Taxol-dependent Transcriptional Activation of IL-8 Expression in a Subset of Human Ovarian Cancer

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ABSTRACT

Taxol is important in the treatment of both primary and drug-resistant ovarian cancer. Although Taxol is known to stabilize microtubules and block cell mitosis, the effectiveness of this drug exceeds that of other antimitotic agents, suggesting it may have an additional mode of action. Stimulation by murine macrophage studies indicating cytokine induction by Taxol, we have investigated proinflammatory cytokine expression in a series of cell lines and recent explants of human ovarian cancer. Taxol induced secretion of interleukin (IL) 8 but not IL-6, IL-1α, or IL-1β in 4 of 10 samples. Induction was dependent on transcriptional activation, and, in contrast to murine macrophage studies, was apparently independent of an active lipopolysaccharide signaling pathway. Confluent cultures secreted as much IL-8 as proliferating cells. Taxol did not induce IL-8 in breast carcinoma, endometrial stromal, or T-lymphocyte or monocyte cultures. We propose that the local expression of this chemokine in vivo may elicit a host response similar in effectiveness to that of cytokine gene therapy. These data are the first to suggest that a chemotherapeutic agent may have a direct effect on transcription of cytokine and/or growth factor genes in ovarian cancer, and that this effect may not be restricted to proliferating tumor cells.

INTRODUCTION

Taxol is the prototype of a new class of drugs with promise in the treatment of several epithelial cancers, including breast, ovary, lung, and colon (1, 2). Taxol, a microtubule stabilizing agent (3, 4), has antimitotic and apoptosis-inducing activity (5), but the therapeutic effect appears to be greater than that of microtubule-disrupting drugs such as vinblastine and colchicine, suggesting that Taxol and recently identified variants such as taxotere, have an additional mode of activity in addition to mitotic arrest. The results of early Phase I trials of Taxol have shown a sporicad influence of Taxol, and Phase II studies were restricted to renal, melanoma, and ovarian cancers (1). The ovarian data were particularly interesting, with 30 and 36% response rates seen with otherwise refractory patients (6, 7). The most promising aspect of these early ovarian studies was the efficacy in platinum-resistant patients, suggesting that Taxol is functioning via a pathway, in part, distinct from that of direct DNA-damaging agents. Subsequently, Holmes et al. (7) carried out a Phase II trial in breast cancer with even more encouraging results: a 56% response rate in metastatic breast cancer. Neutrophils appears to have been the major side effect encouraging the combined therapy with granulocyte colony-stimulating factor (8). In breast and ovary, the available data suggest a lack of cross-reactivity with either doxorubicin or platinum (9).

Several reports have shown that in mice Taxol is a potent stimulator which can induce a series of LPS-dependent macrophage-derived cytokines such as IL-1α, IL-1β, TNF-α, and IP-10 (10–15). Macrophages from C3H/HeJ mice which carry a mutation in the LPS3 gene fail to activate microtubule-associated protein kinase and are unable to either produce TNF-α or down-regulate TNF-α receptors in response to LPS or Taxol (10, 16). These data demonstrate that Taxol and LPS may share a functionally important signal transduction pathway. Curiously, human monocytes and macrophages appear to be refractory to Taxol (15). The experiments reported below demonstrate that Taxol induces IL-8 transcription and secretion but not other cytokines such as IL-1α, IL-1β, or IL-6 in long-term human ovarian cancer cell lines as well as ovarian cells recently explanted from tumors. In contrast, Taxol did not induce IL-8 synthesis in any breast carcinoma lines, normal T lymphocytes, or monocytes. The induction of IL-8 in both proliferating and confluent cultures suggests that in vivo Taxol responsiveness may depend on direct tumor cell destruction as well as modification of the local tumor environment through release of IL-8, a cytokine with proinflammatory and growth-modulating properties.

