Activity of a New Vascular Targeting Agent, ZD6126, in Pulmonary Metastases by Human Lung Adenocarcinoma in Nude Mice

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ABSTRACT

ZD6126 (ANG453) is a novel vascular targeting agent that selectively disrupts the cytoskeleton of endothelial cells in tumor. In mouse s.c. xenograft models, ZD6126 was found to induce selective occlusion of tumor blood vessels, cessation of tumor blood flow, and death of tumor cells because of the starvation of oxygen and nutrition. Here, we investigated whether ZD6126 inhibited the metastatic formation of human nonsmall cell lung cancer cells. PC14PE6 (adenocarcinoma) and H226 (squamous cell carcinoma) cells were injected into the tail vein of nude mice, and lung metastases were estimated. ZD6126 treatment involved either a single dose on 24 h before killing or daily doses from day 14 until the end of the experiment. Single treatment with i.p. injection of 200 mg/kg ZD6126 caused bleeding and necrotic changes in the tumor by 24 h. Histological analysis revealed that apoptotic tumor cells were markedly increased in the ZD6126-treated group. Moreover, ZD6126 induced the apoptosis of CD31-positive vascular endothelial cells in tumors but not in the normal lung parenchyma. When mice were treated daily with 100 mg/kg ZD6126 from day 14 until the end of the experiment, the lung weight was significantly less in the ZD6126-treated group than that of the control group, despite no difference in the number of metastatic nodules. These data suggest that ZD6126 could demonstrate its antitumor activity against both already established and early phase of lung cancer metastasis by causing the selective apoptosis of tumor endothelial cells and destruction of the tumor vasculature.

INTRODUCTION

Lung cancer is a leading cause of malignancy-related death worldwide, and >90% of deaths from lung cancer can be attributed to metastasis. One of the major obstacles in treating metastases is that tumors are biologically heterogeneous and contain subpopulations of cells with different angiogenic, invasive, and metastatic properties. To produce metastasis, tumor cells must complete a series of sequential and selective steps (1, 2). Failure to complete even one step eliminates the cells from the process (3). Therefore, therapy of cancer metastasis can be targeted not only against tumor cells but also against host homeostatic factors that facilitate this process, and this host microenvironment seems to be important when treating cancer metastases.

Although tumors 1–2 mm in diameter can receive all nutrients by diffusion, further growth depends on the development of an adequate blood supply, i.e., angiogenesis. Many studies have consistently reported that the angiogenesis plays an essential role in progression of solid tumors and metastases, including lung cancer, and inhibition of angiogenesis may provide a novel and more general approach for treating metastases by manipulation of the host environment (4–7).

To inhibit angiogenesis, two major strategies could be considered: (a) one is to block the angiogenic factor that is produced by either tumor cells or host cells; and (b) the other is to disrupt tumor endothelial cells directly and arrest blood flow in tumors. Most antiangiogenic agents, such as matrix metalloproteinase inhibitors, have been developed on the basis of the former concept. However, effects with antiangiogenic agents seen to date are far from adequate, and other approaches are being studied (8, 9). In this study, we focused on an alternative strategy known as “vascular targeting” therapy.

It has been reported that tumor blood vessels differ significantly from vessels in normal tissues, e.g., tumors contain a chaotic network of tortuous thin-walled vessels, within which there is a significant proportion of neovasculature because of a relatively high proportion of proliferating endothelial cells (10, 11). These specific features of neovasculature might provide targets with selectivity for tumors, including adhesion molecule expression, altered coagulation control, and increased permeability (10). As a therapeutic approach, vascular targeting aims to exploit the distinctive features of 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 a tumor normally resistant to conventional therapies (13–15). This vascular targeting strategy is therefore distinct from antiangiogenic approaches that aim primarily to prevent new vessel formation to restrict tumor growth.

ZD6126 is a novel vascular targeting agent that was developed for its tubulin-binding properties and its ability to induce vascular damage in tumors. It is a phosphate produrg of the tubulin binding agent N-acetylcolchinol (ZD6126 Phenol) that inhibits microtubule polymerization. Release of ZD6126 Phenol by phosphatases in vivo leads to the selective disruption of the cytoskeleton of tumor endothelial cells. This results in selective occlusion of tumor blood vessels, cessation of tumor blood flow, and death of tumor cells because of the starvation of oxygen and nutrition in mouse s.c. xenograft models (16). However, the effect of ZD6126 on metastatic tumor is not well understood.

