Inhibition of tumor angiogenesis by oral etoposide

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Abstract. The chemotherapeutic agent etoposide is a topoisomerase II inhibitor widely used for cancer therapy. Low-dose oral etoposide, administered at close regular intervals, has potent anti-tumor activity in patients who are refractory to intravenous etoposide; however, the mechanism remains unclear. Since endothelial cells may be more sensitive than tumor cells to chemotherapy agents, we determined the effects of etoposide alone and in combination with oral cyclooxygenase-2 inhibitors and peroxisome-proliferator activated receptor γ ligands on angiogenesis and tumor growth in xenograft tumor models. Optimal anti-angiogenic (metronomic) and anti-tumor doses of etoposide on angiogenesis, primary tumor growth and metastasis were established alone and in combination therapy. Etoposide inhibited endothelial and tumor cell proliferation, decreased vascular endothelial growth factor (VEGF) production by tumor cells and suppressed endothelial tube formation at non-cytotoxic concentrations. In our in vivo studies, oral etoposide inhibited fibroblast growth factor 2 and VEGF-induced corneal neovascularization, VEGF-induced vascular permeability and increased levels of the endogenous angiogenesis inhibitor endostatin in mice. In addition, etoposide inhibited Lewis lung carcinoma (LLC) and human glioblastoma (U87) primary tumor growth as well as spontaneous lung metastasis in a LLC resection model. Furthermore, etoposide had synergistic anti-tumor activity in combination with celecoxib and rosiglitazone, which are also oral anti-angiogenic and anti-tumor agents. Etoposide inhibits angiogenesis in vitro and in vivo by indirect and direct mechanisms of action. Combining etoposide with celecoxib and rosiglitazone increases its efficacy and merits further investigation in future clinical trials to determine the potential usefulness of etoposide in combinatorial anti-angiogenic chemotherapy.

Introduction

Daily administered, low-dose, cytotoxic, chemotherapeutic drugs were initially shown by Browder et al to preferentially target the endothelium of the tumor vasculature (1). When cyclophosphamide was administered in low frequent doses, as opposed to the maximally tolerated dose every three weeks, potent tumor suppression was achieved as a result of endothelial cell apoptosis. This anti-angiogenic, or metronomic, chemotherapeutic approach avoids the development of tumor cell resistance by targeting the proliferating endothelial cells required for tumor neovascularization (2-4). Furthermore, the greater sensitivity of endothelial cells in comparison to tumor cells allows for significantly lower doses of the drug to be effective, thus improving tolerability (5,6). Anti-angiogenic chemotherapy has entered clinical trials for various vascular tumors refractory to conventional chemotherapy (4,7-9). In our study, 40% of children with recurrent or progressive cancer, treated with daily low-dose oral etoposide alternating every 21 days with daily low-dose oral cyclophosphamide combined with daily oral thalidomide and celecoxib, exhibited a prolonged or persistent progression-free disease status (7).

Etoposide (VP16), a topoisomerase II inhibitor, is a semisynthetic derivative of podophyllotoxin introduced in cancer clinical trials in 1971 and FDA-approved in 1983. It is an alkaloid cytotoxic drug that binds to and inhibits topoisomerase II-DNA function in ligating cleaved DNA molecules, resulting in the accumulation of single- or double-strand DNA breaks and stops the cell cycle at the late S and G2 phases (10). Daily oral etoposide is effective for the treatment of several tumors, including non-small cell lung cancer, recurrent medulloblastoma and neuroblastoma, after these tumors have developed resistance to the maximally tolerated doses of intravenous etoposide (11,12). Additionally, platinum-resistant epithelial ovarian cancer, metastatic breast cancer and pediatric recurrent sarcomas have been successfully treated with oral etoposide (13-15). When compared to intravenous administration, treatment with oral etoposide increased the response rate in patients with small-cell lung and advanced breast cancers (16,17). However, the mechanism by which low-dose oral etoposide inhibits the growth of tumors resistant to maximally tolerated higher-dose intravenous etoposide has not been extensively studied.

