Combination Therapy Including a Gelatinase Inhibitor and Cytotoxic Agent Reduces Local Invasion and Metastasis of Murine Lewis Lung Carcinoma

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Abstract

The efficacy of combination therapy including an oral gelatinase inhibitor (CT1746) and cytotoxic agent was analyzed using the murine Lewis lung carcinoma model. Primary tumors, pulmonary metastases, and sera from tumor-bearing animals had increased gelatinase B activity that was inhibited by CT1746 levels achievable in vivo. The combination of CT1746 and cyclophosphamide (CTX) was significantly more effective than either single agent in inhibiting tumor growth (CT1746/CTX, 30.9 ± 1.7 days; CT1746, 26 ± 0.3 days; CTX, 19.5 ± 1.1 days; P < .001) and reducing the number and size of pulmonary metastases (CT1746/CTX, 5 ± 2 (15% metastases > 3 mm); CT1746, 15 ± 4 (55% > 3 mm); CTX, 11 ± 3 (63% > 3 mm); no treatment, 24 ± 5 (62% > 3 mm); P < .001). These data support the notion of combining matrix metalloproteinase inhibitors and cytotoxic agents to treat certain epithelial malignancies.

Introduction

Lung cancer remains largely incurable because of the early dissemination of tumor cells and the lack of effective therapy for metastatic disease. Current therapeutic approaches based on killing tumor cells with cytotoxic agents have had only modest benefits. A complementary approach to the treatment of lung cancer involves modifying the tumor microenvironment and reducing the propensity for tumor cell invasion, neovascularization, and metastasis. One mechanism for modifying the tumor microenvironment would be to inhibit local matrix metalloproteinase (MMP) activity. This approach holds particular promise because MMPs degrade the structural support network for malignant cells and promote the neovascularization of tumor cell deposits (1-3).

In an experimental metastasis model, tumor cells transfected with human gelatinase A were more capable of colonizing the lung after their injection into the tail veins of nude mice, and treatment reversed this with a gelatinase inhibitor reversed this effect (4). In similar studies, transfection with gelatinase B also conferred metastatic ability (5). Furthermore, tumor cell attachment and angiogenesis are also influenced by gelatinase activity (6, 7). For example, antibodies directed against gelatinases A and B reduced the endothelial cell invasion and capillary tube formation necessary for the development of tumor vasculature (7, 8).

Gelatinase expression and activity have been specifically implicated in human NSCLC pathophysiology. For example, human NSCLC cell lines with increased gelatinase expression were more invasive in a rodent model (9). In addition, primary NSCLC specimens had significantly greater gelatinase A and B activity than adjacent normal lung tissue (10). Furthermore, in patients with NSCLC, circulating levels of gelatinase A were correlated with the stage of disease at presentation and responses to cytotoxic therapy (11). Taken together, these data provide a rationale for assessing the therapeutic benefits of a gelatinase inhibitor in human NSCLC.

Additional studies using rodent lung cancer models provide the basis for combining a MMP inhibitor with standard chemotherapeutic agents. In these early studies, the combination of a weak broad spectrum MMP inhibitor (minocycline), an inhibitor of angiogenesis (TNP-470), and a standard chemotherapeutic agent was more effective than any of the single agents in reducing local invasion and distant metastases (12). These preliminary studies suggested that combined modality therapy with cytotoxic agents and specific MMP inhibitors might be more effective than either modality alone. For these reasons, we have evaluated the combination of an oral gelatinase inhibitor (CT1746) and standard chemotherapy using the murine Lewis lung carcinoma model.

Materials and Methods

Lewis Lung Carcinoma Model

The Lewis lung tumor (13) was carried in male C57BL/6 mice (Taconic Farms, Germantown, NY). Tumor cells (2 X 10⁶) were prepared from a brei of several stock tumors and implanted s.c. into the hind legs of 8-10-week-old mice (12).

Gelatin Zymography

On day 18, specimens from a cohort of tumor-bearing and normal control animals were obtained for gelatin zymography. Whole blood was obtained from anesthetized tumor-bearing and control animals by direct cardiac puncture. Circulating cells were removed by centrifugation, and sera were snap frozen at −70°C. The animals were then euthanized and primary tumors, uninvolved gastrocnemius muscle, and lungs containing metastatic tumor deposits were obtained from the tumor-bearing cohort, and normal gastrocnemius muscle and lung were obtained from a normal control group. Thereafter, tissue specimens were snap frozen at −70°C and subsequently homogenized in nonreducing sample buffer. After protein concentrations of tissue lysates and sera were determined (Bio-Rad protein assay, Bio-Rad, Hercules, CA), 10-µg aliquots were diluted in sample buffer and size fractionated in duplicate with 8.3% polyacrylamide gels embedded with 1 mg/ml gelatin (Sigma, St. Louis, MO; Ref. 14). The duplicate gels were washed with 2.5% Triton X-100 for 2 h and incubated overnight in 50 mM Tris-HCl (pH 7.6) and 10 mM CaCl₂ at 37°C in the presence or absence of 100 mM 1,10-phenanthroline. Gels were subsequently stained with Coomassie brilliant blue at 65°C for 30 min and destained with 10% acetic acid until gelatinolytic bands were clearly visible. Thereafter, gels were dehydrated in 20% ethanol/10% glycerol and preserved in cellophane (Integrated Separation Systems, Natick, MA).

