Interleukin-6: A Potential Biomarker of Resistance to Multitargeted Receptor Tyrosine Kinase Inhibitors in Castration-resistant Prostate Cancer

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OBJECTIVE To determine if cellular interleukin-6 production predicts response to tyrosine kinase inhibitors (TKIs). As clinical experience using TKIs in patients with castration-resistant prostate cancer (CRPC) matures, Phase II trials show a heterogeneous response to sunitinib in CRPC patients. Change in serum prostate-specific antigen (PSA) level has proven unreliable for prediction of CRPC response to TKIs. Interleukin-6 (IL-6), a critical mediator of prostate cancer pathogenesis, has been shown to rise in patients with disease progression. As such, we investigated whether cellular IL-6 production can predict TKI response in both in vitro and in vivo models.

METHODS IL-6 mRNA levels and protein expression were examined by reverse transcriptase–polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. Apoptosis was examined using the terminal dUTP nick-end labeling assay. For in vivo studies, a CRPC xenograft model in C.B17/Icr-scid mice was used.

RESULTS PC-3 and DU-145 CRPC cell lines exhibited a heterogeneous response to sunitinib and pazopanib. Dose-dependent reduction of IL-6 was observed in TKI-sensitive DU-145 cells. In contrast, the TKI-resistant PC-3 cells failed to suppress IL-6 secretion. Instead, in the presence of tumor necrosis factor-alpha, IL-6 rose significantly upon administration of TKIs. Findings of in vitro experiments were confirmed in an in vivo mouse model of CRPC.

CONCLUSION Sensitivity of CRPC cells to TKIs is heterogeneous. These findings are consistent with results of recently published Phase II clinical trials using sunitinib in patients with CRPC. A substantial rise in IL-6 occurs both in vitro and in vivo in the presence of TKIs in resistant PC-3 cells but not in TKI-sensitive DU-145 cells. These findings suggest that IL-6 may represent a biomarker for TKI resistance in patients with CRPC. UROLOGY 78: 968.e7–968.e11, 2011. © 2011 Elsevier Inc.

Angiogenic inhibition using small-molecule receptor tyrosine kinase inhibitors (TKIs) represent a clinically relevant strategy for patients with advanced tumors, such as renal cell carcinoma (RCC) and gastrointestinal stromal tumors (GISTs). Since introduction of these targeted therapies, median survival for patients with metastatic RCC has increased to nearly 24 months.[1] Given their efficacy in RCC and GIST, TKIs are being tested clinically for many other solid tumors.[2]

Castration-resistant prostate cancer (CRPC) is the second leading cause of cancer death among American men.[3] Current treatment options, including docetaxel-based chemotherapy and more recently, Sipuleucel-T, have been shown to improve survival by only 2-4 months.[4,5] Furthermore, CRPC is highly vascular and possibly angiogenesis-dependent.[6] Xenograft models of CRPC in mice have confirmed sunitinib's activity against DU-145 castration-resistant prostate cancer cells.[7] More recently, initial clinical experience with sunitinib in CRPC patients has been reported.[8,9] In Phase II trials, a heterogeneous response to sunitinib was noted in both docetaxel-resistant and chemotherapy-naïve patients, with a significant proportion of tumors demonstrating at least partial radiographic response. In the most recent study of subjects with docetaxel-resistant CRPC, 11.1% showed 30% tumor reduction by Response Evaluation Criteria in Solid Tumors (RECIST), whereas another 44.4% of patients demonstrated quantifiable but less significant tumor response.[9] Notably, clinical response did

Funding Support: *This work was supported in part by National Institutes of Health, Grants RO1 CA108890 and CA134463 (VMK); American Institute for Cancer Research, Grant (RGU); Department of Defense, physician Research Training Award (AK).*