MATERIALS AND METHODS

Taxol Treatment of Carcinoma Cell Lines. Initial studies were carried out with four ovarian cancer cell lines (OVCA 420, 429, 432, and 433) which have been established in Dr. Robert Bast’s laboratory and the origins of which have been described previously (17, 18). Cells were seeded in 24-well plates (Costar, Inc.) and grown in DMEM media supplemented with endotoxin-free 10% fetal bovine serum until 60–75% of confluence. Immediately before use, the medium was removed, the cells were washed once with sterile endotoxin-free PBS, and then treated with Taxol or the vehicle, DMSO (or methanol in some experiments), as indicated. Stock Taxol was maintained at −70°C at a concentration of 50 mg/mL. The final concentration of DMSO never exceeded 0.1%. At various times after treatment, culture supernatants were removed for ELISA analysis, or cells were treated for RNA isolation as described below. Confluent cultures were obtained as described above, except cells were allowed to cease proliferation as judged by the lack of mitotic figures and plate density. Cultures were then left for an additional 2 or 5 days prior to Taxol exposure. Although subconfluent cultures exhibited numerous mitotic cells following Taxol exposure, examination of confluent cultures by phase-contrast microscopy indicated the entire plate was a homogeneous monolayer in which mitoses were entirely absent following exposure to Taxol. Six recently explanted ovarian cancer cell lines were cultured in 24-well plates (Corning) and grown in RPMI 1640 supplemented with 15% fetal bovine serum. Cells (5 × 104) were set up per well, and 30 μM Taxol was added to the cells in log phase. As a control, 0.1% DMSO was used. To check for possible endotoxin contamination, media were assayed several times and found to contain <3 pg/mL endotoxin using the limulus assay. These cell lines were used within 4 months of establishment from ascites sources, and they are in the range of 10–20 passages. All were derived from patients who had previously failed

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3 The abbreviations used are: LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor.

4 S. Vogel, personal communication.
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Various chemotherapy protocols. There was no obvious correlation of constitutive or Taxol-induced IL-8 production with prior clinical history.

ELISA Analyses. IL-1β and IL-1α analyses were carried out with commercial kits from Endogen according to the instructions supplied by the manufacturer (19, 20). IL-8 was determined, also using reagents supplied from Endogen (rabbit antihuman IL-8) and Cappel (goat antirabbit alkaline phosphatase; Ref. 21).

Northern Blot Analyses. Total RNA was isolated from Taxol- and DMSO-treated cell lines using the guanidinium isothiocyanate/CsCl method as detailed (22). Purified RNA (3–5 μg) was loaded into each lane of the denaturing agarose gels (23). IL-8 and β-actin cDNA probes were hybridized to Northern blots as reported (24).

Nuclear Run-On Analyses. Nuclear run-on analyses were carried out according to the method described by us previously (25). The nuclei were extracted from each treatment group of OVCA 420 cells (1.5 × 10⁷). The control plasmid pEMSV (pEMSV scribe 2: Harold Weintraub, Fred Hutchinson Cancer Research Center, Seattle, WA) and plasmids of IL-1α, IL-1β, TNF-α, and IL-8 were denatured and slot blotted to Nytran membranes according to the directions of the manufacturer (Schleicher and Schuell). Total cpm were equilibrated for all samples and hybridized to slot-blotted cDNA for 60 h at 42°C. Hybridization and wash conditions were as described previously (25). After autoradiography, the filters were exposed to a PhosphorImager screen (Molecular Dynamics) for quantitation of nuclear run-on activity.

RESULTS

Taxol Stimulates IL-8 Expression in Human Carcinoma Cells Independent of LPS Responsiveness. Taxol is known to function as a microtubule-stabilizing agent, and its principle mode of antineoplastic action is assumed to be inhibition of mitotic spindle formation (3)

Fig. 1. Taxol induces both IL-8 protein (A) and mRNA transcripts (B). mRNA levels were detected using Northern blot transfer analyses, whereas IL-8 protein in the media was quantitated using an ELISA described in “Materials and Methods.” Treatment with 10–30 μM of Taxol for 6 h resulted in a substantial induction of IL-8 protein in an early passage T84 colon carcinoma line. Data shown are from two similar experiments. Note that the T84 cells secreted IL-8 in the pg/ml range. A kinetic analysis shows that IL-8 mRNA was induced by treatment of these cells with 30 μM Taxol for 1 h, and the response was at maximum 6 h after treatment (B). Samples without Taxol did not produce IL-8; control pretreatment cells (Lane 6) and 6-h methanol-treated cells (Lane 7).

| Table 1 IL-8 production* by SW620 cells |
|------------------------------|------------------|
| Taxol (μM) | LPS (ng/ml) |
| 0 | 1.4 | 15.5 | 50.5 | 67.0 |
| 3 | 1.4 | 12.0 | 46.5 | 56.5 |
| 30 | 1.0 | 11.5 | 35.5 | 46.0 |

* IL-8 was measured as pg/ml using an ELISA.
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Fig. 2. Summary of the IL-8 responses to Taxol of several human carcinoma cell lines, endometrial stromal cells, monocytes, and T lymphocytes. IL-8 secretion was determined using an ELISA. Four of 10 ovarian cell lines respond to Taxol and 2 constitutively secreted IL-8. DMSO; T, Taxol. Dots, data points from one cell line. ○, DMSO group; ●, Taxol group. In the case of primary cell lines, a dot represents a separate cell population. All of these cells were treated with DMSO or Taxol for 18 h.

were observed as early as 30 min after treatment, and these increased to a maximum level after 2 h of treatment. Fluctuation in the level of actin mRNA was also observed, albeit in a different manner since it decreased on Taxol treatment, especially after 2 h. This may be expected perhaps due to the effect of Taxol on cytoskeletal organization. The levels of 28S rRNA remained identical and served as a control for equal loading. Culture supernatants from the same test samples were measured for IL-8 production and indicated a 5-fold enhanced secretion of IL-8 after 6 h of treatment compared to the cells treated with DMSO for 6 h (Fig. 4, A and B).

