Sensitization of Tumor-Associated Endothelial Cell Apoptosis by the Novel Vascular-Targeting Agent ZD6126 in Combination with Cisplatin

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ABSTRACT

Purpose: ZD6126 is a novel vascular-targeting agent that selectively disrupts the tubulin cytoskeleton of endothelial cells. In the immature vessels characteristic of tumor vasculature, this leads to endothelial cell contraction, blood vessel congestion, and, consequently, tumor cell death. ZD6126 has been shown to delay tumor growth in a range of xenograft models. The antitumor effect of ZD6126 can be increased in combination with cisplatin or radiation therapy, although the precise mechanism of this enhancement has not been demonstrated. ZD6126 treatment has also been shown to inhibit lung metastasis, and the present study has explored the potential to increase the antitumor effect of ZD6126 by combining with cisplatin, and the underlying mechanisms have been investigated.

Experimental Design: Human lung adenocarcinoma PC14PE6 cells were injected into the tail vein of nude mice. Five weeks after injection, animals were treated with ZD6126 (200 mg/kg i.p.), cisplatin (6 mg/kg i.v.), or a combination of the two agents. The animals were sacrificed 24 hours later, and the extent of lung metastases and the presence of apoptotic cells were assessed.

Results: Histologic analysis revealed that the ZD6126/ cisplatin combination resulted in a 2- to 4-fold increase in the total number of tumor-associated apoptotic cells compared with either treatment alone. ZD6126 alone induced apoptosis of tumor-associated endothelial cells in tumors, and the extent of apoptosis was increased 2-fold in combination with cisplatin. The lung weight was significantly reduced, and the number of metastatic nodules significantly was lower in the combined treatment group than in the control group.

Conclusions: These data suggest that the antitumor effect of the vascular-targeting agent ZD6126 can be increased by use in combination with cisplatin, which increases the incidence of endothelial cell apoptosis.

INTRODUCTION

Lung cancer is a leading cause of malignancy-related death worldwide, and >90% of deaths from lung cancer can be attributed to metastasis (1). The ability to control metastasis would, therefore, result in improved patient prognosis and quality of life. Management of metastasis with current treatment options is limited, and one major obstacle to therapeutic success appears to be the biological heterogeneity of tumors due to subpopulations of cells with different angiogenic, invasive, and metastatic properties (2). Of these, angiogenic variation is thought to be the most problematic because the growth and production of metastases depends on angiogenic ability (3). Therefore, targeting the tumor vasculature is considered to be an attractive strategy for the treatment of metastatic malignant diseases (4). Antiangiogenic agents inhibit the activity of proangiogenic factors, thereby preventing or delaying tumor development and metastases by inducing tumor dormancy rather than regression of the established tumor (5–8). Bevacizumab, a neutralizing antibody directed against vascular endothelial growth factor, has demonstrated significant clinical efficacy in patients with advanced colorectal and renal cancers and has established antivascular therapy as a highly promising treatment approach (9, 10).

In contrast to antiangiogenic approaches, vascular-targeting agents disrupt existing tumor vasculature leading to vessel occlusion and arrested blood flow. As a large number of tumor cells depend on a relatively small number of blood vessels, interruption of the vascular supply could have significant anti-tumor effects. Tumor blood vessels differ significantly from those found in normal tissues and contain a chaotic network of tortuous thin-walled vessels with a significant degree of neo-vasculature and a relatively high proportion of proliferating endothelial cells (2, 11). Vascular-targeting agents exploit the distinctive features of the tumor vasculature to irreversibly arrest blood flow in tumors (12). The resulting ischemia leads to a rapid cascade of secondary tumor cell death and the destruction of central areas of the tumor, which are normally most resistant to conventional therapies (13–15). ZD6126 is a novel vascular-targeting agent that is rapidly converted by serum phosphatases to ZD6126 phenol (N-acetylcolchinol), which disrupts the tubulin cytoskeleton of endothelial cells, resulting in conformational changes in immature, but not mature, endothelial cells. ZD6126 appears to be the most problematic because the growth and production of metastases depends on angiogenic ability (3). Therefore, targeting the tumor vasculature is considered to be an attractive strategy for the treatment of metastatic malignant diseases (4). Antiangiogenic agents inhibit the activity of proangiogenic factors, thereby preventing or delaying tumor development and metastases by inducing tumor dormancy rather than regression of the established tumor (5–8). Bevacizumab, a neutralizing antibody directed against vascular endothelial growth factor, has demonstrated significant clinical efficacy in patients with advanced colorectal and renal cancers and has established antivascular therapy as a highly promising treatment approach (9, 10).