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Submitted for publication: April 18, 2011, accepted (with revisions): July 9, 2011

not correlate well with decreases in prostate-specific antigen (PSA),[8,9] indicating that PSA is a suboptimal biomarker for CRPC response to TKIs.[8-10]

An appropriate biomarker of TKI response/resistance would be relevant to future clinical trials.[10] Data from a recent Phase II trial in advanced hepatocellular carcinoma suggest that a rise in serum interleukin-6 (IL-6) levels in patients treated with sunitinib strongly correlate with rapid disease progression.[11] Furthermore, recent data from a model using Kasumi-1 acute myelogenous leukemia (AML) cells, indicate that IL-6 is able to induce sunitinib resistance through the janus kinase 2(JAK2)/signal transducer and activator of transcription 5 (STAT5) signaling pathway.[12] Because IL-6 is a critical mediator of prostate cancer pathogenesis at the molecular level[13] and has been shown to rise in patients with disease progression,[14,15] here we investigate whether cellular IL-6 production can predict TKI response in both an in vitro and an in vivo model of CRPC.

MATERIAL AND METHODS

Cells and Materials

Cell lines were obtained from ATCC (Rockville, MD). The cells were resuscitated and cultured in our laboratory for <6 months after resuscitation in RPMI 1640 medium (Bio-Whittaker, Walkersville, MD) supplemented with 10% FCS (HyClone, Logan, UT), gentamicin (50 mg/L), sodium pyruvate (1 mM), and nonessential amino acids (0.1 mM).

Reagents

Tumor necrosis factor-alpha (TNF-α) was obtained from Sigma (St. Louis, MO). Sunitinib was obtained from LC Laboratories (Woburn, MA). Pazopanib was obtained from Eton BioScience (San Diego, CA).

Measurement of IL-6

IL-6 levels in cell culture supernatants, cell lysates, and plasma samples were determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). For intracellular IL-6 assessment, cells were lysed in 1% Tween 20/phosphate-buffered saline containing a proteinase inhibitor cocktail (Roche Applied Science, Madison, WI). Protein concentrations were measured with BCA protein assay reagents (Pierce, Rockford, IL).

Real-Time Polymerase Chain Reaction Analysis

Total RNA was isolated from cells using MINI RNA isolation II Kit (Zymo-Research, Orange, CA) and purified using DNA-Free RNA Kit (Zymo-Research). Reverse transcription (RT) of 2 μg RNA was subsequently carried out using 200 U of SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA was amplified by real time polymerase chain reaction using IL-6 TaqMan Gene Expression Assay (ID# Hs00174131_m1).

GAPDH Gene Expression Assay (ID# Hs99999905_m1) was used as an endogenous control. Each sample was run in triplicate using TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Reactions were carried out in an Applied Biosystems 7500 Real-Time PCR System. Analysis of IL-6 expression was carried out using the 2(-Delta Delta C(T)) method ($2^{-\Delta\Delta Ct}$).

Luciferase Reporter Assay

Cells were transfected with pNF-κB-luc (Stratagene, La Jolla, CA) and ether GFP (Clontech, Mountain View, CA) (sunitinib experiments) or pRL-TK (Promega, Madison, WI) (pazopanib experiments) plasmids. GFP and pRL-TK plasmids were used to monitor transfection efficacy. Transfections were performed using TransIT-Prostate transfection kit (mirus Bio, Madison, WI). Twenty-four hours after transfection, cells were treated with either sunitinib or pazopanib for 3 hours followed by treatment with TNF-α (20 ng/mL) for an additional 4 hours. Samples were assayed for firefly and *Renilla* luciferase activities using the Dual-Glo Luciferase assay System (Promega) and normalized as instructed by the manufacturer. GFP expression was assessed using a BioTek (Winooski, VT) microplate fluorimeter with excitation and emission filters of 485/20 and 528/20 nm, respectively.

Measurement of Apoptosis

DNA fragmentation was detected using APO-BRDU kit (The Phoenix Flow Systems, Inc., San Diego, CA).

