High Basal Level Gene Expression of Thymidine Phosphorylase (Platelet-derived Endothelial Cell Growth Factor) in Colorectal Tumors Is Associated with Nonresponse to 5-Fluorouracil

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ABSTRACT

The gene expression levels of the nucleoside cleavage enzyme/angiogenic factor thymidine phosphorylase (TP), also known as platelet-derived endothelial cell growth factor, were measured by quantitative reverse transcription-PCR in 38 pretreatment biopsies of colorectal tumors from patients who were subsequently treated with 5-fluorouracil (5-FUra) and leucovorin (LV). The range of TP gene expression (relative mRNA levels) in those tumors nonresponsive to 5-FUra was much broader than that of the responding tumors. In contrast to in vitro studies that had shown that an increased intracellular level of TP potentiates the activity of 5-FUra by converting it to the more cytotoxic nucleoside form 5-fluoro-2'deoxyuridine, tumors with the highest basal TP expressions were nonresponders to 5-FUra/LV therapy. The mean TP mRNA level in the nonresponding tumors was 2.6-fold higher than that of the responding patients. We had previously shown that high expression of thymidylate synthase (TS), the target enzyme of 5-FUra, was also a predictor of nonresponse to 5-FUra (L. Leichman et al., J. Clin. Oncol., 15: 3223–3229, 1997). TP and TS expressions were found to be independent variables in these tumors, so that low expression levels of both TS and TP in tumors predicted a very high response rate (11 of 14) to 5-FUra/LV as well as a significantly longer survival, whereas none (0 of 24) of the patients with high expression of either TP or TS were responders.

INTRODUCTION

TP (EC 2.4.2.4) catalyzes the reversible interconversion of thymine and thymidine using deoxyribose-1-phosphate and inorganic phosphate as second substrates (1, 2) and in addition, also catalyzes a deoxyribosyl transfer reaction from one nucleoside to another (3). It has been proposed that one role for TP is to control the intracellular levels of thymidine, which at higher concentrations becomes toxic to cells and causes replication errors in DNA (4). However, the physiological role of this nucleoside cleavage reaction remained unclear until the surprising finding was made that TP and the angiogenesis factor, platelet-derived endothelial cell growth factor, are actually one and the same molecule (5-9). Platelet-derived endothelial cell growth factor had been recognized several years before this discovery as a novel factor that had chemotactic activity in endothelial cells (10). TP was found to be angiogenic and to promote tumor growth in model systems (10-12). The up-regulation of TP correlates with MVD in breast cancer (13), colorectal cancer (14, 15), gastric cancer (16, 17), and renal cell carcinoma (18). Consistent with the hypothesis that increased tumor vascularization (as indicated by higher MVD) leads to a greater metastatic propensity, higher TP levels were found to be associated with more invasive bladder tumors (4), increased frequency of hepatic metastases from gastric cancers (17), and a more malignant phenotype of ovarian cancer (19). High TP is an indicator of worse prognosis in colorectal cancer, even after adjustment for Dukes’ stage (15), in gastric cancer (17) and in breast cancer (13). The thymidine-cleaving catalytic activity of TP is essential for its angiogenic effects (20), and there is evidence that the deoxyribose-1-phosphate thus generated serves as a direct molecular signal for initiating or promoting angiogenesis (11).

TP received attention in connection with fluoropyrimidine cancer chemotherapy over 30 years ago. TP was found to degrade the powerful TS-directed agentFdUrd to the less potent 5-FUra (21), and thus early interest in TP centered on the design of inhibitors that would minimize the cleavage of FdUrd in vivo and thereby maximize its clinical efficacy. A number of moderately effective inhibitors of TP were synthesized (22), but to the best of our knowledge, they were never tested clinically in...
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TP levels can affect sensitivity of cells to fluoropyrimidines. Thus, TP not only serves as an indicator of angiogenic potential because TP is often preferentially expressed in tumor tissue (30), these results provide a rationale for using TP-activated drugs to achieve more tumor-selective activity. Thus, TP not only serves as an indicator of angiogenic potential and as a prognostic factor but also may play an important role in cancer chemotherapy as a target for antiangiogenic agents as well as an activating enzyme for prodrugs of 5-FUra (31).