The aim of this study was to assess the antitumor effect of ZD6126 on lung cancer metastasis. Specifically, using a lung metastasis model, we have examined the effect of ZD6126 on both early and advanced phases of lung metastases. In addition, immunohistochemical technique was used to determine the effect of ZD6126 on tumor cells and endothelial cells in the metastatic lung tumor, and possible underlying mechanisms of action of this novel compound are discussed.

MATERIALS AND METHODS

Cell Lines. The human lung adenocarcinoma cell line PC14PE6 was a kind gift from Dr. I. J. Fidler (17). The human lung squamous cell carcinoma cell line H226 was obtained from Dr. J. D. Minna (University of Texas Southwestern Medical School, Dallas, TX; Ref. 18). PC14PE6 cells were maintained in RPMI 1640, and H226 cells were maintained in DMEM. Both media were supplemented with 10% fetal bovine serum and penicillin-streptomycin. Cells were cultured in a humidified CO2 incubator at 37°C. HMEC3 (Kurabo, Japan).
Osa, Japan) were maintained in HuMedia-MvG with growth supplements (Kurabo) and used for in vitro assays at passage 2–5.

Reagents. ZD6126 was synthesized by AstraZeneca (Cheshire, United Kingdom). Cisplatin, VP-16, and Paclitaxel were provided by Bristol-Myers Squibb (Princeton, NJ). Docetaxel and Vincristine were from Aventis Pharma (Strasbourg, France) and Eli Lilly (Indianapolis, IN), respectively.

Animals. Male athymic BALB/c nude mice, 5 weeks old, were obtained from CLEA Japan (Osaka, Japan) and maintained under specific pathogen-free conditions throughout this study. Experiments were performed according to the guidelines of our university.

Experimental Metastasis in Vivo. For production of lung metastasis, 1 × 10⁶ PC14PE6 or 5 × 10⁵ H226 cells suspended in 0.2 ml of PBS were injected i.v. into the tail vein of nude mice (17). After the injection of PC14PE6 or H226 cells, mice were humanely killed on days 35 (PC14PE6) or 56 (H226), respectively, for measurement of pulmonary nodules.

Histology and Immunohistochemistry. After killing the mice, the lungs were fixed in Bouin’s solution or cut into 5-mm fragments and placed into either buffered 10% formalin solution or OCT compound (Miles Laboratories, Elkhart, IN) to be snap frozen in liquid nitrogen for immunohistochemical analysis. TUNEL method was performed after the protocol of 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 permethylated 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 deoxynucleotidyltransferase enzyme. The reaction was stopped with 2 × SSC and washed, and localized green fluorescence of apoptotic cells was detected by fluorescence microscopy. For double staining for TUNEL and CD31, frozen tissue sections were fixed with cold acetone. After washing the slides, TUNEL method was first performed as described, and then samples were incubated for 10 min at room temperature with protein-blocking solution consisting of PBS and 5% fetal bovine serum. Excess blocking solution was drained, and the samples were incubated for 18 h at 4°C with a 1:200 dilution of rat antimonoclonal antibody (PharMingen, San Diego, CA). The samples were then rinsed with PBS and incubated for 60 min at room temperature with the appropriate dilution of Texas Red-conjugated goat antirat IgG (Vector Laboratories, Burlingame, CA). Sections (4-µm thick) of formalin-fixed, paraffin-embedded tissues were also stained with H&E for routine histological examination.

Cell Proliferation Assay. Cell proliferation was measured by the MTT dye reduction method (19). Briefly, 2 × 10³ cells/100 µl were plated into each well of 96-well plates in medium and incubated at 37°C under 5% CO₂ in humidified air. After 24 h, 100 µl of various concentration of anticancer drugs or ZD6126 were added and incubated for an additional 72 h. After incubation, 50 µl of stock MTT solution (2 mg/ml; Sigma, St. Louis, MO) were added to all wells, and the cells were incubated for 2 h at 37°C. The media containing MTT solution were removed, and the dark blue crystals were dissolved by adding 100 µl of DMSO. Absorbance was measured with an MTP-120 microplate reader (Corona Electric, Ibaraki, Japan) at test and reference wavelengths of 550 and 630 nm, respectively.

Statistical Analysis. The significance of differences of in vivo data was analyzed by the Mann-Whitney t test, and the significance of differences of in vitro data was analyzed by Student’s t test (two-tailed).

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CA4P, Combretastatin A4 phosphate.