In vivo Studies

For in vivo experiments, 6-week-old male C.B17/Icr-scid mice (n = 5 mice per group) were inoculated intraperitoneally with 5 × 106 PC-3 cells using a 27-gauge needle. All animal procedures were done according to local guidelines on animal care and with appropriate institutional certification. Ten days after tumor cell inoculation, animals were given sunitinib p.o. (40 mg/kg) followed by an i.v. injection of TNF-α (0.1 mg/kg) into the tail vein. Three hours after TNF-α injection, blood was collected from the retro-orbital plexus under anesthesia from both experimental and control groups. IL-6 levels in plasma samples were examined by ELISA.

RESULTS

Concomitant treatment with sunitinib and TNF-α resulted in apoptosis in DU-145 cells, while PC-3 cells were resistant (Fig. 1A). A dose-dependent reduction of IL-6 levels in sunitinib-treated DU-145 cell lines was observed (Fig. 1B). Meanwhile, the sunitinib-resistant PC-3 cells did not only fail to suppress IL-6 excretion in the presence of sunitinib, but coadministration of sunitinib and TNF-α resulted in IL-6 levels rising more than 5-fold when compared with IL-6 levels from cells treated with TNF-α alone (Fig. 1C). Similar results were seen with the TKI pazopanib (Fig. 1D). Higher levels of IL-6 protein were also documented intracellularly by ELISA when cells were treated with sunitinib or pazopanib in the presence of TNF-α (Fig. 1E).

Despite the rise in IL-6 protein levels in PC-3 cells, both IL-6 mRNA levels (Fig. 2A) and NF-κB activity are suppressed in both PC-3 cells (Fig. 2B) and DU-145 cells (data not shown). Addition of IL-6 at 100 ng/mL to the cell culture medium did not induce sunitinib resistance in the TKI-sensitive DU-145 cell line (Fig. 3A). Furthermore, the addition of neutralizing IL-6 antibody (10-fold

