tissue would result in the preferential intratumor activation of Capecitabine. In addition, irradiation (a standard of care for the treatment of GBM) is shown to produce a tumor-associated induction of TP expression, which could result in improved Capecitabine efficacy. Most importantly, the induction of TP is shown to occur in distant (nonirradiated) tumors in the same animal. Although the combination of 5-FU plus radiotherapy has been shown to produce additive efficacy, preclinical studies in xenograft models suggest that the effect of Capecitabine plus radiotherapy is synergistic (18, 20, 36). In addition, because 5-FU is a well-established radiosensitizing agent, potentially higher intratumoral levels of 5-FU (achieved by higher intratumoral TP levels) represent an opportunity to maximize the antitumor efficacy through the careful optimization of timing, dose, and administration of Capecitabine and irradiation. The pharmacogenomic approach used in this study may provide the basis for the use of molecular markers in the rational design of new clinical treatment paradigms.

Acknowledgments

We thank Jennifer Trevor, Kim Hoyle, and Suzanne Randall for technical assistance. We also thank Dr. Gretchen Cloud and Dr. Mark Carpenter for their aid with statistical analyses.

References

1. Galanis, E., and Buckner, J. C. Chemotherapy of brain tumors. Curr. Opin. Neurol., *13:* 619–625, 2000.

2. Macarthur, D. C., and Buxton, N. The management of brain tumours. J. R. Coll. Surg. Edinb., *46:* 341–348, 2001.

3. Prados, M. D., and Levin, V. Biology and treatment of malignant glioma. Semin. Oncol., *27:* 1–10, 2000.

4. Hildebrand, J., Sahmoud, T., Mignolet, F., Brucher, J. M., and Afra, D. Adjuvant therapy with dibromodulcitol and BCNU increases survival of adults with malignant gliomas. EORTC Brain Tumor Group. Neurology, *44:* 1479–1483, 1994.

5. Brem, H., Piantadosi, S., Burger, P. C., Walker, M., Selker, R., Vick, N. A., Black, K., Sisti, M., Brem, S., Mohr, G., *et al*. Placebo-controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas. The Polymer-brain Tumor Treatment Group. Lancet, *345:* 1008–1012, 1995.

6. Subach, B. R., Witham, T. F., Kondziolka, D., Lunsford, L. D., Bozik, M., and Schiff, D. Morbidity and survival after 1,3-bis(2-chloroethyl)-1-nitrosourea wafer implantation for recurrent glioblastoma: a retrospective case-matched cohort series. Neurosurgery (Baltimore), *45:* 17–22; discussion 22–13, 1999.

7. Wang, M. L., Yung, W. K., Royce, M. E., Schomer, D. F., and Theriault, R. L. Capecitabine for 5-fluorouracil-resistant brain metastases from breast cancer. Am. J. Clin. Oncol., *24:* 421–424, 2001.

8. Bajetta, E., Carnaghi, C., Somma, L., and Stampino, C. G. A pilot safety study of capecitabine, a new oral fluoropyrimidine, in patients with advanced neoplastic disease. Tumori, *82:* 450–452, 1996.

9. Budman, D. R., Meropol, N. J., Reigner, B., Creaven, P. J., Lichtman, S. M., Berghorn, E., Behr, J., Gordon, R. J., Osterwalder, B., and Griffin, T. Preliminary studies of a novel oral fluoropyrimidine carbamate: capecitabine. J. Clin. Oncol., *16:* 1795–1802, 1998.

10. Ishikawa, T., Fukase, Y., Yamamoto, T., Sekiguchi, F., and Ishitsuka, H. Antitumor activities of a novel fluoropyrimidine, N4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine (capecitabine). Biol. Pharm. Bull., *21:* 713–717, 1998.

11. Heggie, G. D., Sommadossi, J. P., Cross, D. S., Huster, W. J., and Diasio, R. B. Clinical pharmacokinetics of 5-fluorouracil and its metabolites in plasma, urine, and bile. Cancer Res., *47:* 2203–2206, 1987.

