Interferon Induces Thymidine Phosphorylase/Platelet-derived Endothelial Cell Growth Factor Expression in Vivo

Della Makower, Scott Wadler, Hilda Haynes, and Edward L. Schwartz

Department of Oncology, Albert Einstein Cancer Center, Albert Einstein College of Medicine, Bronx, New York 10467

ABSTRACT

The enzyme/cytokine thymidine phosphorylase/platelet-derived endothelial cell growth factor (TP/PD-ECGF) has diverse functions within cells, including the regulation of steady-state thymidine levels, the conversion of the cancer chemotherapeutic agent 5-fluorouracil (Fura) to an active metabolite, and the mediation of angiogenesis in normal and malignant cells. Although the levels of TP/PD-ECGF vary substantially among different tissues and are generally found to be elevated in tumors, little is known about the control of its expression in vivo in humans. In this study, peripheral blood mononuclear cells were obtained from patients prior to and during treatment with IFN and Fura and analyzed for TP/PD-ECGF expression. Sixteen of 21 patients (76%) exhibited an average 3-4-fold increase of TP/PD-ECGF protein levels after treatment with either IFN-α or -β, with the remaining patients having either a decrease (four patients) or no change (one patient) at the sampling times examined. Expression in vivo increased rapidly within 1–2 h of IFN treatment and remained elevated for up to 48 h after its administration. The increase in TP/PD-ECGF protein was accompanied by a concomitant increase in TP/PD-ECGF mRNA levels. TP/PD-ECGF mRNA expression in cells in vitro was induced by IFN but not by pharmacologically relevant concentrations of Fura, suggesting that the IFN was responsible for the induction seen in the patients. This study demonstrates that IFN induces TP/PD-ECGF expression in vivo by regulation of the level of mRNA expression.

INTRODUCTION

TP catalyzes the reversible synthesis of thymidine and inorganic phosphate from thymine, using deoxyribose-1-phosphate as cosubstrate (1–3). In addition to its presumed role in thymidine metabolism and homeostasis, TP also readily catalyzes the conversion of the cancer chemotherapeutic drug Fura to 5-fluoro-2-deoxyuridine; subsequent action of thymidine kinase generates the active metabolite fluorodeoxyuridylate (reviewed in Ref. 4). Transfection and elevated expression of TP in human colon carcinoma cells increased the intracellular levels of fluorodeoxyuridylate and potentiated the sensitivity of the cells to the cytotoxic actions of Fura (5). Treatment of colon carcinoma cells with IFN also increased their sensitivity to Fura in vitro (6–9), an action that has been attributed to the ability of the cytokine to increase the activity of TP (8, 9). The induction of TP activity by IFN was accompanied by increased levels of TP mRNA, suggesting that TP is a member of a family of genes, the expression of which is transcriptionally regulated by IFN (9).

TP has been reported to be identical to a tumor angiogenic factor, PD-ECGF, and the enzyme activity of TP/PD-ECGF was closely associated with its angiogenic activity (10–12). TP/PD-ECGF expression showed substantial variation in different human tissues and has been found to be increased in carcinomas of the stomach, colon, and ovary, when compared to nonneoplastic regions of these organs (13–18). Recent reports examining TP/PD-ECGF levels in biopsies of solid tumors suggested that elevated expression may have been associated with increased tumor invasiveness and metastasis and shorter survival time (in the absence of chemotherapy; Refs. 19 and 20).

Although IFN-α and -β have been shown to induce TP/PD-ECGF expression in vitro, it is not known whether these factors exert similar effects in vivo, nor has any information regarding potential mechanisms of induction been reported. In this study, we demonstrate that IFN induced a rapid and sustained increase in TP/PD-ECGF mRNA and protein levels in peripheral blood mononuclear cells obtained from patients with advanced malignancies.

