ABSTRACT

Tumor tissues include malignant cells and a stroma made up of mainly inflammatory cells, endothelial cells, and fibroblasts. To differentiate the effects of treatment on angiogenic cytokine secretion in tumor tissue, exponential and stationary phase human CaKi-1 renal cell carcinoma cells, human SW2 small cell lung carcinoma cells, human umbilical vein endothelial cells (HUVECs), murine NIH-3T3 fibroblasts, and murine RAW264.7 macrophages were exposed to gemcitabine, paclitaxel, carboplatin, and the protein kinase Cβ inhibitor LY317615, and secretion (24 h) of tumor necrosis factor-α, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and transforming growth factor (TGF)-β was determined by a Luminex FlowMetrix assay. After 72 h of exposure, exponential RAW, 3T3, and SW2 cells were sensitive to gemcitabine; exponential and stationary SW2 and HUVECs were sensitive to paclitaxel; and exponential and stationary HUVECs were most sensitive to LY317615. None of the cells secreted detectable tumor necrosis factor-α. Generally, exponentially secreted cells secreted higher levels of cytokines than stationary cells (exponential cells secreted ~10 times less TGF-β). Only malignant cells secreted VEGF (80–300 pg/10⁶ cells). VEGF secretion by exponential SW2 cells decreased in an anticancer agent concentration-dependent manner. Every cell type secreted TGF-β at levels comparable with human, carboplatin, paclitaxel, and the protein kinase Cβ inhibitor LY317615.

INTRODUCTION

Similar to normal tissues, solid tumors have a structure that includes a parenchyma and a stroma. In solid tumors, the parenchyma is composed of neoplastic cells. The stroma induced by neoplastic cells and in which these cells are dispersed is essential for tumor growth. The stroma, composed of nonmalignant supporting tissues, includes connective tissue, blood vessels, and frequently inflammatory cells (1–6). Thus, malignant tumor tissue is composed of many cell types, including blood vessels composed of endothelial cells, infiltrating macrophages, and fibroblasts. Although whole tumor response to therapy has been intensively investigated, less is known about the contribution of the various cell types to response to treatment or to growth factor and cytokine production and how these change in response to treatment.

Angiogenesis is the formation of new blood vessels from preexisting vascular networks. Tumor-induced angiogenesis is essential for continued tumor growth and metastasis formation (7–9). Numerous angiogenic factors that regulate this complex process have been identified. VEGF² is a potent, multifunctional, and endothelial cell-specific growth factor. It stimulates vasodilation and cell proliferation, increases permeability and migration, and promotes endothelial cell survival (10, 11). Other cytokines promoting angiogenesis include TGF-β, TNF-α, and bFGF (12).

Plasma levels of multiple cytokines can be determined in one small volume sample through Luminex FlowMetrix technology (13–16). The current study examines cytokine production by exponentially growing and stationary phase human SW2 SCLC, human CaKi-1 renal cell carcinoma cells, HUVECs, murine RAW264.7 macrophages, and murine NIH-3T3 fibroblasts after exposure to gemcitabine, carboplatin, paclitaxel, and the protein kinase Cβ inhibitor LY317615.

MATERIALS AND METHODS

Drugs. Carboplatin and paclitaxel were purchased from Sigma Chemical Co. (St. Louis, MO). Carboplatin was prepared as 125 mM stock in PBS. Gemcitabine was prepared as 5 mM stock in PBS. Paclitaxel was prepared as 20 µM stock in DMSO. LY317615 was prepared as 25 mM stock in DMSO. Stock solutions were stored at −20°C until use.

Cell Lines. The human SW2 SCLC cell line originated from the pleural fluid of a patient with SCLC in 1979 (17, 18). The human CaKi-1 renal cell carcinoma cell line, the murine RAW264.7 macrophage line, and the murine NIH-3T3 fibroblast cell line were purchased from American Type Culture Collection (Manassas, VA). HUVECs were purchased from BioWhittaker Technologies (Walkersville, MD). The human SW2 SCLC were grown in RPMI 1640 supplemented with 0.2% sodium bicarbonate and 10% fetal bovine serum. HUVECs were maintained in endothelial growth medium (BioWhittaker Technologies). The human CaKi-1 cells were maintained in McCoy’s medium supplemented with 10% fetal bovine serum. The murine RAW264.7 cells were grown in RPMI 1640 containing 10% fetal bovine serum. The murine NIH-3T3 cells were maintained in DMEM containing 10% fetal bovine serum. All media contained 1% penicillin-streptomycin (Life Technologies, Grand Island, NY).