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lial cells (16). A single dose of ZD6126 has been shown to induce hemorrhage and necrosis in established lung metastases and lead to apoptosis of endothelial cells in tumor tissue but not in the normal lung parenchyma (17).

Despite the potent antivascular effects of ZD6126, a narrow rim of viable tumor cells is often observed at the periphery of the tumor after treatment (16). The residual tumor tissue can rapidly reestablish the tumor mass, but because of its high proliferation rate, it is still a good target for conventional anticancer therapies. Combined treatment of ZD6126 with the cytotoxic agents paclitaxel or cisplatin or irradiation has demonstrated additional growth delay in tumor xenografts. However, the mechanism of action by which these combined therapies cause an enhanced antitumor effect has yet to be established (16, 18, 19).

The aim of this study was to assess the benefit of combining the vascular-targeting agent ZD6126 and platinum-based chemotherapy, cisplatin, on lung cancer metastasis. An immunohistochemical technique was used to determine the effect of ZD6126 and cisplatin on tumor cells and endothelial cells in the metastatic PC14PE6 lung tumor model.

MATERIALS AND METHODS

Cells. Cells from the human lung adenocarcinoma cell line PC14PE6 were kindly donated by Dr. Isaiah J. Fidler (20). The cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin-streptomycin and were cultured in a humidified CO2 incubator at 37°C. Human dermal microvascular endothelial cells (KURABO, Osaka, Japan) were maintained in HuMedia-MvG with growth supplements (KURABO) and used for in vitro assays at passage 2 to 5 (17).

Reagents. ZD6126 was provided by AstraZeneca (Cheshire, United Kingdom) and cisplatin by Bristol-Myers Squibb (Princeton, NJ).

Animals. Male, 5-week-old athymic BALB/c nude mice were obtained from CLEA (Tokyo, Japan) and maintained under specific pathogen-free conditions throughout the study. Experiments were performed according to guidelines set out by the University of Tokushima School of Medicine.

Experimental Metastasis In vivo. For production of lung metastases, human lung adenocarcinoma PC14PE6 cells (1 × 10⁶) were suspended in 0.2 mL of PBS and injected into the tail vein of nude mice (20). For evaluation of apoptosis of tumor and endothelial cells, animals received cisplatin (6 mg/kg i.v.), ZD6126 (200 mg/kg i.p.), or a combination of the two agents (in which case ZD6126 was administered 1 hour after cisplatin therapy) 5 weeks after PC14PE6 cell injection. After 24 hours (day 35), the mice were killed, and the frozen sections of the lung with metastatic nodules were prepared. A, frozen sections of lung metastasis stained immunohistochemically with the terminal deoxynucleotidyl transferase-mediated nick end labeling method. B. The number of apoptotic cells was increased in the combined treatment group. Data show the means ± SD of the five independent areas (×200). **P < 0.01.

Histology and Immunohistochemistry. Tissue from the excised lungs was cut into 5-mm slices and placed into either buffered 10% formalin solution or OCT compound (Sakura Fine-technical Co., Tokyo, Japan) to be snap-frozen in liquid nitrogen for immunohistochemical analysis. Terminal deoxynucleotidyl transferase-mediated nick end labeling staining was performed using the Apoptosis Detection System (Promega, Madison, WI). Briefly, the frozen tissue sections (9-μm thick) were fixed with...
PBS containing 4% formalin. The slides were washed with PBS and permeabilized with 20 μg/mL proteinase K. The samples were then equilibrated, and DNA strand breaks were labeled with fluorescein-12-dUTP by adding nucleotide mix and terminal deoxynucleotidyl transferase enzyme. The reaction was stopped with saline sodium citrate, and the localized green fluorescence of apoptotic cells was detected by fluorescence microscopy. Double staining for endothelial cells and the presence of apoptosis was performed on frozen tissue sections with an anti-CD-31 monoclonal antibody (PharMingen, San Diego, CA) and Texas Red-conjugated secondary antibody (Vector Laboratories, Burlingame, CA), followed by terminal deoxynucleotidyl transferase-mediated nick end labeling staining.