MATERIALS AND METHODS

Patients and Blood Samples. The aim of this study was to determine the effects of IFN on the expression of TP/PD-ECGF. Patients were enrolled consecutively from several clinical trials using IFN-α or IFN-β treatment. All of the patients had biopsy-proven advanced gastrointestinal or head and neck malignancies and had enrolled on a clinical trial at the Albert Einstein Cancer Center. The patients all met commonly used Phase II eligibility requirements, including adequate bone marrow, renal and hepatic function, adequate performance status and nutritional status, and the presence of measurable disease. All of the patients provided informed consent.

IFN-α2a (Roche) and IFN-α2b (Schering) were administered at doses of 9 or 10 MU, respectively; one patient (patient 10) received a dose of 5 MU. IFN-β1b (Berlex) was used at a
Table 1  Effect of IFN on TP/PD-ECGF protein expression

Samples were obtained from patients with the indicated malignant diseases and who were enrolled in clinical trials in which they were to receive treatment with the indicated types and doses of IFN-α or IFN-β. All patients were also treated with FUra, and some received CDDP or HU, as indicated. A blood sample was obtained immediately prior to the first dose of IFN, and additional samples were obtained at timed intervals after the first dose of IFN; in some patients, additional samples after the fourth, fifth, or seventh doses were also obtained. The time of day when the first dose of IFN was given is indicated, when known. Mononuclear cells were purified, and extracts were prepared and analyzed by electrophoresis and immunoblotting as described in “Materials and Methods.” Detection was by chemiluminescence with Lumiphos. Films were scanned on a Molecular Dynamics scanning densitometer to determine band densities, and the ratio of the post-IFN sample:pre-IFN sample was calculated to determine the fold-change for individual patients (the post-IFN band showing the greatest change from baseline was used). NA, information not available.

<table>
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<tr>
<th>Patient</th>
<th>Primary tumor</th>
<th>IFN used</th>
<th>Dose</th>
<th>Other chemotherapy</th>
<th>Time of day for first dose of IFN</th>
<th>Fold change in TP/PD-ECGF</th>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>First dose</td>
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Fig. 1 Protein immunoblots of TP/PD-ECGF during treatment with IFN in vivo. Blood samples were obtained at the indicated times before and after administration of the first dose of IFN, as indicated. Peripheral blood mononuclear cells were isolated as described in “Materials and Methods.” Cell extracts were prepared, and 25 μg of protein were subjected to SDS-PAGE, transferred to membrane filters, and probed with anti-human TP/PD-ECGF antibody. Immunoblots were visualized by chemiluminescence after exposure of the blots to X-ray film. Patient numbers correspond to those in Table 1; purified recombinant human TP/PD-ECGF was run in Lane 11.

Preparation of Cell Extracts. Mononuclear cells were purified from whole blood by centrifugation on a Ficoll/Hypaque gradient. For protein immunoblots, cells were washed twice with PBS and suspended in 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA. The cells were sonicated on ice and centrifuged. Protein levels in the supernatant were quantitated, and the samples were stored at −70°C until analysis. For RT-PCR analysis, the cells were placed immediately in Trizol (Life Technologies, Inc.) and stored at 5°C.

Protein Immunoblots. Samples were suspended in loading buffer and were subjected to electrophoresis (25 μg protein/
density was proportional to the amount of cDNA used in the assay. An aliquot of the cDNA was used for the simultaneous amplification of glyceraldehyde-3-phosphate dehydrogenase as a control.

RESULTS

Characteristics for the 21 patients studied are summarized in Table 1. Primary tumors included carcinomas of the colon (seven patients); esophagus (five); pancreas (three); stomach (two); and head and neck, rectum, liver, and bile duct (one patient each). Four patients (patients 8, 9, 11, and 16) had been treated previously with radiotherapy and FUra, and one (patient 11) had previously received FUra and IFN-β. All of the other patients were previously untreated. Fifteen patients received IFN-α, and six received IFN-β. Four patients were treated on a Phase I protocol of FUra, HU, and IFN-α; however, one of these patients (patient 11) did not receive HU as per the protocol. Two patients were treated with CDDP, FUra, and IFN-α. One patient (patient 20) received FUra, N-(phosphonacetyl)-L-aspartate, and IFN-α.