We had found previously that intratumoral levels of expression of the mRNA for the target enzyme TS predicted the sensitivity of colorectal and gastric tumors to 5-FUra protocols (32). High levels of TS expression were associated with nonresponse to 5-FUra, whereas those tumors with low TS expression had a higher response rate than the overall one. However, because a number of nonresponding patients also had low TS, we were interested in finding other response determinants that might be used to identify those low TS tumors not responding to 5-FUra. Because of the aforementioned studies showing that changes in TP levels can modulate the activity 5-FUra, we thought that intratumoral basal TP levels could be a predictor of clinical efficacy of 5-FUra, provided that there was sufficient variation among individuals in the expression of this enzyme. Because all previous studies had reported that increasing TP levels had resulted in greater sensitivity of cells or tumors to 5-FUra, our initial hypothesis was that those tumors with higher levels of TP would tend to be more sensitive to 5-FUra-based chemotherapy than those with lower levels of TP. Accordingly, we used quantitative RT-PCR to measure TP expression (relative mRNA levels) in a group of colorectal tumors in which we had already determined TS expression values and knew the responses to 5-FUra-based therapy. As discussed below, the results were the opposite of what was expected from the in vitro data.

**MATERIALS AND METHODS**

**Clinical Methods.** Eligibility criteria for this trial and tumor response evaluation parameters were described previously in detail (32). In brief, eligible patients had: (a) a diagnosis of disseminated or recurrent colorectal cancer with a measurable lesion accessible for biopsy; (b) a Southwest Oncology Group performance status of 0 to 2 with adequate hematological, hepatic, and renal function; (c) no prior infusional 5-FUra; and (d) a lesion that was bidimensionally measurable either by physical or radiological examination. Following placement of an indwelling venous access, patients were treated with 5-FUra (200 mg/m²/day) as a continuous infusion administered by ambulatory infusion pump (Pharmacia Deltec Cadd pump, Minneapolis, MN) with LV (20 mg/m² by i.v. push weekly).

The initial cycle was given for 4 weeks, followed by a 1-week rest, with subsequent cycles consisting of 3 weeks continuous therapy and a 1-week rest. Response rates and toxicity profiles for this regimen have been published (33).

After two cycles (8 weeks) of treatment, measurable disease was reassessed. Response criteria were the standard definitions used for national cooperative group trials (34). To be classified as a responder, a tumor had to have a 50% reduction in the sum of the products of the perpendicular diameters of the indicator lesion without growth of other disease or the appearance of new lesions. Those with responses or stable disease were continued on protocol until progression was documented.

Liver metastases represented the most common site of disease on which a biopsy was performed and assessed for response, whereas in some cases, nodal metastases or peritoneal metastases were the disease sites evaluated. All specimens were obtained by core-needle biopsy prior to administration of 5-FUra/LV. This technique uses a coaxial system in which fine-needle aspiration is used to confirm cytological evidence of cancer within moments of the aspiration. The fine needle is withdrawn from a sheath and a core needle is inserted through the sheath without losing position. The core-needle material is used for gene expression analysis. Demographic characteristics of these patients were also previously published (32).

**Laboratory Methods.** The procedure for RT-PCR quantitation of gene expression has been described in detail previously (35, 36). In brief, the method involves isolation of mRNA from each tumor, preparation of cDNA using reverse transcriptase and random hexamers, and PCR amplification of the specific cDNA of interest in a range of concentrations that gives rise to a linear curve of the resulting PCR products. An internal standard gene (e.g., β-actin) from the same cDNA solution is amplified by PCR separately. We found previously that with β-actin as the reference gene, a good linearity is obtained between gene expression values determined by RT-PCR and protein content determined by immunohistochemistry (37). Once the concentration ranges for linear amplification are established for the cDNA of the target gene and the reference gene, the ratio of the slopes generates an empirical number proportional to the amount of mRNA of interest in the tissue normalized to total RNA.