**Taxol Response Is Independent of Target Cell Proliferation.** To determine whether inhibition of mitosis may be important for IL-8 induction, we investigated the response of proliferating and confluent nondividing OVCA 420 cells. Although numerous mitotic cells were observed in the Taxol-treated subconfluent cultures, they were totally absent in the similarly treated cultures kept confluent for 2 or 5 days. As shown in Fig. 4C, Taxol induced IL-8 synthesis in all samples with similar kinetics, regardless of the state of confluence of the OVCA 420 cells. Taxol-treated cultures that were confluent for 2 or 5 days did produce more IL-8 than proliferating cells; however, this pattern is also observed in the DMSO-treated group. This indicates that the induction of IL-8 by Taxol is likely to be independent of the growth of the cell and may be dependent on a signaling pathway distinct from regulation of mitosis.

**IL-8 Induction Is Due to Transcription.** Nuclear run-on analyses were performed to examine whether the induction of IL-8 in OVCA 420 cells was likely to result from transcriptional activation. The nuclear run-on data indicate a marked increase (3-fold) of IL-8 transcription by Taxol and a lesser increase in the related chemokine GRO (Fig. 5). As a positive control, IL-1β induced transcription of IL-8 and GRO 17- and 12-fold, respectively. However, Taxol had no influence on IL-1α, IL-1β, or TNF-α transcription levels.

**DISCUSSION**

Taxol is a novel chemotherapeutic agent that has promise in the treatment of several cancers, including ovary and breast cancers (1, 2). Although the principle activity of this compound is thought to be stabilization of microtubules (3), there is evidence that activities in addition to an antimitotic process may be important. For example, there is recent evidence that Taxol can be associated with programmed cell death (apoptosis) (5, 28, 29), and may mediate this event via induction of p34<sup>cdc2</sup> kinase (5) or down-regulation of bcl-2.
expression and subsequent inhibition of several other genes associated with cell growth such as c-myc and c-jun. In addition, studies on a pair of sensitive and resistant ovarian cancer cell lines suggested that Taxol disrupted the normal association of p53 with microtubules. This suggests that Taxol might have a more direct influence on cell survival than one resulting from mitotic arrest alone. Taxol has also been shown to have a reversible influence on granulosa cell steroidogenesis. Finally, Taxol stabilization of microtubules might also be expected to modify the association of microtubule-associated protein kinases with microtubules, thereby altering intracellular signaling in response to a variety of stimuli.

The most convincing evidence that Taxol could have an indirect effect on tumor cell survival comes from murine studies which demonstrate that Taxol can be a potent inducer of proinflammatory cytokines in macrophages. Taxol has been shown to induce a series of LPS-sensitive mediators of inflammation including IL-1α, IL-1β, TNF-α, and IP-10 in murine macrophages. We now report that Taxol induction of proinflammatory genes in human cell lines is restricted as to factor and cellular target. In addition, Taxol is not a general modulator of cytokine genes in human monocytes (current study). Instead, Taxol can selectively induce IL-8 in some long-term human ovarian carcinoma lines and recently isolated ovarian epithelial cancer cell lines. However, Taxol does not induce IL-8 in either breast or stromal cells. IL-1α, IL-1β, and IL-6 did not change in several carcinoma cell lines as well as stromal cells according to the ELISA and bioassay, yet Taxol induced IL-1β in several breast carcinoma lines. It will be relevant to determine whether additional genes important in the regulation of cell growth or modulation of the extracellular matrix are sensitive to Taxol stimulation. Our preliminary PCR screening and the nuclear run-on data described here indicate that the only additional cytokine gene activated by Taxol in the OVCA 420 cell line is the related CXC chemokine GRα'. MIP-1β, a CC chemokine, was not transcriptionally activated (data not shown). More extensive screening studies are in progress.