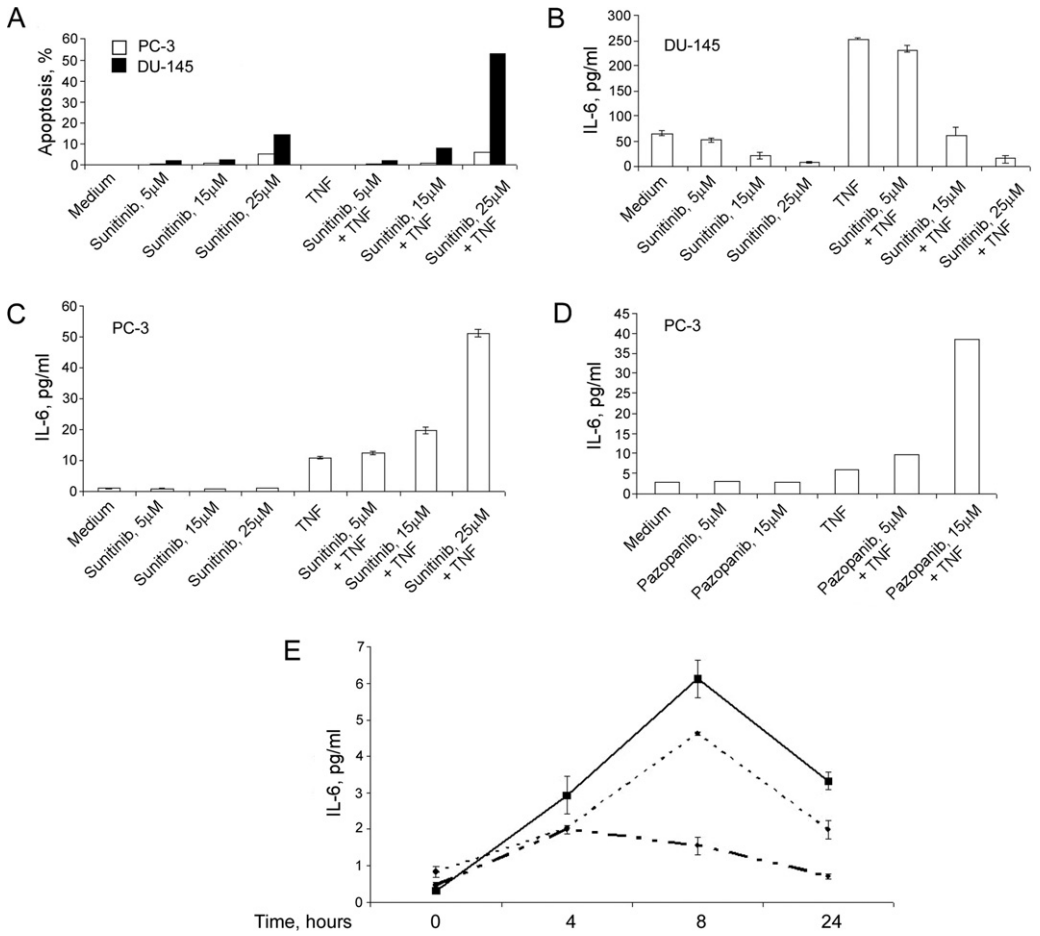

Figure 1. **(A)** Assessment of apoptosis using the terminal dUTP nick-end labeling (TUNEL) assay in PC-3 and DU-145 cells. **(B)** IL-6 protein levels in cell culture supernatant of DU-145 cells treated with sunitinib. **(C)** IL-6 levels in cell culture supernatant of PC-3 cells treated with sunitinib. **(D)** IL-6 protein levels in cell culture supernatant of PC-3 cells treated with pazopanib. *(E)* Intracellular IL-6 protein levels in PC-3 cells treated with either sunitinib and TNF-α (*solid line*), pazopanib and TNF-α (*dashed line*), or TNF-α only (*heavy dashed line*).

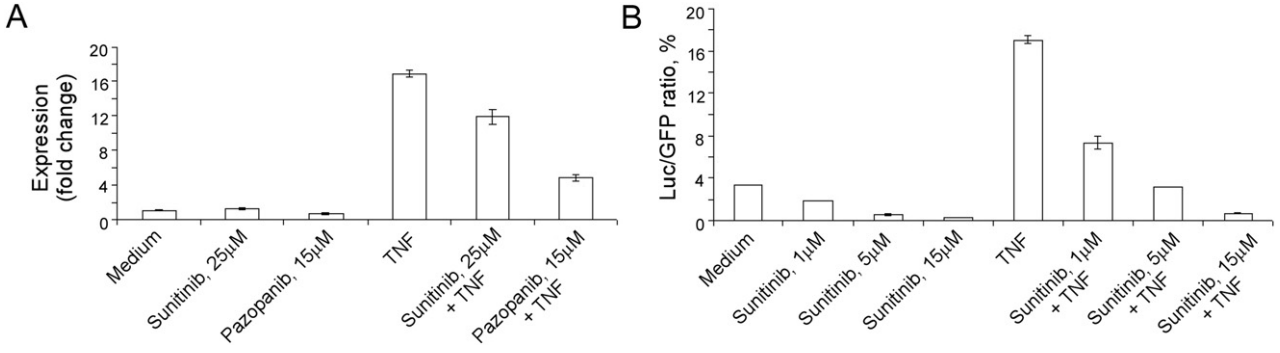

Figure 2. **(A)** IL-6 mRNA levels in PC-3 cells treated with sunitinib. **(B)** The effect of sunitinib on the NF-κB transcriptional activity in PC-3 cells.

excess of concentration sufficient to induce 50% inhibition of biological activity) failed to restore sensitivity of PC-3 cells to sunitinib (Fig. 3B).