TP/PD-ECGF was detected on immunoblots as a Mr 47,000–55,000 doublet band (Fig. 1), consistent with previous reports (21). Samples were obtained from all of the patients immediately prior to, and at least once after the first dose of IFN. In some instances, samples were obtained prior to and after subsequent doses of IFN. The majority of the specimens were run at least twice on separate gels, and the relative levels of expression measured had good reproducibility. TP/PD-ECGF was detected in all of the patients examined, with the constitutive level showing substantial variation among the different individuals. Induction of TP/PD-ECGF levels in the cells obtained from patients was observed readily in many instances with both IFN-α (patients 3, 7, and 9) and IFN-β (patient 2) treatment (Fig. 1). In other instances, TP/PD-ECGF levels decreased with IFN treatment (patient 8).

Band intensity was quantitated by densitometric analysis, and the change in expression with IFN treatment was expressed relative to the baseline pretreatment level for each individual patient. As indicated in Table 1, TP/PD-ECGF levels increased up to 8-fold with IFN therapy. Using a 2-fold or greater increase as the arbitrary cutoff for increased expression, 15 of 21 patients (71%) had an increase in expression with the first dose of IFN, and 16 of 21 patients (76%) had an increase with either the first or subsequent dose of IFN; in both cases, the frequency was statistically significant (P < 0.05 by χ² test). Four individuals (patients 5, 8, 12, and 19) had a decrease in expression of TP/PD-ECGF to 50% or less compared to pretreatment baseline, and in only one instance (patient 17) was there less than a 2-fold change in expression in either direction when analyzed at two different times of IFN administration. There was no difference in frequency of induction at the first dose between patients who received IFN-α (12 of 15) and those who received IFN-β (4 of 6), nor in the average fold increase in expression (3.64 ± 0.56 versus 2.53 ± 0.94, respectively). There appeared to be no relationship between the time of day at which samples were obtained and pretreatment TP/PD-ECGF expression or any effect of IFN on expression. All of the patients were administered IFN during the late morning to afternoon period, with two-thirds of the patients receiving IFN during a 6-h midday period. Neither visual inspection nor calculated correlation coefficient
revealed a significant relationship between time of day and either baseline TP/PD-ECGF ($r = 0.14$) or the fold increase in expression with IFN ($r = -0.27$).

Although all of the patients were being treated with combinations of IFN and FUra and other cytotoxic anticancer drugs, there was one instance in which increased expression was observed with IFN administration alone. In patient 14, a 5.5-fold increase in TP/PD-ECGF band density occurred when IFN-α was given with FUra and CDDP (first dose), and a 2.4-fold increase was observed at the fourth dose of IFN-α, which was given alone. To further address the question of whether the FUra the patients were receiving could have been responsible for the induction of TP/PD-ECGF, or alternatively, if it was capable of modulating the induction of expression by IFN, the interaction of the two agents was evaluated in vitro using HT29 colon carcinoma cells. Cells were treated with 2 μM FUra, the maximal steady-state plasma concentration of FUra observed in a series of patients treated with the same dose and schedule of FUra as in the patients in this study (22). As illustrated in Fig. 2, FUra treatment over a 48-h period did not induce TP/PD-ECGF expression; nor did FUra significantly affect the IFN-α- and IFN-β-induced increase in TP/PD-ECGF expression.