12. Diasio, R. B., Beavers, T. L., and Carpenter, J. T. Familial deficiency of dihydropyrimidine dehydrogenase. Biochemical basis for familial pyrimidinemia and severe 5-fluorouracil-induced toxicity. J. Clin. Investig., *81:* 47–51, 1988.

13. Ishikawa, T., Sekiguchi, F., Fukase, Y., Sawada, N., and Ishitsuka, H. Positive correlation between the efficacy of capecitabine and doxifluridine and the ratio of thymidine phosphorylase to dihydropyrimidine dehydrogenase activities in tumors in human cancer xenografts. Cancer Res., *58:* 685–690, 1998.

14. Tsukamoto, Y., Kato, Y., Ura, M., Horii, I., Ishitsuka, H., Kusuhara, H., and Sugiyama, Y. A physiologically based pharmacokinetic analysis of capecitabine, a triple prodrug of 5-FU, in humans: the mechanism for tumor-selective accumulation of 5-FU. Pharm. Res., *18:* 1190–1202, 2001.

15. Schuller, J., Cassidy, J., Dumont, E., Roos, B., Durston, S., Banken, L., Utoh, M., Mori, K., Weidekamm, E., and Reigner, B. Preferential activation of capecitabine in tumor following oral administration to colorectal cancer patients. Cancer Chemother. Pharmacol., *45:* 291–297, 2000.

16. Nishimura, G., Terada, I., Kobayashi, T., Ninomiya, I., Kitagawa, H., Fushida, S., Fujimura, T., Kayahara, M., Shimizu, K., Ohta, T., and Miwa, K. Thymidine phosphorylase and dihydropyrimidine dehydrogenase levels in primary colorectal cancer show a relationship to clinical effects of 5′-deoxy-5-fluorouridine as adjuvant chemotherapy. Oncol. Rep., *9:* 479–482, 2002.

17. Eda, H., Fujimoto, K., Watanabe, S., Ura, M., Hino, A., Tanaka, Y., Wada, K., and Ishitsuka, H. Cytokines induce thymidine phosphorylase expression in tumor cells and make them more susceptible to 5′-deoxy-5-fluorouridine. Cancer Chemother. Pharmacol., *32:* 333–338, 1993.

18. Sawada, N., Ishikawa, T., Fukase, Y., Nishida, M., Yoshikubo, T., and Ishitsuka, H. Induction of thymidine phosphorylase activity and enhancement of capecitabine efficacy by taxol/taxotere in human cancer xenografts. Clin. Cancer Res., *4:* 1013–1019, 1998.

19. Endo, M., Shinbori, N., Fukase, Y., Sawada, N., Ishikawa, T., Ishitsuka, H., and Tanaka, Y. Induction of thymidine phosphorylase expression and enhancement of efficacy of capecitabine or 5′-deoxy-5-fluorouridine by cyclophosphamide in mammary tumor models. Int. J. Cancer, *83:* 127–134, 1999.

20. Sawada, N., Ishikawa, T., Sekiguchi, F., Tanaka, Y., and Ishitsuka, H. X-ray irradiation induces thymidine phosphorylase and enhances the efficacy of capecitabine (Xeloda) in human cancer xenografts. Clin. Cancer Res., *5:* 2948–2953, 1999.

21. Blanquicett, C., Johnson, M., Heslin, M., and Diasio, R. B. Housekeeping gene (HKG) variability in normal and carcinomatous colorectal and liver tissues; applications in pharmacogenomic gene expression studies. Anal. Biochem., *303:* 209–214, 2002.

22. Johnson, M. R., Wang, K., Smith, J. B., Heslin, M. J., and Diasio, R. B. Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. Anal. Biochem., *278:* 175–184, 2000.

23. Andratschke, N., Grosu, A. L., Molls, M., and Nieder, C. Perspectives in the treatment of malignant gliomas in adults. Anticancer Res., *21:* 3541–3550, 2001.

24. Diasio, R. B., and Johnson, M. R. The role of pharmacogenetics and pharmacogenomics in cancer chemotherapy with 5-fluorouracil. Pharmacology (Basel), *61:* 199–203, 2000.