Gene expression values are reported only if the PCR of serial dilutions of the cDNA solution generates a set of distinct bands with intensities that are linear with the concentration of cDNA. Slopes of the lines are obtained from at least three data points, so that each reported gene expression value represents the average of a minimum of three separate PCR reactions within the linear amplification range. When the initially chosen cDNA concentrations for a particular determination give PCR products clearly outside of the proportional linearity region, the determination is repeated, with adjusted cDNA concentrations, until the data points are in the linear range and the correlation coefficient for linearity for a set of at least three consecutive serial dilutions plus the zero point is greater than 0.90. This method has been used by this group (32, 37) as well as by others (38) to quantitate various gene expressions in tumor biopsy specimens.

For RT-PCR quantitation of TP, the following primers were used: T7-CTTCGTGGCCGCTGTGGTG, corresponding
to bases 1921–1939 of the genomic sequence located in exon 2 (with “T7” designating the T7 RNA polymerase clamping sequence TAATACGACTCATATAGGGAGA attached to the 5′ end), and TATCCAGAGCCAGAGCAGA, corresponding to bases 2929 to 2949 of the genomic sequence located in exon 4. (β-actin primers BA67 and BA68 were described previously; Ref. 35). The PCR reaction was performed as described previously (35, 36), except that cycling conditions were modified to best suit the TP primers. The cycling conditions for TP amplification were 15 s at 96°C, 30 s at 65°C, and 30 s at 72°C for 31 cycles.

Statistical Methods. Fischer’s Exact test was used to compare responding and nonresponding patients according to baseline characteristics.

RESULTS

TP expression was determined by quantitative RT-PCR (35, 36) in tumor tissue specimens from 38 colorectal cancer patients analyzed previously for TS expression levels (32). Data on demographics and tumor sites for these patients were also reported in the previous paper (32). No associations were apparent between TP expression and age, sex, ethnicity, or tumor site. Tumors were categorized as either responding or not responding to a regimen of 5-FUra and LV (see “Materials and Methods” for definition of clinical response criteria). Fig. 1 shows the distributions of TP expression for the two groups. The mean TS expression of the nonresponders was 2.8-fold larger than that of the responders (20.3 versus 7.3, respectively; P = 0.004). The range of TP expressions among the responding tumors was much narrower (1.0–16.3, 16-fold) compared with that of the nonresponders (0.4–82.6, 205-fold). None of the patients (10 of 38) with TP >18 responded (P = 0.037).

For tumor response prediction, it was of interest to determine the relationship between TP and TS expressions. That is, if TS and TP were co-regulated, there would be little or no additional benefit for response prediction from measuring two gene expressions, whereas if the genes were independently regulated, some of the low TS patients might be identified as nonresponders by their high TP expression. Fig. 2 shows that there is no apparent correlation between the expressions of these genes (r² = 0.01), with a number of low TS tumors having high TP.

Longer periods of survival have been observed for colorectal cancer patients whose tumors respond to chemotherapy (39). In the present study, patients with TP expression above the median of expression value of 9.8 showed a tendency to survive longer than those below the median, but the difference was not statistically significant (P = 0.3), probably because the individual medians for the responding and nonresponding groups were not very different (8.7 and 11.8, respectively; Fig. 3A). However, if survival is compared for those patients with TP values above or below the nonresponse threshold of TP = 18, there was a statistically significant divergence of the curves (P = 0.046; Fig. 3B). The greatest increase in survival was seen for that group of patients with both TS and TP below their respective nonresponse cutoff values (P = 0.0023; Fig. 3C). Thus, low TS and TP expression values identify tumors not only with a high probability of response to therapy with 5-FUra but also considerably better survival prognosis compared with the situation when either or both genes are highly expressed.