The in vivo data were also analyzed using an alternative approach so as to directly compare the levels of expression between patients both pre- and post-IFN administration. Samples that were available from 14 patients were run on gels and analyzed together; in this instance, all of the band densities were expressed in arbitrary units relative to that of the patient with the lowest level of expression (patient 10), which was set equal to 1.0 (Fig. 3). Pretreatment TP/PD-ECGF levels (Fig. 3, open columns) varied substantially between patients; nevertheless, further increases with IFN treatment (Fig. 3, solid columns) were seen in patients with both low (e.g., patients 7, 15, and 18) and high (patients 6 and 9) baseline levels.
IFN administration, increased further at 6 h, and remained elevated at an average of 3 times baseline for up to 48 h. A similar pattern was seen when only patients showing an increase in expression were examined (Fig. 4), with induction observed as soon as 1 h (the earliest time point examined) and reaching a maximal increase of 4-fold over baseline.

To evaluate the molecular basis for the increase in TP/PD-ECGF protein expression in vivo, additional samples were obtained from two patients and used for analysis of both protein by immunoblot and mRNA expression by RT-PCR (Fig. 5). TP/PD-ECGF protein levels in patient 20 increased with time after IFN-α treatment for up to 48 h. TP/PD-ECGF mRNA was not detected in this patient by RT-PCR prior to IFN treatment, was detected at low levels at 2 and 4 h, and was detected readily at 24 and 48 h similarly, in patient 21, both TP/PD-ECGF protein and mRNA levels were higher in samples obtained after IFN-α treatment than in the pretreatment sample. The TP/PD-ECGF mRNA levels in patient 20 showed a direct correlation with protein levels over the time period examined, whereas the relationship between the parameters was less consistent in patient 21.

**DISCUSSION**

Recent studies have prompted renewed interest in the enzyme/cytokine TP/PD-ECGF. As the enzyme responsible for the conversion of Fura to 5-fluorodeoxyuridine, the level of TP expression has been shown to be a determinant of the sensitivity of human colon carcinoma cells to the cytotoxicity of Fura (5). TP/PD-ECGF expression was induced in vitro by IFN under conditions in which it also increased the sensitivity of the cells to Fura (8, 9). TP/PD-ECGF expression was quite variable among different human tumors, but in several studies, TP/PD-ECGF has generally been found to be expressed at higher levels in the tumors when compared to the corresponding normal tissue (15–18). Higher levels of TP enzyme activity were also found in plasma of cancer patients when compared to levels in plasma of healthy subjects (14). In addition to its direct role in mediating Fura anabolism, the level of TP/PD-ECGF expression could influence Fura cytotoxicity via an effect on the levels of circulating thymidine, which antagonizes the actions of Fura.

TP/PD-ECGF has also been found to be an angiogenic factor (10–12), and its induction by IFN might be anticipated to have a negative impact on tumor therapy. Data suggested that higher expression of TP/PD-ECGF in tumor specimens from patients was correlated with more extensive tumor neovascularization, evidence of greater tumor aggressiveness, and shorter survival times (19, 20). Much remains to be determined, however, as to the role of TP/PD-ECGF in tumor biology and its relationship to the several other angiogenic factors that have been identified. A recent study of TP/PD-ECGF expression in breast cancer biopsies suggested that elevated expression was more likely to be associated with early tumor angiogenesis and might be less important in regulating tumor vascularity in larger, higher-grade carcinomas (20). Similarly, the actions of IFN are likely complex as the cytokine has been shown to down-regulate the expression of another angiogenic factor, basic fibroblast growth factor, in human carcinoma cell lines (23). Although the net effect on tumor angiogenesis of IFN’s actions on TP/PD-ECGF and basic fibroblast growth factor is not known, clinical studies have shown that IFN-α has an antiangiogenic effect in hemangiomas (24).