Cell Growth Inhibition Assay. The growth of each cell line was determined after exposure of the cells to various concentrations of LY317615 (0, 0.61, 1.85, 5.55, 16.6, and 50 µM), carboplatin (0, 3.0, 9.25, 27.7, 83.3, and 250 µM), gemcitabine (0, 0.123, 0.37, 1.11, 3.33, and 10 µM), or paclitaxel (0, 0.617, 1.85, 5.55, 16.66, and 50 nM) for 72 h. For the exponential growth phase experiments, 5 × 10³ cells were plated in 200 µL of medium supplemented as described above in a 96-well plate in triplicate and incubated at 37°C in a humidified atmosphere of 5% CO₂. The next day, the compounds were added over a concentration range in DMSO or PBS, and the cultures were incubated for 72 h. After 48 h, the supplemented medium was replaced with serum-free medium for the final 24 h. For the stationary growth phase experiments, 5 × 10³ cells were plated for CaKi-1, SW2, RAW264.7, and NIH3T3 and 1.5 × 10⁴ cells for HUVECs into 96-well plates. For the stationary phase cells, the cells were incubated in complete medium for 72 h to produce confluent cultures. The exponential phase cells were plated once the stationary phase cells were confluent. The next day, the compounds were added over a concentration range in DMSO or PBS and incubated for 72 h. After 48 h, the medium was replaced with serum-free medium for the final 24 h. At 72 h, WST-1 tetrazolium salt solution (Roche Diagnostics, Indianapolis, IN; 20 µl) was added to each well. One h later, the absorbance of each well at 440 nm
using a SpectraMax 250 (Molecular Devices Corp., Sunnyvale, CA) was determined. The data are presented as growth fraction defined as the fraction of the WST-1 absorbance compared with untreated control cultures ± SE for three independent experiments.

**Growth Factor and Cytokine Determinations.** Parallel 96-well plates were prepared as described above and were used for the multiplex assay analysis for the measurement of VEGF, TNF-α, bFGF, and TGF-β. The multiplex kits were custom designed in-house and manufactured by R&D Systems (Minneapolis, MN). The medium was collected from triplicate wells and stored at −80°C until analysis. Each 96-well filter plate (Millipore, Bedford, MA) was blocked with 100 μl of blocking buffer for 30 min and filtered by vacuum at 2 psi. The 25× multiplex bead mix was mixed using a vortex mixer for 1 min and then mixed using a sonic mixer for 30 s before diluting. The bead mix was diluted in wash buffer, and 50 μl were immediately added to each well. Wash buffer was filtered through vacuum at 2 psi.

Each standard was dissolved in medium supplied in kits. The standards and samples were added to the filter plates containing the bead mix in 50 μl in triplicate. To measure TGF-β, 250 μl of sample were acidified with 50 μl of 1 N HCl and incubated for 10 min. The samples were neutralized with 50 μl of 1.2 N NaOH/0.5 M HEPES and further diluted with RDzM for a 1:4 dilution. The beads are light sensitive, and therefore the plates were covered with foil. The plates were placed on a shaker at 4°C overnight to allow binding of growth factors to the bead-bound antibodies. The next day, the medium was vacuum filtered at 2 psi, and 50 μl of detection antibody were added to each well. The wells were washed four times with 100 μl of wash buffer. Then, the plates were incubated on a shaker at room temperature for 1 h in the dark. The plates were again washed four times with 100 μl of wash buffer. Fifty μl of streptavidin-phycocerythrin were then added to each well. The plates were shaken for 15 min at room temperature in the dark. The wells were washed four times with 100 μl of wash buffer. The beads were then resuspended in 150 μl of wash buffer for analysis. Plates were wrapped in aluminum foil and maintained at 4°C until analysis. Immediately before analysis, the plates were again shaken to ensure complete resuspension of beads. The fluorescence intensity of the detection antibody was determined using a BioPlex System (Bio-Rad Laboratories, Hercules, CA). Fluorescence intensity readings for 100 beads/cytokine were collected for each standard and sample dilution. The lower limit of detection for each assay is 5.6 pg/ml. The standard curves for three independent experiments for each of the four cytokines are shown in Fig. 1.