DISCUSSION

In this study, we found that TP gene expressions in colorectal tumors varied over a range of more than 200-fold, and that those tumors with the highest basal levels of TP gene expression were nonresponsive to a 5-FUra-based protocol, whereas the response rate among tumors with lower TP expressions was greater than the overall response rate. The apparent discrepancy between these results and the concept that cells with higher TP activity would be more sensitive to 5-FUra could be explained in several ways: (a) the relationship between the amount of TP mRNA and TP protein levels in the tumors remains to be established. If some of the regulation of TP protein expression takes place at the translational level, as is the case with TS (40), high TP gene expression may not be precisely reflected in the TP protein levels. Alternately, even if TP protein...
levels were proportional to gene expression, the rate of conversion of 5-FUra to FdUrd by TP could be limited by the availability of deoxyribose-1-phosphate in tumors. The addition of deoxyribose donor cosubstrates of TP has been shown to greatly increase the incorporation of thymine into DNA as well as the growth-inhibitor potency of 5-FUra (41). However, although both of these possibilities might explain a lack of increased 5-FUra sensitivity in tumors with high TP expression, they would not readily account for the observed inverse relationship. Our present hypothesis is that the inverse association between TP expression levels and response to 5-FUra is a consequence of the role of TP as an angiogenesis factor. As noted in the introduction (13–18), the development of angiogenesis and increased MVD is associated with high TP protein expression. An inverse relationship was reported recently in gastric cancers between frequency of apoptotic cells in tumors and MVD, indicating that angiogenesis is accompanied by reduction of apoptotic propensity in tumor cells (42). It has been shown that hypoxia, which is known to stimulate the development of angiogenesis (43), can select for cells that are apoptosis resistant (44). These observations suggest that high TP gene expression in tumors is a marker for other genetic and biochemical changes associated with the development of a more aggressive and malignant tumor phenotype that has increased resistance to cytotoxic agents due to the loss of apoptotic potential.

Because RT-PCR quantitation is a homogeneous solution methodology, each gene expression value is an average that includes contributions from tumor cells as well as from any non-tumor tissue infiltrating the specimen. The conclusions of this study could have been confounded: (a) if the specimens were severely heterogeneous with appreciable amounts of non-tumor tissue; and (b) if TP expressions in the nonneoplastic cells of the specimen were much greater than in the tumor cells. Recognizing the potential of tumor heterogeneity to skew the results of this study, we made every effort to analyze only specimens that were judged by pathologists to consist of predominantly (>80%) tumor tissue. In addition, a number of studies have found that normal cell expressions of TP are much less than those of the cancer cells (4, 17, 45, 46). In view of these data, the presence of a minor percentage of normal tissue in a specimen can only underestimate somewhat the real tumor TP expression rather than overestimate it, and thus when a high TP expression value is observed in a specimen, it very likely is a true indicator of high TP expression in the tumor cells.

The pattern of TP expressions as a function of response or nonresponse resembled that of TS expression determined previously (32). In both cases, tumors not responding to 5-FUra/LV had a much broader range of gene expressions compared with the responders, suggesting the occurrence of some event in the nonresponding tumors that confers increased genetic instability in the form of dysregulated gene expression. Similarly to the TS pattern, there was a cutoff value of TP expression (TP/β-actin ≈ 18), above which there were no 5-FUra-responsive tumors, whereas the set of tumors with expressions below the cutoff value comprised both responders and nonresponders. Thus, low TP and TS expressions do not uniformly identify responding tumors but do predict a higher response rate. However, the fact that TS and TP are not coordinately regulated provided the opportunity to test whether overall response prediction could be improved by using these two determinants simultaneously. In fact, we found that four of the nonresponding patients found previously to have TS expression below the nonresponse cutoff value of 4.1 could be positively identified as nonresponders by
their high TP expression. The response data for each individual determinant as well as both together are summarized in Table 1. As shown in Table 1, combining both determinants results in a substantially better prediction of response and nonresponse than using either gene expression separately. If these results can be confirmed in a clinical trial with a larger set of patients, data on TS and TP expressions in tumors may permit a more rational decision on whether to proceed with 5-FUra-based therapy as first-line treatment. In case of unfavorable 5-FUra response indices, the alternative drug CPT-11, which also has appreciable activity against colorectal cancer, is now available. Encouraging in this regard are preliminary results from this laboratory showing that tumors with high TS levels that have failed 5-FUra can still respond to CPT-11. Furthermore, it is anticipated that patients will be more likely to respond to CPT-11 when given as primary therapy, compared with its use after 5-FUra failure. Thus, consignment of patients for either 5-FUra or CPT-11 treatment according to their TS and TP levels may provide an effective way of increasing the overall response rate to chemotherapy using presently available agents without the need for expensive and time-consuming development of new drugs or treatments.

REFERENCES


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