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We hypothesize that tumor endothelium is a potential target of low-dose oral etoposide, since the primary tumor and metastatic growth are dependent on angiogenesis (18). This hypothesis is supported by observations that etoposide inhibits the proliferation of endothelial cells (19). In fact, endothelial cells were found to be more sensitive to etoposide than tumor cells in vitro (20), suggesting that the anti-tumor effect of etoposide may, in part, be mediated through the endothelium. Therefore, we investigated the role of etoposide in tumor angiogenesis. We report that etoposide inhibits primary tumor growth and metastasis through anti-angiogenic and direct anti-tumor effects. Oral administration of etoposide allows it to be easily incorporated into chemotherapy regimens and supports its addition to the growing class of oral anti-angiogenic drugs for cancer therapy.

Materials and methods

Cells and reagents. Bovine capillary endothelial (BCE) cells were maintained on gelatinized plastic in Dulbecco's modified Eagle's medium (DMEM) low glucose + 10% bovine calf serum. Human umbilical vein endothelial cells (HUVECs) were maintained in EBM-2 media. Lewis lung carcinoma ( LLC), fibrosarcoma (T241), glioblastoma (U87), breast (MDA-MB 231) and K1000 [a tumor cell line that expresses and secretes high levels of fibroblast growth factor 2 (FGF2)] cells were cultured in DMEM + 10% heat-inactivated FBS + 1% penicillin streptomycin glutamine. For in vitro studies, etoposide (VP-16) (Sigma, St. Louis, MO, USA) coated 24-well plates. The animal experiments or vehicle on (5 x 10^4 cells/well) with varying concentrations of etoposide (20), suggesting that the anti-tumor effect of etoposide may, in part, be mediated through the endothelium. Therefore, we investigated the role of etoposide in tumor angiogenesis. We report that etoposide inhibits primary tumor growth and metastasis through anti-angiogenic and direct anti-tumor effects. Oral administration of etoposide allows it to be easily incorporated into chemotherapy regimens and supports its addition to the growing class of oral anti-angiogenic drugs for cancer therapy.

Vascular endothelial growth factor (VEGF) ELISA. Tumor cells that were known to secrete high levels of VEGF (U87 glioblastoma and LLC) were plated at 15 x 10^4 cells per well (6-well plates), and 24 h later were treated with etoposide or vehicle. Medium containing the drugs was changed on Days 3 and 5. On Day 6, the medium was collected, and VEGF was assayed by ELISA (R&D Systems Inc., Minneapolis, MN, USA).

Angiogenesis assays. Endothelial cell proliferation was assayed as described (21) at 15 x 10^3 cells per well. For tumor cell proliferation, cells were plated at 5 x 10^4 cells per well. Endothelial cell tubes were formed by combining HUVECs (5 x 10^4 cells/well) with varying concentrations of etoposide or vehicle on Matrigel- (Collaborative Biochemical, Bedford, MA, USA) coated 24-well plates. The animal experiments were performed in accordance with IRB-approved protocols at Children's Hospital Boston.

For the corneal neovascularization assay, 80 ng FGF2 or 160 ng VEGF pellets were implanted into C57BL/6 mice (Jackson Labs, Bar Harbor, ME, USA) (22). Etoposide was administered daily over 6 days by gavage in 0.5% methylcellulose, and control mice received vehicle (0.5% methylcellulose).

For tumor studies, LLC was injected subcutaneously as described (21). Glioblastoma (U87) and T241 fibrosarcoma were injected subcutaneously (1 x 10^6 cells in 0.1 ml PBS) into 6-week-old male severe combined immunodeficient (MGH, Boston, MA, USA) or C57BL/6 mice, respectively. Once tumors were 100-150 mm^3, mice were randomized into treatment and vehicle groups. Etoposide, celecoxib, rosiglitazone and/or cyclophosphamide were administered by daily gavage for 14-40 days. Tumors were measured every 3-7 days, and the volume was calculated as width^2 x length x 0.52.

For metastasis studies, LLC tumors were resected 15 days after implantation as described (21). After LLC resection, mice were treated with etoposide or vehicle for 16 days when control mice became terminally ill. On the last day of treatment, the statistical difference between the treatment and control groups was determined by the Student's t-test. A p-value < 0.05 was accepted as significant.