**RESULTS**

The growth inhibition for each cell type after 72-h exposure to a concentration range of gemcitabine, paclitaxel, carboplatin, or LY317615 was determined for cells in exponential growth and for cells in stationary phase. The human CaKi-1 renal cell carcinoma cells were overall most resistant to each of the agents (Fig. 2). Gemcitabine (10 μM), paclitaxel (50 nM), and carboplatin (250 μM) exposure did not reach an IC50 for exponentially growing or stationary phase CaKi-1 cell proliferation inhibition. However, exposure of exponentially growing CaKi-1 cells to LY317615 produced an IC50 of 30 μM, whereas stationary phase CaKi-1 cells were unaffected by LY317615 concentrations up to 50 μM. The human SW2 SCLC cells responded to each of the four agents tested, with similar effects in exponentially growing cells and stationary phase cells. The IC50s for SW2 cells exposed to gemcitabine and paclitaxel were 2–4 and 3 μM, respectively. For carboplatin and LY317615, the IC50s for exponentially growing and stationary phase SW2 cells were 120 and 20 μM, respectively. The normal exponentially growing HUVECs were especially responsive to paclitaxel and LY317615. Paclitaxel decreased cell proliferation in HUVECs, producing an IC50 of 3 nM, whereas the IC50 after exposure to LY317615 was 6 μM. HUVECs were more sensitive to paclitaxel and LY317615 than to either of the tumor cell lines; however, HUVECs were not very responsive to gemcitabine or carboplatin. Exponentially growing and stationary phase NIH3T3 cells were relatively resistant to LY317615, carboplatin, and paclitaxel. The exponential phase NIH-3T3 cells were responsive to gemcitabine with an IC50 of 0.5 μM. Exponentially growing murine RAW264.7 macrophages were responsive to gemcitabine with an IC50 of 0.1 μM, to paclitaxel with an IC50 of 25 nM, to carboplatin with an IC50 of 120 μM, and to LY317615 with an IC50 of 20 μM. Stationary phase RAW264.7 cells reached an IC50 of 2.8 μM when exposed to gemcitabine but did not reach an IC50 after exposure to paclitaxel, carboplatin, or LY317615.

The secreted levels of each cytokine (VEGF, bFGF, TNF-α, and TGF-β) into cell culture medium (24 h) were determined de novo and during exposure to each agent (Figs. 3–5). Only the malignant cells, CaKi-1 renal cell carcinoma cells and SW2 SCLC cells, secreted detectable levels of VEGF in cell culture. Exponentially growing CaKi-1 and SW2 cells secreted similar amounts of VEGF into culture medium (200–250 pg/106 cells; Fig. 3). In each cell line, the exponentially growing cells produced 2-fold higher levels of VEGF than the stationary phase cells. There were only small changes in the VEGF secreted by the exponentially growing or stationary phase CaKi-1 cells after exposure to each agent. VEGF production by exponentially growing SW2 cells decreased in a concentration-dependent manner.

![Fig. 1. Standard curves for Luminex multiplex assay for VEGF, bFGF, TGF-β and TNF-α. Bars, SE for 10 individual curves. The limit of detection is 5.6 pg/ml.](cancerres.aacrjournals.org)
when exposed to gemcitabine, paclitaxel, or LY317615, with VEGF concentrations decreasing to 60, 10, and 60 pg/10^6 cells, respectively. The decrease in VEGF levels occurred at the two highest concentrations of gemcitabine and LY317615. There was a marked decrease in VEGF secretion after exposure to paclitaxel, although the decrease in SW2 cell proliferation was similar after exposure to paclitaxel and gemcitabine. There was no decrease in VEGF secretion from stationary phase SW2 cells under the conditions tested.