**Cell Proliferation Assay.** Cell proliferation was measured by the MTT dye reduction method (21). Briefly, 2 × 10^3 PC14PE6 cells/100 μL were plated into 96-well plates in medium and incubated at 37°C. After 24 hours, 100 μL of media containing increasing concentrations of ZD6126 were added and incubated for 2 hours. Cells were then washed and fresh media containing 1 μg/mL cisplatin added and incubated for 70 hours. After this, 50 μL of stock MTT solution (2 mg/mL; Sigma, St. Louis, MO) were added and incubated for 2 hours. The medium containing MTT solution was removed and the residual dark blue crystals dissolved in 100 μL of DMSO. Absorbance was measured using an MTP-120 microplate reader (Corona Electric, Ibaraki, Japan) at test and reference wavelengths of 550 and 630 nm, respectively.

**Statistical Analysis.** In vivo data were analyzed by the Mann-Whitney U test; in vitro data were analyzed by the Student’s t test (two-tailed).

**RESULTS**

**Effect of ZD6126 and Cisplatin on Established Metastatic Lung Tumors.** Single-dose treatment with ZD6126 or cisplatin resulted in a 3- to 7-fold increase in total apoptotic cell count compared with controls (Fig. 1). When both agents were
combined, the number of apoptotic cells was significantly greater than that seen with either agent alone. Apoptosis of endothelial cells in the normal lung parenchyma was barely detectable.

**Combined Effect of ZD6126 and Cisplatin on Endothelial Cell Proliferation In vitro.** Using the MTT assay, an effect of cisplatin, but not ZD6126, on PC14PE6 cell proliferation has been shown (Fig. 4A). Treatment with ZD6126 alone inhibited the proliferation of human dermal microvascular endothelial cells in a dose-dependent manner (Fig. 4B). Maximal inhibition of the proliferation of human dermal microvascular endothelial cells was observed when treatment with ZD6126 and cisplatin was combined (P < 0.01).

**Therapeutic Effect of ZD6126 and Cisplatin on Lung Cancer Metastasis.** We finally examined the therapeutic effect of ZD6126 on lung metastases by PC14PE6 cells. PC14PE6 cells were injected i.v. into nude mice. Daily i.p. administration of ZD6126 (100 mg/kg) commenced 14 days after tumor cell inoculation (because at this time, the PC14PE6 cells progress to micrometastases in the lung) and continued until mice were killed. Cisplatin (6 mg/kg) was administered i.v. on day 14. Treatment with ZD6126 or cisplatin alone resulted in a reduction in lung weight, although the decrease in the number of lung metastases did not reach statistical significance (Fig. 5, Table 1). Combining ZD6126 and cisplatin therapies, however, caused significant inhibition both of the number and volume of metastatic tumors. Treatment with both drugs was well tolerated, and no loss of body weight was observed throughout the study.

**DISCUSSION**

In the present study, we have confirmed previous reports of ZD6126-mediated necrosis in established lung metastases (17). In addition, we have demonstrated that treatment with ZD6126 alone can lead to apoptosis of endothelial cells in tumors, and therapy with cisplatin alone results in apoptosis of tumor cells. Tumor-associated endothelial cell apoptosis and growth inhibition induced by ZD6126 was enhanced in combination with cisplatin, and this regimen reduced the tumor burden in the lungs.

**Fig. 3** Effect of ZD6126 and cisplatin (CDDP) on endothelial cell apoptosis in lung metastases. Sequential staining for CD31 and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) were performed in tumor-bearing lung sections from each treatment group (n = 5, each group). Treatment with ZD6126 alone resulted in a 15-fold increase of apoptotic endothelial cells in tumors over the control group. By contrast, apoptosis of endothelial cells in the CDDP-treated group was minimal. When both agents were combined, there was a 30-fold increase in the number of apoptotic endothelial cells observed compared with the control group. Data show the means ± SD of four independent areas (×200). *P < 0.05; **P < 0.01. HPF, high power field.