Table 1 Effect of single administration of ZD6126 on the formation of lung metastasis by lung cancer cell lines in nude mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Group</th>
<th>Mice with tumor/total</th>
<th>No. of lung metastasis</th>
<th>Lung weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC14PE6</td>
<td>A</td>
<td>4/4</td>
<td>50.5 (28–73)</td>
<td>487 (332–632)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3/3</td>
<td>25 (13–34)</td>
<td>508 (367–523)</td>
</tr>
<tr>
<td>H226</td>
<td>A</td>
<td>5/5</td>
<td>&lt;150</td>
<td>275 (238–310)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>5/5</td>
<td>&lt;150</td>
<td>273 (243–306)</td>
</tr>
</tbody>
</table>

* PC14PE6 or H226 cells were injected into the tail vein of nude mice on day 0. Experimental lung metastasis was determined on days 35 (PC14PE6) or 56 (H226).
  
1 Group A: PBS-treated (control) group. Group B: The mice were treated with ZD6126 (200 mg/kg) 24 h before killing.
nearly undetectable. No difference in the number of CD31-positive cells was observed between control and ZD6126-treated tumors (microvessel density was 15 and 15.3, respectively). These findings suggest that ZD6126 selectively leads to tumor endothelial cell apoptosis, whereas it has a minimum effect on normal endothelial cells.

Selective Inhibition of Endothelial Cell Proliferation by ZD6126 in Vitro. To confirm the selectivity of ZD6126 against tumor endothelial cells, we next examined the effect of ZD6126 on the proliferation of PC14PE6 and H226 cells and HMVECs in vitro using MTT assay. Because endothelial cells in tumor blood vessels divide rapidly, whereas those in normal tissues do not, we decided to use growing HMVECs as a mimicry of tumor endothelial cells. ZD6126 inhibited proliferation of HMVECs at lower concentrations, which could not affect proliferation of PC14PE6 or H226 cells (Fig. 5A), indicating that ZD6126 selectively inhibited the endothelial cell proliferation in vitro. On the other hand, no significant difference was seen in other anticancer drugs that target tubulin function or are used for non-small cell lung cancer (Fig. 5, B–F). IC₅₀s of ZD6126 concentration against PC14PE6 and H226 cells and HMVECs were 241.1, 311.5, and 48.7 ng/ml, respectively.

Effect of Continuous Administration of ZD6126 on Metastatic Lung Tumors. 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 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. Treatment with 50 mg/kg ZD6126 had no effect on the number of metastatic nodules or lung weight (Table 2). Treatment with 100 mg/kg ZD6126 inhibited lung weight, although the reduction in the number of lung metastases did not reach statistical significance. These results suggest that ZD6126 inhibits the tumor growth by disrupting the feeding vessels, although it does not have a strong effect to kill tumor cells. Treatment with ZD6126 was well tolerated, and loss of body weight was not observed throughout this study.

DISCUSSION

The facts that we have shown in this study are: (a) single administration of ZD6126 could induce the hemorrhage and necrosis of already established lung metastases; (b) ZD6126 could lead to apoptosis of endothelial cells in the tumors but not in the normal lung parenchyma; (c) ZD6126 affected endothelial cells in the tumors more selectively rather than tumor cells; and (d) continuous administration of ZD6126 reduced tumor burden in the lung but not the number of metastatic nodules.
The process of metastasis consists of linked sequential steps. More than a century ago, Paget proposed that metastasis is attributable to the specific affinity of certain tumor cells (the “seed”) for the milieu provided by certain organs (the “soil”; Ref. 20). This seed and soil hypothesis consists of the concepts that neoplasms exhibit heterogeneous biological and metastatic properties, and the outcome of metastasis depends on the properties of both tumor cells and host factors. Accordingly, when treating cancer, we must always consider the host organ microenvironments which surround the tumor. If metastases occur in multiple organs, the character of each metastatic tumor might be different. Indeed, we have reported previously the evidence showing that antimetastatic effect of macropage colony-stimulating factor may be specific to particular organs, suggesting the influence of organ microenvironment on antimetastatic effect of macrophage colony-stimulating factor (21). Therefore, models of cancer metastasis are important to evaluate the effect of anticancer agents. In this study, we have assessed the antitumor effect of ZD6126 using a lung metastasis model. This model seems to resemble the clinical situation better than s.c. xenografts.