Miles vascular permeability assay. One to two days prior to the experiment, mice were shaved to expose the skin. Mice were anesthetized with intraperitoneally injected Avertin and injected with 1% Evan's blue dye, either by tail vein or through the orbital plexus. VEGF (50 µl of 1 ng/µl) and 50 µl of saline or PBS with 0.05% gelatin were injected intradermally using a 30-gauge needle into the skin overlaying the back. Similar experiments were performed by injecting 5 µl of VEGF, saline or PBS intra-dermally into the ears. After 10 min, the animals were euthanized, and the skin was opened and exposed to assess the intensity of Evan's blue dye extravasations. The areas of blue skin (vascular leak) were removed and placed into formamide for 5 days. The intensity of the reaction was quantified by reading the samples at a wavelength of 620 nm on a SpectraMax plate reader.

Immunohistochemistry. For PECAM1, the sections of tumors were treated with 40 µg/ml proteinase K (Roche Diagnostics Corp.) for 25 min at 37°C. PECAM1 was amplified using tyramide signal amplification direct and indirect kits (PerkinElmer Life Sciences, Boston, MA, USA). For computer-enhanced imaging of tumors, histological sections were analyzed for vessel density by computerized densitometric imaging (Corel Photo Paint and IP Lab software). The degree of vascularization was quantified over the entire tumor section and expressed as a ratio of vessel area (PECAM1) to tumor area. Total fields scored per tumor were 67-70. For control and etoposide-treated tumors, 4 animals/group were evaluated.

Results

Etoposide has direct and indirect anti-angiogenic and anti-tumor activity in vitro

Direct effects. To investigate the effects of etoposide on endothelial cell proliferation, we stimulated the proliferation of BCE cells with FGF2, a potent mitogen for BCE cells, in a standard proliferation assay. Etoposide inhibited FGF2-induced proliferation of BCE cells in a dose-dependent manner, with a maximal inhibition of 80% after a 72-h incubation period at 2.5 µM, a concentration easily achieved orally in humans (Fig. 1A). Similarly, etoposide inhibited VEGF-induced proliferation of HUVECs up to 80% at 2.5 µM (Fig. 1B). We next determined whether etoposide inhibits tumor cells at similar doses as those applied to endothelial cells. Etoposide inhibited the proliferation of human tumor cells, including glioblastoma (U87) and breast (MDA-MB 231),...
differentially because of the primary resistance of these cell lines (Fig. 1c and d). Murine tumor cell lines, LLC and T241 fibrosarcoma demonstrated sensitivity to etoposide (Fig. 1E-G).

**Indirect effects.** To determine whether etoposide inhibits angiogenesis by down-regulating tumor-secreted growth factors, we measured VEGF levels in tumor-conditioned media via ELISA. The tumor cell lines glioblastoma (U87 resistant to etoposide) and LLC (sensitive to etoposide) secreted substantial amounts of VEGF: 20,000 and 938 pg/10^6 cells, respectively. Etoposide inhibited VEGF secretion in U87 cells by 51% and in LLC cells by 36% (Fig. 1H). The inhibitory effect of etoposide on VEGF secretion in vitro suggests a potential anti-angiogenic mechanism in vivo via decreased tumor cell production of this angiogenic mitogen.

Etoposide inhibits endothelial cell tube formation and FGF2- and VEGF-induced corneal neovascularization. To investigate whether etoposide has an effect on vessel morphogenesis, we seeded HUVECs on Matrigel, where they formed branching, anastomosing tubes that mimicked capillary-like structures (Fig. 2A). Etoposide inhibited tube formation in a dose-dependent manner (Fig. 2B-D), consistent with previous studies (23). To optimize the anti-angiogenic doses of etopo-
Etoposide inhibits vascular permeability and raises endostatin levels in vivo.