The demonstration of the up-regulation of TP/PD-ECGF by IFN treatment in the patients examined is important not only for the understanding of the actions of IFN as a therapeutic agent, but also because it suggests that IFN could have a similar function in its role as an endogenous mediator of cellular biology. In an immunohistochemical study of TP/PD-ECGF levels in normal human tissues, expression was found to be particularly high in macrophages, perhaps reflecting the colocalization of these cells at sites of wound healing and inflammation along with the cytokines and factors that mediate these processes (25). Normal stomach and colon epithelial cells were found to stain negative for TP/PD-ECGF, although fibroblasts and macrophages associated with these organs stained strongly positive (25). Although TP/PD-ECGF expression was observed in the epithelial cells of colon and other gastrointestinal carcinomas, staining was higher and more consistently found in the stromal...
components of the tumors (26). Indeed, the density of macrophages in certain solid tumors has been shown to be positively correlated with tumor angiogenesis, reflecting evidence suggesting that macrophages may mediate angiogenesis by secreting proangiogenic cytokines (27). Hence, the elevated levels of TP/PD-ECGF expression in certain solid tumors may be attributed more to the inflammatory and stromal cells than to the epithelial elements.

The increase in TP/PD-ECGF mRNA levels in cells from patients treated with IFN confirm similar observations made in human colon carcinoma cells in vitro (28). It is not known from the present studies whether the increase in TP/PD-ECGF mRNA in vivo was due to transcriptional activation of expression and/or to changes in mRNA stability; at least one other IFN-inducible gene has been shown to be subject to both transcriptional and posttranscriptional regulation (29). Recent studies have begun to define the mechanisms for the transcriptional regulation of IFN-inducible genes and have demonstrated that the IFN-mediated signal transduction pathways share similarities, and in some instances, overlap with those of other cytokines (reviewed in Ref. 30). Hence, the level of TP/PD-ECGF expression in vivo likely reflects the consequences of multiple extracellular influences.

The levels of expression of a number of enzymes involved in FUra metabolism, most notably the catabolic enzyme dihydroxypropyrimidine dehydrogenase, have been shown to be subject to circadian rhythm (31). This phenomenon was unlikely to have been a factor in the observed effect in our study, for the following reasons: (a) in a previous study, TP/PD-ECGF was the only one of four enzymes studied that did not show circadian variability (32). TP/PD-ECGF also does not show cell cycle variability in human lymphocytes in vitro, in contrast to the changes observed in thymidine kinase activity (33); (b) the blood sampling in all of the patients began within a narrow period of time in the early afternoon, and sampling began during a 6-h period in two-thirds of the patients; and (c) the comparison of a sample drawn prior to treatment with one drawn 24 h later in most of the patients minimizes any impact circadian variability would have. On the basis of the lack of effect on HT29 cells observed in vitro, it was also unlikely that the induction of TP expression was a consequence of the concurrent treatment with FUra that the patients received. This was in contrast to the increase in thymidylate synthase levels that occurred after FUra treatment (34, 35).

The sustained elevated levels of TP/PD-ECGF observed after a single injection of IFN were similar to the changes seen with this gene in vitro and with another IFN-inducible gene, 2’-5’-oligoadenylate synthetase in vivo (36, 37). These results differ, however, from the kinetics observed with other IFN-inducible genes in vitro (e.g., see Refs. 29, 38, and 39). Due to the ease of obtaining peripheral blood, the difficulty in obtaining serial tumor biopsies, and the high expression of TP/PD-ECGF in both lymphocytes and tumor tissue, TP/PD-ECGF levels were measured in peripheral blood mononuclear cells as a surrogate for TP/PD-ECGF in tumor tissue. Additional studies are planned using tumor biopsies to determine whether peripheral mononuclear TP/PD-ECGF levels do indeed reflect those in the primary and metastatic tumors. Alternatively, peripheral blood TP/PD-ECGF levels may in fact be an independent determinant of response to FUra, based on the substantial metabolism of FUra and thymidine that likely occurs in both mononuclear cells and platelets (40). Furthermore, as discussed above, some components of the peripheral mononuclear cell population may in fact reflect or indeed contribute to tumor angiogenesis (26, 27). Because TP/PD-ECGF expression may be mechanistically related to the actions of FUra, the incorporation of temporal pharmacodynamic data into the development of future clinical trials would provide for a more rational design of studies, the aim of which is the biochemical modulation of FUra antitumor activity.

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REFERENCES


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