In an in vivo mouse model in male C.B17/Icr-scid mice with diffuse peritoneal implants, after injection with TNF-α, statistically significant higher plasma levels of IL-6 were present in mice who received oral sunitinib (40 mg/kg) compared with mice who did not (Fig. 4).

COMMENT

Castration-resistant prostate cancer cell lines PC-3 and DU-145 respond differently to *in vitro* sunitinib treat-

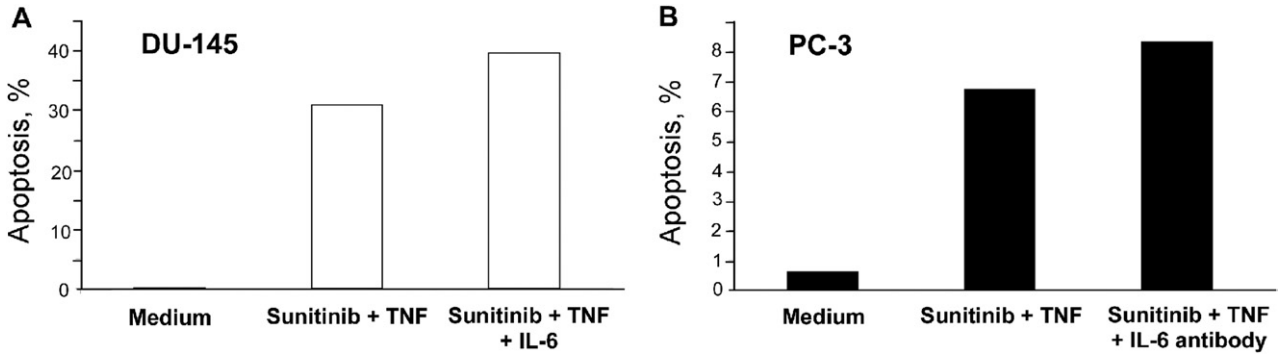

Figure 3. **(A)** Administration of IL-6 failed to rescue DU-145 cells from sunitinib-mediated apoptosis. **(B)** Administration of IL-6 antibody did not sensitize sunitinib-resistant PC-3 cells to sunitinib and TNF-α. Degree of apoptosis was assessed by the TUNEL assay. Representative data from 1 of 3 experiments is shown.

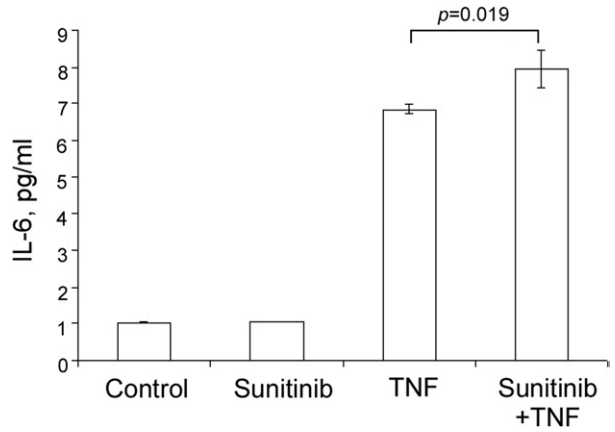

Figure 4. Plasma IL-6 levels in a PC-3 xenograft C.B17/Icr-scid mouse model. Ten days after mice were inoculated intraperitoneally with PC-3 cells, animals were given sunitinib p.o. (40 mg/kg) followed by an i.v. injection of TNF-α (0.1 mg/kg). Three hours after TNF-α injection, blood was collected from the retro-orbital plexus. IL-6 levels in plasma samples were examined by ELISA.

ment. Concomitant treatment with sunitinib and TNF-α induced significant apoptosis in DU-145 cells, whereas PC-3 cells were resistant to such conditions (Fig. 1A). TNF-α was used to simulate in vivo conditions, because TNF-α levels are elevated in prostate cancer tumors in situ[15] and in the serum of CRPC patients,[16] but not in human prostate cancer cell line supernatants.[17] Moreover, TNF-α serum levels appear elevated in patients with metastatic RCC who progress on sunitinib therapy.[18]