Treatment Regimens

Lewis lung carcinomas were implanted s.c. into the hind legs of an additional series of male C57BL/6 mice. Mice were subsequently treated with the gelatinase inhibitor CT1746 alone, one of two standard chemotherapeutic agents, and a combination of an MMP inhibitor (CT1746) and standard chemotherapy using the murine Lewis lung carcinoma model.
agents (CDDP or CTX) alone, or the combination of CT1746 and CDDP or CTX.

**Gelatinase Inhibitor.** A screen for orally active gelatinase inhibitors led to the identification of CT1746 (N1-[2-(S)-(3,3-dimethylbutanamidyl)]-N4-hydroxy-2-(R)-[3-(4-chlorophenyl)-propyl]succinamide; Ref. 15; synthesized by Andrew T. Millican, Celltech Therapeutics Ltd., Slough, Berkshire, United Kingdom). CT1746 has Kis against human gelatinase A, gelatinase B, stromelysin 1, collagenase, and matrilysin of 0.04, 0.17, 10.9, 122, and 136 nM, respectively (Kis performed by Jimi P. O’Connell, Celltech Therapeutics Ltd.). It has negligible activity (> 40 μM) against other classes of metalloproteinase such as neprilysin (EC 24.11), meprin, pepidyl dipeptidase A (ACE), and aminopeptidase N, and, at a concentration of 1 mM, has no apparent cytotoxicity. In previous studies of mice given 10 mg/kg CT1746 by gavage, blood levels of the gelatinase inhibitor were ~200 nM for at least 8 h following CT1746 treatment (15). For this reason, CT1746 formulated in propylene glycol was administered p.o. (100 mg/kg) by gavage twice daily on days 4–18, 7–18, or 12–18 following tumor implantation in the current experiments. Vehicle (propylene glycol) alone was administered twice daily on days 4–18 after tumor implantation.

**Chemotherapy.** Full-dose single-agent CDDP or CTX was administered on an optimal schedule (12). Tumor-bearing mice received 10 mg/kg CDDP i.p. (Sigma) on day 7 or 150 mg/kg CTX i.p. on days 5, 7, 9, and 11 after tumor implantation.

**Combined Gelatinase Inhibitor and Chemotherapy.** Single-agent CDDP or CTX was administered in optimal doses as above and CT1746 was administered on days 4–18, 7–18, or 12–18 after tumor implantation. In each experiment, cohorts of six animals were treated with each regimen and schedule. The entire experiment was repeated three times.

**Analysis of Therapeutic Efficacy**

Tumor-bearing mice had tumor measurements taken three times per week. Two animals per group were sacrificed on day 20 for pathological analysis. The therapeutic efficacy of CT1746 alone, optimal dose single-agent CDDP or CTX, or combined CT1746 and CDDP or CTX was assessed using the parameters of tumor growth delay and incidence and size of pulmonary metastases.

**Tumor Growth Delay.** The growth of s.c. Lewis lung carcinomas was measured three times per week using calipers. Tumor diameters were measured and tumor volumes were calculated as hemiellipsoid (long diameter × short diameter × π/6) in treated and untreated C57BL/6 animals. Tumor growth delay was defined as the number of extra days required for the local s.c. tumor to reach a volume of 500 mm³ in treated animals as compared to untreated control animals.

**Pulmonary Metastases.** Treated and untreated tumor-bearing C57BL/6 mice were sacrificed on day 20 after s.c. tumor implantation for analysis of pulmonary metastases. In each animal, lungs were removed and surface lung metastases were counted manually. A total of 12 lungs were analyzed from each treated and untreated cohort of animals. Individual surface lung metastases were also measured and scored as being ≤ or > 3 mm.

**Results and Discussion**

**Gelatinase Activity in Lewis Lung Carcinoma**

The well-characterized syngeneic Lewis lung carcinoma model was chosen for these studies because the tumor is relatively resistant to many cancer therapies and avidly metastasizes to the lungs after s.c. implantation. To determine whether gelatinases A and/or B might be implicated in the growth and dissemination of Lewis lung carcinoma, we compared gelatinase activity in primary tumors, pulmonary metastases, and sera from tumor-bearing animals with that in muscle, lung, and sera from untreated control animals (Fig. 1). Gelatinase A and B activities were identified by molecular weight (Fig. 1a) and inhibition of gelatinolytic activity with the MMP inhibitor 1,10-phenanthroline (Fig. 1b).