All cells types studied secreted TGF-β in cell culture (Fig. 4). Exponentially growing CaKi-1 and SW2 cells secreted similar amounts of TGF-β (near 800 pg/10^6 cells in 24 h). These TGF-β levels were near 7-fold higher and 15-fold higher than those secreted by stationary phase CaKi-1 cells and stationary phase SW2 cells, respectively. The HUVECs also secreted high levels of TGF-β, with the exponentially growing cells producing 325 pg/10^6 cells and stationary phase HUVECs producing 220 pg/10^6 cells. There were no
observed changes in TGF-β secretion by the CaKi-1 renal cell carcinoma cells or the SW2 SCLC cells after exposure to each of the agents. Small decreases in TGF-β levels were seen in exponential growing HUVECs after exposure to paclitaxel and LY317615, decreasing to 200 pg/10⁶ cells, in good agreement with the growth-inhibitory effects of paclitaxel and LY317615 in HUVECs. Exponentially growing RAW264.7 cells and exponentially growing NIH-3T3 cells secreted similarly high levels of TGF-β of ~1000 pg/10⁶ cells. Stationary phase RAW264.7 cells and stationary phase NIH-3T3 cells secrete ~10-fold less TGF-β than do the exponentially growing cells.

SW2 SCLC cells, CaKi-1 renal cell carcinoma cells, and HUVECs secreted measurable levels of bFGF. The RAW264.7 cells and NIH-3T3 cells did not secrete measurable bFGF (Fig. 5). Both exponent-
tially growing and stationary phase CaKi-1 cells secreted similarly high levels of bFGF in culture of 20 pg/10⁶ cells. Both exponentially growing and stationary phase HUVECs secreted ~8 pg/10⁶ cells. SW2 cells secreted less bFGF (2.5 pg/10⁶ cells). Unlike VEGF and TGF-β, the amount of bFGF secreted was very similar in exponentially growing and stationary phase cells. An increase in bFGF secretion was observed upon exposure of exponentially growing CaKi-1 cells to gemcitabine, paclitaxel, and LY317615 with values at 50, 40, and 70 pg/10⁶, respectively. Production of bFGF by stationary phase CaKi-1 cells was unchanged by exposure to the agents. The bFGF secretion by HUVECs was unchanged by exposure to the agents. In contrast to the effects on bFGF production in CaKi-1 cells, paclitaxel exposure caused a concentration-dependent decline in bFGF secretion by exponentially growing SW2 cells from 2.5 to 0.75 pg/10⁶ cells. The other agents had little effect on the secretion of bFGF by HUVECs.

DISCUSSION

Tumors are complex tissues with varied interacting cells within the mass as well as networks of interaction with the host in general. The malignant cells initiate and sustain the tumor; however, “normal” stromal cells are active participants in the malignant growth process. In many, if not most tumors, stromal cells outnumber malignant cells (1–5). Solid tumors and slow-growing lymphomas have low growth fractions and are likely to contain large populations of noncycling cells. In cell culture, these noncycling cells can be modeled using stationary phase cultures containing a large fraction of noncycling but potentially clonogenic cells (18–21). This study examines the potential contribution of several cell types to angiogenic cytokine secretion under proliferating and nonproliferating conditions and in response to treatment. The cells used to model the stromal compartment of the tumor were well-established cell lines, NIH-3T3 fibroblasts and RAW264.7 macrophages, or well-differentiated normal cells, HUVECs. It is likely that in the microenvironment of the malignant disease process, the stromal cells may be induced to express a more dedifferentiated phenotype. The current study focuses on the response of each stromal cell type to the stress produced by exposure to each chemotherapeutic agent. It is anticipated that a similar biological response to chemotherapy-induced stress would be elicited from NIH-3T3 fibroblasts and tumor fibroblasts, from RAW264.7 macrophages and tumor macrophages and from HUVECs and tumor endothelial cells to make the results of the study meaningful. For all cell types studied, exponentially growing cells were either more sensitive or equally as sensitive to each treatment than were stationary phase cells. It is well known that stationary cells are less sensitive to most anticancer agents than exponentially growing cells, with the recognized exceptions being bleomycin and carmustine, which are more toxic toward stationary phase cells (22–24). Metastatic renal cell carcinoma remains one of the most treatment-resistant malignancies in humans and is highly resistant to systemic therapy (25–27). No chemotherapeutic agent is considered a standard of care for treatment of metastatic renal disease (26, 27). CaKi-1 renal cell carcinoma cells were most resistant to each of the four agents. Interestingly, RAW264.7 macrophages and NIH-3T3 fibroblasts were quite sensitive to gemcitabine, whereas HUVECs were more sensitive to paclitaxel and LY317615.