We next determined whether etoposide (VP16) affects VEGF-induced vascular permeability, a standard test of in vivo VEGF activity (24). In response to VEGF, control mice displayed Evan's blue extravasation into the subcutaneous skin and ears (Fig. 3A and B). Evan's blue dye leakage in dorsal skin and ears after injection with VEGF or saline in control mice (n=10 mice/group) and etoposide (VP16)-treated mice (n=10 mice/group). (C and D) Spectrophotometric analysis of extravasated Evan's blue of the skin and ear is represented in bar graphs (average ± standard deviation). (E and F) H&E sections of skin from SCID mice treated with etoposide (VP16) or vehicle. (G) Etoposide, but not cyclophosphamide (cytoxan), elevated the endostatin level in vivo.

Etoposide inhibits VEGF-induced vascular permeability and raises endostatin levels in vivo. We next determined whether etoposide (VP16) affects VEGF-induced vascular permeability, a standard test of in vivo VEGF activity (24). In response to VEGF, control mice displayed Evan's blue extravasation into the subcutaneous skin and ears (Fig. 3A) 80-82% greater than that of etoposide-treated mice (Fig. 3B).
There was also a decrease in vascular leakage between the two saline groups in the etoposide-treated mice, presumably representing the inhibition of basal circulating VEGF. Spectrophotometric analysis of extravasated Evans blue in both the skin and ear of etoposide-treated mice exhibited a dramatic reduction in VEGF-induced vascular permeability (Fig. 3C and D). Immunohistochemical analysis (H&E staining) revealed that the area of skin edema was greatly reduced in the etoposide-treated mice when compared to the vehicle-treated mice (Fig. 3E and F). Together, these results indicate that daily low-dose oral etoposide is a potent inhibitor of VEGF-dependent signaling.

Since etoposide raises biologically active endostatin levels in vitro (25), we examined whether the administration of etoposide raises endostatin levels in vivo. Mice treated with etoposide exhibited a 75% increase in plasma levels of endostatin (Fig. 3G). Another oral chemotherapeutic agent, cyclophosphamide, had no effect on endostatin levels in the plasma (Fig. 3G).

Systemic therapy with etoposide inhibits primary tumor growth and metastasis. In order to examine the anti-angiogenic effect of daily, low-dose, oral etoposide (VP16) on the growth of primary tumors, we treated established subcutaneous tumors of 100-150 mm³ volume grown in mice. We utilized the optimal doses of etoposide identified in the corneal neovascularization assay. Oral etoposide at 40 and 80 mg/kg/day inhibited the growth of LLC by 29 and 56%, respectively (Fig. 4A), and T241 fibrosarcoma by 55 and 79%, respectively (Fig. 4B); 80 mg/kg of etoposide inhibited glioblastoma (U87) by 95% (Fig. 4C). There was no evidence of significant weight loss or other drug-related toxicity in any of the mice. To determine whether etoposide inhibited primary tumor growth by inhibiting angiogenesis, we measured the microvessel density in the treated and control tumors. A decrease in the microvessel density during treatment with an angiogenesis inhibitor suggests an anti-angiogenic effect on tumor growth (18). Etoposide treatment reduced microvessel density in the etoposide-treated tumors (Fig. 4D and E).
density relative to that in the control tumors, thus indicating the presence of its anti-angiogenic efficacy (Fig. 4D-F).

Etoposide, when administered in mice via liposomes, was found to inhibit the formation of lung nodules in a metastatic tail vein model (26). The tail vein model illustrates only the homing step of tumor cells from circulation to an organ. By contrast, the LLC metastasis model is a model of spontaneous lung metastasis with all of the steps involved in metastasis including the invasion of tumor cells from the primary tumor to the circulation. Removal of the primary LLC was found to decrease the circulating angiogenesis inhibitor angiostatin, resulting in rapid growth of lung metastasis (21). In the present study, mice were treated for 15 days with oral daily etoposide (80 mg/kg/day) or vehicle after removal of the primary LLC. In mice treated with vehicle, growing invasive metastasis almost entirely replaced the normal lung tissue, leading to lung weights in these mice of 505±42 mg. In marked contrast, mice treated with oral etoposide (80 mg/kg/day) had a lung weight of 216±19 mg vs. normal lung weights of 152±10 mg (Fig. 4G).