Among several steps to complete metastasis, angiogenesis takes one of the most critical parts (11). The blockade of angiogenesis might prevent the growth of tumor cells at both the primary and secondary sites and thus prevent the emergence of metastases and lead the tumor to dormancy. The tumor dormancy would result in the prolongation of survival and improvement of quality of life for cancer patients with advanced stage. From this point of view, dozens of molecular-targeted agents (mostly angiogenesis targeted) have been developed to overcome the cancer metastasis, and many experimental studies have shown that angiogenesis-targeted agents are effective enough to control tumor cell growth and metastases (22–25). Once they proceed to clinical phase studies, however, the outcome thus far is not adequate and leaves room for improvement (8, 9). What makes this discrepancy? In view of this conflict, several studies have demonstrated that down-regulation of the expression of angiogenic factors, such as basic fibroblast growth factor, vascular endothelial growth factor, or fibroblast growth factor-binding protein, after the tumor had reached a certain size did not affect further tumor growth, although it resulted in a marked inhibition of small tumors (26–28). Antiangiogenic therapy seems to be effective on the early phase, when tumors grow from undetectable size (8, 9). At this phase, the formation of tumor-associated blood vessels begins, and thus antiangiogenic agents seem to control tumor growth. However, in most cases we come across in the clinical situation, tumors have already grown large enough that we could diagnose them by X-ray. Antiangiogenic agents might have less effect at this time because angiogenesis has been already completed to a certain extent. Thus, antiangiogenic therapies are thought to be more efficient in preventing or delaying tumor relapse and/or the appearance of metastases by inducing tumor dormancy rather than in causing tumor regression (29–33).

From this point of view, ZD6126 has a completely new aspect. Besides the antitumor effect of continuous administration from early phase of metastasis, ZD6126 has a direct effect on tumor endothelial cells and leads them to undergo apoptosis in established lung metastases. This event would result in the cessation of tumor blood flow and indirect death of tumor cells because of the starvation of oxygen and nutrition. Tumor cell death was detected by an apoptosis stain that would stain both apoptotic and necrotic cells, so it is not clear from this study if the tumor cell death was because of apoptosis or necrosis.

Previous studies with ZD6126 have indicated by H&E staining that after rapid blood vessel blockade by forming the thrombus formation and detachment of endothelial cell coverage, tumor cells undergo necrosis rather than apoptosis (34). H&E staining of the treated metastases in this study also indicated tumor cell death because of necrosis, which is the expected route of cell death if the tumor cells die because of nutrient starvation after occlusion of the tumor blood vessels. Thus, one can expect ZD6126 to affect established tumors, which we usually meet in the clinical situation.

Another feature of ZD6126 is that this compound attacks tumor endothelial cells but not normal endothelial cells. Furthermore, it inhibits the growth of endothelial cells more selectively rather than tumor cells. Although other tubulin-binding inhibitors, such as docetaxel, also have an antiangiogenic activity (35), its effect was not selective on endothelial cells. The mechanism of the selectivity of ZD6126 on lung cancer metastasis.

### Table 2: Effect of continuous administration of ZD6126 on the formation of lung metastasis by PC14PE6 cells in nude mice

<table>
<thead>
<tr>
<th>ZD6126 (mg/kg)</th>
<th>Mice with metastasis</th>
<th>No. of lung metastasis</th>
<th>Lung weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9/10</td>
<td>77 (0–93)</td>
<td>550 (168–663)</td>
</tr>
<tr>
<td>50</td>
<td>10/10</td>
<td>83 (47–108)</td>
<td>473 (321–600)</td>
</tr>
<tr>
<td>100</td>
<td>9/10</td>
<td>57 (0–102)</td>
<td>333 (250–484)</td>
</tr>
</tbody>
</table>

PC14PE6 cells were injected into the tail vein of nude mice on day 0. Experimental lung metastasis was determined on days 35.

*Statistically significant difference (P < 0.05) compared with the value obtained in the absence of treatment.
ZD6126 against tumor endothelial cells remains unclear, but mutations of tubulin may be responsible for this phenomenon. Additional examinations are warranted to clarify this mechanism.

Concerning the therapeutic efficacy of ZD6126, total tumor volume but not the number of metastatic nodules was reduced by continuous administration. Continuous administration of ZD6126 in addition to inducing acute antivascular effects may also inhibit formation of new blood vessels (angiogenesis). The data in Table 2 indicate that the effect of ZD6126 might result in the delay of each tumor growth.

Several vascular targeting agents, such as CA4P, have been developed and evaluated their antitumor activity. ZD6126, however, seems to be less toxic than CA4P. Indeed, the data from CA4P Phase I studies indicate ataxia and neurotoxicity as dose-limiting toxicities (36). On the other hand, neither ataxia nor neurotoxicity have been observed in the ZD6126 Phase I study (final data from ZD6126 Phase I study will be presented at ASCO 2002).

In conclusion, ZD6126 is a new vascular targeting agent that selectively targets the tumor-associated endothelial cells and leads to tumor death by effects on tumor vasculature involving induction of endothelial cell apoptosis. Besides the therapeutic efficacy on early stage tumor, ZD6126 could be distinct from other antiangiogenic agents in view of the efficacy against established metastatic tumor. ZD6126 is currently in Phase I clinical trials.

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