In general, cells maintained cytokine production in the face of treatment, although cell growth fraction declined upon exposure to these agents. For all cases, exponentially growing cells secreted higher amounts of VEGF and TGF-β than stationary phase cells, in agreement with the notion that cell populations with a greater proliferative thrust in vivo require more rapid production of vasculature. VEGF secretion came only from the tumor cells, CaKi-1 renal cell carcinoma cells and SW2 SCLC cells. Consistent with this finding, nude mice bearing s.c. SW2 or CaKi-1 tumors produce high plasma levels of VEGF, whereas non-tumor-bearing mice have no detectable levels of VEGF in circulation. We have found that circulating levels of VEGF in human tumor-bearing nude mice range between 5 and 200 pg/ml (n = 12), and that plasma VEGF levels in mice bearing SW2 or CaKi-1 tumors were 100–200 pg/ml (28). The circulating levels of bFGF in nude mice bearing human tumor xenografts ranged between 1 and 50 pg/ml, and animals bearing SW2 and CaKi-1 tumors had plasma levels of 9–20 pg/ml. The TGF-β plasma levels in nude mice bearing human tumor xenografts ranged between 20 and 60 ng/ml, and animals bearing SW2 and CaKi-1 tumors had TGF-β plasma levels of 40–50 ng/ml. In the current cell culture study, the growth factor levels measured were within the scope of levels measured in tumor-bearing animals.

Marked decreases in the secretion of VEGF by the SW2 SCLC cells were seen after exposure to relatively high concentrations of paclitaxel, LY317615, and to a lesser extent, gemcitabine. There was no apparent relationship between the agent causing the greatest growth inhibition of SW2 cells and those resulting in the largest decrease in VEGF secretion by the cells. Thus, especially in the case of LY317615, the decrease in VEGF secretion is likely a result of the mode of action of the compound. Interestingly, although the growth inhibition by paclitaxel was the same for both exponentially growing and stationary phase SW2 cells, VEGF secretion only decreased from the exponentially growing cells.

At higher concentrations, both paclitaxel and LY317615 were quite growth inhibitory toward HUVECs. The secretion of TGF-β by cells was only noticeably decreased (~2-fold) in HUVECs exposed to higher concentrations of paclitaxel and LY317615. Changes in TGF-β secretion did not occur in any other condition, despite growth inhibition of the cells.

Interestingly, although the CaKi-1 renal cell carcinoma cells were only slightly growth inhibited by exposure to the agents, when in exponential growth, CaKi-1 cells increased secretion of bFGF (except carboplatin) when exposed to treatment.

Inhibition of the secretion or activity of angiogenic cytokines may result in tumor growth delays and be a component in the response of tumors to treatment. Surprisingly, only the exponentially growing SW2 SCLC cells responded to therapy by decreasing secretion of an angiogenic cytokine. Other cell types maintained secretion of the angiogenic cytokines through therapy. Under the experimental conditions, no cells secreted TNF-α, only malignant cells secreted VEGF, only malignant cells and HUVECs secreted bFGF, and all of the cells secreted TGF-β. Although VEGF and bFGF may be more selective for cancer, TGF-β may be a more universal player in tumor tissue. Further exploration of the role of stromal cells in tumor growth and progression is warranted.

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An *In Vitro* Tumor Model: Analysis of Angiogenic Factor Expression after Chemotherapy

Kristan Keyes, Karen Cox, Patti Treadway, et al.


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