Initial murine studies linked the Taxol signaling pathway with that induced by LPS. More recent evidence has revealed a discordance between the effect of Taxol and other family members, at least one of which has demonstrated clinical efficacy. Taxotere appears to be clinically active in ovarian cancer yet fails to stimulate the LPS family of inflammatory cytokines in murine macrophages, indicating that an intact LPS-mediated pathway might not be required for Taxol-induced cytokine synthesis. Although restricted to a pair of cell lines, our data comparing the early passage LPS-unresponsive T84 cells with the LPS-sensitive SW620 cells support the concept that IL-8 activation by Taxol may not depend on the same...
signaling pathway as in murine macrophages. Additional studies are required to examine this question in more detail.

Taxol is primarily regarded as a mitotic inhibitor (3) and an inducer of apoptosis (5), yet we were able to stimulate equivalent levels of IL-8 from cells maintained in a state of confluence for 5 days or at half confluence and exhibiting numerous mitotic figures. Since the confluent cultures failed to show mitotic figures in response to Taxol exposure, it suggests that the drug has an additional mode of activity independent of the mitotic apparatus. In support of this, Donaldson et al. (5) have suggested that a mitotic block may not be sufficient to induce apoptosis and an additional p34cdc2 kinase activation step may be important. Our data also imply that dormant tumor cells in vivo may be responsive and influential in Taxol therapy insomuch as local production of cytokines such as IL-8 may influence prognosis.

The major question raised by these studies is what is the role of IL-8 in tumor-host interaction? Could IL-8 facilitate therapy or enhance the rate of tumor invasion and metastasis? IL-8 is a member of a new superfamily of CXC cytokines highly homologous to several known connective tissue-activating proteins with proinflammatory activities including βTGF, PF4, and GRO/MGSA (34). IL-8 is known to exhibit multiple activities including chemotactic activity for neutrophils, T cells, and basophils, adhesion induction of monocytes (34, 35), and the proliferative activation of endothelial cells (36). The latter role suggests that it may have direct effects on tumor angiogenesis. In addition, IL-8 has also been implicated as a negative regulator of colony formation by granulocyte-macrophage colony-stimulating factor-stimulated myeloid progenitors (37).

There are several recent reports that suggest IL-8 may play a direct role in enhancing metastatic spread (38) or provide an antitumor effect independent of neutrophils (39). In addition, IL-8 appears to have a mitogenic activity in melanoma cells (38), an activity which is likely to be mediated via the type II IL-8/GRO receptor (39). Finally, there is also evidence that GRO may modulate the level of collagen secretion from fibroblasts (40), raising the potential for modulation of invasiveness and/or extracellular matrix remodeling in the tumor environment. However, the potential role of IL-8 in vivo has not been clearly identified. It will be important to determine whether IL-8 directly influences tumor growth and vasculature or functions as a proinflammatory cytokine to counter tumor growth. We believe that both direct tumor cell destruction by Taxol as well as the significant induction of either a growth factor or proinflammatory response may be important variables in the outcome of successful Taxol therapy in ovarian cancer. Our previous suggestion that the initial response of patients with stage III and IV ovarian cancer correlated with a strong intratumor inflammatory infiltrate in the residual tumor sites (41) inferred that lymphocytes, macrophages, and neutrophils played an effective role either in tumor stasis or destruction. Nicoletti et al. (42) reported that Taxol has significant antitumor activity in a nude mouse model of human ovarian cancer. In their studies, Taxol treatment caused a significant lymphocytic infiltrate to both omentum and liver implants, supporting our concept that Taxol therapy has a proinflammatory component.

Recent studies involving chemotherapy/immunotherapy by cytokine gene transfection have pointed to several directions of host-tumor interactions which impact on the inflammatory cell compartment and the tumor per se. First, expression of several different cytokines via gene delivery can induce a host recognition response capable of rejecting established nonimmunogenic tumors (reviewed in Ref. 43). Second, it is now established that tumors may be controlled in either a positive or negative fashion by autocrine growth factors (44). In particular, GRO has been reported to convert preneoplastic melanomas into malignant tumors (32).

As discussed above, it is also possible that IL-8 induction by Taxol has a negative influence on survival. A recent report has shown that Taxol has a unique dose-response cytotoxic effect (45). We also observed that exposure to Taxol for 72 h exhibits a sharp decline in cell survival at low concentrations of the drug, followed by an increase in cell survival at high concentrations of Taxol (data not shown). It seems probable that growth factors or cytokines secreted in the medium upon high-dose Taxol treatment can antagonize Taxol cytotoxicity. Clinical evaluation combined with laboratory studies of patient samples recovered during therapy will be required to determine the degree to which Taxol induction of IL-8 and/or additional cytokines, in the responsive subset of patients, modifies the therapeutic index.

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