We demonstrated a dose-dependent reduction of IL-6 levels in sunitinib-treated DU-145 cell lines (Fig. 1B). Similar reduction in IL-6 levels was observed in the CA-HPV-10 prostate cancer cell line. In contrast, the sunitinib-resistant PC-3 cells failed to suppress IL-6 excretion in the presence of sunitinib. Moreover, when sunitinib was coadministered with TNF-α, IL-6 levels rose more than 5-fold when compared with IL-6 levels from cells treated with TNF-α alone (Fig. 1C). Comparable results were noted with pazopanib (Fig. 1D), which is a novel TKI that is currently in early clinical trials for treatment of CRPC.[19] Similar to the rise of IL-6 in the extracellular supernatant, higher levels of IL-6 protein were observed intracellularly. ELISA revealed rising intracellular IL-6 levels over an 8-hour period with TNF-α stimulation in the presence of either sunitinib or pazopanib (Fig. 1E).

In an attempt to understand the mechanisms involved in IL-6 elevation, we investigated IL-6 mRNA levels and the status of NF-κB activity in the presence of TKIs. We showed that although protein levels rise in PC-3 cells, both IL-6 mRNA levels (Fig. 2A) and NF-κB activity, a known regulator of IL-6 synthesis, are suppressed in both TKI-resistant PC-3 cells (Fig. 2B) and TKI-sensitive DU-145 cells.[20] Nishioka et al recently showed that IL-6 expression is induced in AML cells in the presence of sunitinib. The authors present evidence that this finding is a result of increased binding of c-Jun to the IL-6 promoter.[12] Induction of IL-6 expression appears to proceed by a different mechanism in CPRC. Unlike in the AML model, IL-6 mRNA levels of CPRC are suppressed in the presence of TKIs (Fig. 2A). These data indicate that IL-6 upregulation in TKI-resistant CRPC occurs at a post-transcriptional level. Furthermore, IL-6 appears to be a marker of TKI resistance and not its cause. Addition of IL-6 to the cell culture medium did not induce sunitinib resistance in the TKI-sensitive DU-145 cell line (Fig. 3A). Furthermore, unlike in the AML model,[12] addition of the neutralizing IL-6 antibody failed to sensitize PC-3 cells to sunitinib-mediated apoptosis (Fig. 3B).

Next, we set out to assess whether results of in vitro experiments could be replicated using the in vivo mouse model of human prostate cancer. Indeed, we were able to reproduce our findings in male C.B17/Icr-scid mice with diffuse peritoneal implants of PC-3 cells. After injection with TNF-α, statistically significantly higher plasma levels of IL-6 were present in mice who received oral sunitinib (40 mg/kg) compared with mice who did not (Fig. 4).

One of the main limitations of this study is the required exogenous administration of TNF-α both in vitro *and* in vivo. Although the use of TNF-α was necessary to simulate clinical conditions, it could potentially be a

confounding variable despite adequate controls. Furthermore, despite the promising results of these in vitro *and* in vivo data, the translation of IL-6 to clinical practice still remains a challenge. Although our experiments appear to establish IL-6 as a marker of treatment efficacy, these findings need to be validated in human studies.

CONCLUSIONS

The sensitivity of CRPC cells to multitargeted TKIs is heterogeneous. These findings are consistent with recently published Phase II trials.[8,9] Here we demonstrate that a substantial rise in IL-6 levels occurs both in vitro and in vivo in the presence of TKIs in the resistant PC-3 cells but not in the TKI-sensitive DU-145 cells. These findings suggest that IL-6 may be a valuable biomarker of resistance to multitargeted receptor TKIs in patients with CRPC.

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