![Fig. 1. Gelatin zymography of specimens from tumor-bearing and normal control mice. Specimens from tumor-bearing mice (primary tumors 1 and 2, lung metastases, and serum (tumor 1, tumor 2)) from tumor-bearing mice as well as specimens from normal control animals (normal muscle, lung, and serum) were size fractionated on gelatin-embedded polyacrylamide gels. Thenceforth, gels were incubated in the absence (a) or the presence (b) of the broad spectrum MMP inhibitor 1,10-phenanthroline prior to staining.](cancerres.aacrjournals.org)
Lewis lung carcinomas prompted us to examine further CT1746 in combination with standard chemotherapy.

**Treatment of Tumor-bearing Animals with a Gelatinase Inhibitor in Combination with Chemotherapy**

In untreated 8–10-week-old male C57BL6 mice given $2 \times 10^6$ Lewis lung carcinoma cells s.c., the following events occurred after tumor cell implantation: (a) primary tumor cells underwent neovascularization by approximately day 4; (b) primary tumors grew to nearly 100 mm$^3$ by day 7; and (c) animals succumbed to metastatic lung carcinoma by days 21–25. For these reasons, the end points of the proposed studies were tumor growth delay and incidence of pulmonary metastases.

**Tumor Growth Delay.** As predicted by previous studies, single-agent CDDP (10 mg/kg) produced a moderate tumor growth delay (4.5 ± 0.3 days) when administered as a single agent (Table 1 and Fig. 2). Single-agent CTX (150 mg/kg on days 5, 7, 9, and 11) was more effective, delaying local tumor growth by 19.5 ± 1.1 days (Table 1 and Fig. 2). Whereas single-agent CT1746 administered on days 4–18 after tumor implantation delayed local tumor growth by 2.6 ± 0.3 days (Table 1 and Fig. 2), CT1746 begun on day 7 or 12 after tumor implantation did not significantly delay local tumor growth (Table 1). Although single-agent CT1746 had a modest effect on local tumor growth, the combination of CT1746 and CDDP or CTX was significantly more active. For example, the optimal combination of CT1746 and CDDP produced a tumor growth delay of 12.4 ± 0.7 (p < 0.001) days, and the optimal combination of CT1746 and CTX delayed tumor growth by 30.9 ± 1.7 days ($P < .001$) (Table 1 and Fig. 2). Of interest, the most effective schedule of administration of CT1746 was one in which the drug was begun on day 4 rather than day 7 or 12 after tumor implantation (Table 1). These data suggest that the gelatinase inhibitor may be most effective when it is administered under conditions of low tumor volume.

**Pulmonary Metastases.** To determine whether treatment with CT1746 and CDDP or CTX might also reduce the implantation and/or growth of metastatic disease, we analyzed the numbers and sizes of pulmonary metastases in animals treated on the above-mentioned schedules (Table 2). As expected, the single agents CDDP and CTX reduced pulmonary metastases to 75% (19 ± 4/24 ± 5) and 46% (11 ± 3/24 ± 5, $P < .001$) of observed baseline values (Table 2 and Fig. 2). With the optimal schedules of administration, single-agent CT1746 also reduced pulmonary metastases to 67% (15 ± 4/24 ± 5, $P < .001$) of baseline values (Table 2 and Fig. 2). However, the combination of CDDP and optimal dose CT1746 further reduced pulmonary metastases to 33% (8 ± 3/24 ± 5, $P < .001$) of controls, whereas that of CTX and optimal dose CT1746 further decreased metastases to 21% (5 ± 2/24 ± 5, $P < .001$) of baseline values (Table 2 and Fig. 2). Taken together, these data suggest that the combination of a gelatinase inhibitor and effective chemotherapeutic agent may also reduce distant metastases.

Because tumors >3 mm are thought to require an effective vasculature, the percentage of metastases >3 mm was assessed as an indicator of tumor angiogenesis (16). As noted in Table 2, the percentage of pulmonary metastases >3 mm was comparable in animals treated with vehicle alone, CTX, CT1746, CDDP, or CT1746 and CDDP. However, the percentage of pulmonary metastases >3 mm was reduced from 55% to 15% in animals treated with CTX and CT1746 (Table 2 and Fig. 2). Since CTX is a more active single agent than CDDP or CT1746 in this animal model (Tables 1 and 2), a certain level of efficacy may be required to impact on tumor angiogenesis. Given the demonstrated importance of tumor angiogenesis in NSCLC (17), the data support the hypothesis that combination therapy with a...
gelatinase inhibitor and effective chemotherapeutic regimen may make inroads in this disease.

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References

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