Etoposide has synergistic anti-tumor activity with oral anti-angiogenic drugs, including celecoxib and rosiglitazone. To determine whether combining other classes of drugs improves the anti-tumor efficacy of etoposide, we utilized the cyclooxygenase-2 (COX2) inhibitor, celecoxib and peroxisome-proliferator activated receptor (PPARγ) ligand rosiglitazone, which are both orally administered and target endothelial and tumor cells (22,27). We administered celecoxib, rosiglitazone and either etoposide or cyclophosphamide at the lowest doses necessary for minimal anti-tumor effect. Oral celecoxib (30 mg/kg/day) significantly enhanced the anti-tumor activity of low-dose oral etoposide (10 mg/kg/day) by 42% (Fig. 5A). When combined, PPARγ agonist rosiglitazone (50 mg/kg/day) and celecoxib (30 mg/kg/day) enhanced the anti-tumor activity of low-dose oral etoposide (10 mg/kg/day) by 69% (Fig. 5B), with no evidence of drug-related toxicity.

Discussion

Most chemotherapy regimens are associated with significant toxicity when administered at maximum tolerated doses. There is now increasing evidence that multi-drug-resistant tumors are effectively targeted by anti-angiogenic chemotherapy (1,2), in which low doses of cytotoxic drugs are given at close, regular intervals, with minimal toxic side effects (4). Therefore, standard chemotherapeutic agents, when modified by frequency and dose, target tumor angiogenesis. The mechanism by which cytotoxic chemotherapy affects the tumor vasculature may include selective killing of endothelial cells, suppression of circulating endothelial precursor cells and/or increasing levels of the endogenous angiogenesis inhibitors, such as thrombospondin-1 (4,28-31), and decreasing levels of increasing levels of the endogenous angiogenesis inhibitors, such as thrombospondin peptide ABT-510 in suppressing tumor growth (43). Recent studies show synergy between PPARγ ligands, such as rosiglitazone, and platinum-based chemotherapeutic agents in inhibiting tumor growth (44). The use of oral etoposide in a number of combinations, such as with other angiogenesis inhibitors, chemotherapy and/or radiation, has demonstrated activity in mouse tumor models and patients (19,25,45,46). Our results show that etoposide has synergistic anti-tumor activity with COX2 inhibitors and PPARγ ligands. COX2 inhibitors, such as celecoxib, have both anti-angiogenic and anti-tumor activities (27); we previously demonstrated that the PPARγ ligand rosiglitazone inhibits primary tumor growth and metastasis by targeting the tumor endothelium (22). The mechanism by which etoposide inhibits tumor angiogenesis may complement the anti-angiogenic effects of COX2 and PPARγ ligands resulting in greater inhibition of endothelial proliferation and a decrease in VEGF secretion.

Already, several human studies support the clinical relevance of oral etoposide. We recently incorporated etoposide as part of a four-drug anti-angiogenic chemotherapy regimen (thalidomide, celecoxib, etoposide and cyclophosphamide), which showed prolonged disease-free status in pediatric patients with recurrent or progressive cancer (7). Similarly, etoposide was part of a four-drug regimen named COMBAT (combined oral maintenance biodifferentiating and anti-angiogenic therapy), which was effective in solid tumors in children which had relapsed (9). This regimen included celecoxib, cis-retinoic acid, metronomic temozolomide and low-dose etoposide. Anti-angiogenic ‘metronomic’ chemotherapy is significantly cost-effective in the treatment of metastatic breast cancer (47). Therefore, oral etoposide, which is very well tolerated, may result in increased patient compliance; it can also be adminis-
tered on an outpatient basis, thereby reducing costs, which is becoming an important issue (48,49).

Our studies suggest that etoposide may be beneficial in treating angiogenic diseases, such as cancer, because of its effect on the endothelium and on angiogenesis pathways. Moreover, the endothelium is also an important target in the treatment of non-neoplastic diseases, such as arthritis, psoriasis and endometriosis. In fact, suboptimal doses of etoposide were found to improve collagen II-induced arthritis without monocyte depletion (50). As an orally administered FDA-approved drug, etoposide is ideally suited for use in combination with other anti-angiogenesis regimes and can complement conventional cancer treatment modalities.

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