**Fig. 4** Effect of ZD6126 and cisplatin on *in vitro* proliferation of human dermal microvascular endothelial cells and PC14PE6 cells. □ indicates ZD6126 single treatment, whereas ○ represent ZD6126 + cisplatin (CDDP; 1 μg/mL). A. PC14PE6 cells. B. human dermal microvascular endothelial cells. Data show the means ± SD of cell counts from triplet wells and are representative of three independent experiments with similar results. *P < 0.05; **P < 0.01.
Use of vascular-targeting therapy to block the blood supply from established vessels to tumor cells is a promising means of controlling metastasis (3). Although a potent vascular-damaging effect is observed after treatment with ZD6126, the antitumor effect is not complete as a narrow rim of viable tumor cells often remains at the periphery (16). This is thought to occur as a result of nutrients and oxygen being obtained by diffusion from the surrounding normal vasculature. Although residual tumor tissue can rapidly reestablish the tumor, its high proliferation rate and host factors (23–25). Therefore, the host organ microenvironment surrounding the tumor should always be considered when treating cancer. The characteristics of metastases in different organs may differ; for example, we have previously shown that the antimetastatic effect of both macrophage colony-stimulating factor (26) and an inhibitor of matrix metalloproteinase (27) are specific to particular organs, although both macrophage colony-stimulating factor and matrix metalloproteinase inhibitors dramatically block the growth of s.c. inoculated tumor cells. For this reason, metastatic models should be used in preference to s.c. xenograft models for evaluating the therapeutic potential of new anticancer agents.

From a clinical point of view, the timing and sequencing of antivascular agents and chemotherapeutic drugs are consider- able. In general, marked enhancements in antitumor activities were demonstrated when the vascular targeting agents were delivered within a few hours after chemotherapy (18, 22). These seem to be reasonable because administration of vascular-targeting agents after chemotherapy may not affect delivery of chemotherapeutic drugs to tumors but rather cause trapping of the drugs in the tumors. Therefore, we administered ZD6126 1 hour after cisplatin treatment in the in vivo experiments. On the other hand, in the in vitro experiments, we treated endothelial cells with ZD6126 for 1 hour and then with cisplatin for 72 hours. ZD6126 is known to be rapidly cleared from plasma (18) and presumably cause trapping of the cisplatin in the tumors (18). To mimic these pharmacodynamics, we chose this experimental condition for in vitro experiments.

This model appears to be important in evaluating the efficacy of anticancer agents in vivo. Tumor cells exhibit heterogeneous biological and metastatic properties, with the outcome of metastasis being dependent on the properties of both tumor cells and host factors (23–25). Therefore, the host organ microenvironment surrounding the tumor should always be considered when treating cancer. The characteristics of metastases in different organs may differ; for example, we have previously shown that the antimetastatic effect of both macrophage colony-stimulating factor (26) and an inhibitor of matrix metalloproteinase (27) are specific to particular organs, although both macrophage colony-stimulating factor and matrix metalloproteinase inhibitors dramatically block the growth of s.c. inoculated tumor cells. For this reason, metastatic models should be used in preference to s.c. xenograft models for evaluating the therapeutic potential of new anticancer agents.

**Table 1** Effect of combined administration of ZD6126 and cisplatin on the formation of lung metastasis by lung cancer cell lines in nude mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals with tumor(s)/total</th>
<th>No. of lung metastases</th>
<th>Lung weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9/9</td>
<td>45 (24–72)</td>
<td>573 (298–683)</td>
</tr>
<tr>
<td>ZD6126</td>
<td>10/10</td>
<td>22 (6–81)</td>
<td>343 (284–628)*</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>10/10</td>
<td>25 (6–71)</td>
<td>407 (267–749)</td>
</tr>
<tr>
<td>ZD6126 + cisplatin</td>
<td>9/9</td>
<td>16 (5–33)*</td>
<td>258 (203–287)*†</td>
</tr>
</tbody>
</table>

NOTE. PC14PE6-bearing nude mice received cisplatin (6 mg/kg i.v.) once on day 14, ZD6126 (100 mg/kg i.p.) daily from day 14 to day 34, or a combination of the two agents. The mice were sacrificed on day 35.

* Statistically significant difference ($P < 0.05$) compared with the value obtained in the absence of treatment.

† Statistically significant difference ($P < 0.05$) compared with the value obtained from each individual treatment (ZD6126 or cisplatin).
In summary, this study has shown that administration of ZD6126 therapy alone could induce apoptosis of tumor-associated endothelial cells but not endothelial cells in normal parenchyma, resulting in hemorrhage and necrosis of established lung metastases. In addition, combined use of ZD6126 with a clinically available anticancer agent, cisplatin, increased apoptosis of tumor-associated endothelial cells and enhanced the anti metastatic effect. Although additional evaluations of the therapeutic potential are necessary, combined use of vascular-targeting agents with conventional chemotherapy may provide a promising therapeutic option for treating patients with advanced lung cancer and metastasis.

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