25. Salonga, D., Danenberg, K. D., Johnson, M., Metzger, R., Groshen, S., Tsao-Wei, D. D., Lenz, H. J., Leichman, C. G., Leichman, L., Diasio, R. B., and Danenberg, P. V. Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase. Clin. Cancer Res., *6:* 1322–1327, 2000.

26. Johnson, M. R., Hageboutros, A., Wang, K., High, L., Smith, J. B., and Diasio, R. B. Life-threatening toxicity in a dihydropyrimidine dehydrogenase-deficient patient after treatment with topical 5-fluorouracil. Clin. Cancer Res., *5:* 2006–2011, 1999.

27. Mori, K., Hasegawa, M., Nishida, M., Toma, H., Fukuda, M., Kubota, T., Nagasue, N., Yamana, H., Hirakawa, Y. S. C. K., Ikeda, T., Takasaki, K., Oka, M., Kameyama, M., Toi, M., Fujii, H., Kitamura, M., Murai, M., Sasaki, H., Ozono, S., Makuuchi, H., Shimada, Y., Onishi, Y., Aoyagi, S., Mizutani, K., Ogawa, M., Nakao, A., Kinoshita, H., Tono, T., Imamoto, H., Nakashima, Y., and Manabe, T. Expression levels of thymidine phosphorylase and dihydropyrimidine dehydrogenase in various human tumor tissues. Int. J. Oncol., *17:* 33–38, 2000.

28. Wild-Bode, C., Weller, M., Rimmer, A., Dichgans, J., and Wick, W. Sublethal irradiation promotes migration and invasiveness of glioma cells: implications for radiotherapy of human glioblastoma. Cancer Res., *61:* 2744–2750, 2001.

29. Goto, H., Kohno, K., Sone, S., Akiyama, S., Kuwano, M., and Ono, M. Interferon γ-dependent induction of thymidine phosphorylase/platelet-derived endothelial growth factor through gamma-activated sequence-like element in human macrophages. Cancer Res., *61:* 469–473, 2001.

30. Knupfer, M. M., Poppenborg, H., Van Gool, S., Domula, M., and Wolff, J. E. Interferon-γ inhibits proliferation and adhesion of T98G human malignant glioma cells *in vitro*. Klin. Padiatr., *209:* 271–274, 1997.

31. Dadie, B., Schartner, J., Vorpahl, J., and Preston, K. Interferon-γ induces apoptosis and augments the expression of Fas and Fas ligand by microglia *in vitro*. Exp. Neurol., *162:* 290–296, 2000.

32. Mizutani, N., Fujikura, Y., Wang, Y. H., Tamechika, M., Tokuda, N., Sawada, T., and Fukumoto, T. Inflammatory and anti-inflammatory cytokines regulate the recovery from sublethal X irradiation in rat thymus. Radiat. Res., *157:* 281–289, 2002.

33. Fujieda, S., Sunaga, H., Tsuzuki, H., Fan, G. K., and Saito, H. IL-10 expression is associated with the expression of platelet-derived endothelial growth factor and prognosis in oral and oropharyngeal carcinoma. Cancer Lett., *136:* 1–9, 1999.

34. Denys, A., Udalova, I. A., Smith, C., Williams, L. M., Ciesielski, C. J., Campbells, A. J., Andrews, C., Kwaitkowski, D., and Foxwell, B. M. Evidence for a dual mechanism for IL-10 suppression of TNF-α production that does not involve inhibition of p38 mitogen-activated protein kinase of NF-κB in primary human macrophages. J. Immunol., *168:* 4837–4845, 2002.

35. Pilkington, G. J. The paradox of neoplastic glial cell invasion of the brain and apparent metastatic failure. Anticancer Res., *17:* 4103–4105, 1997.

36. Wilke, H. Future treatment options with Capecitabine in solid tumours. Eur. J. Cancer, *38:* 21–25, 2002.

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Mol Cancer Ther 2002;1:1139-1145.