Rapamycin Inhibits the Growth and Metastatic Progression of Non-Small Cell Lung Cancer

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ABSTRACT

Purpose: Lung cancer has a dismal prognosis and comprises 5.5% of post-transplant malignancies. We explored whether rapamycin inhibits the growth and metastatic progression of non-small cell lung cancer (NSCLC).

Experimental Design: Murine KLN-205 NSCLC was used as the model tumor in syngeneic DBA/2 mice to explore the effect of rapamycin on tumor growth and metastatic progression. We also examined the effect of rapamycin on cell cycle progression, apoptosis, and proliferation using murine KLN-205 NSCLC cells and human A-549 NSCLC cells as targets. The in vivo and in vitro effects of cyclosporine and those of rapamycin plus cyclosporine were also investigated.

Results: Rapamycin but not cyclosporine inhibited tumor growth; s.c. tumor volume was 1290 ± 173 mm³ in untreated DBA/2 mice, 246 ± 80 mm³ in mice treated with rapamycin, and 1203 ± 227 mm³ in mice treated with cyclosporine (P < 0.001). Rapamycin but not cyclosporine prevented the formation of distant metastases; eight of eight untreated mice and four of six mice treated with cyclosporine developed pulmonary metastases whereas only one of six mice treated with rapamycin developed pulmonary metastases (P = 0.003). In vitro, rapamycin induced cell cycle arrest at the G₁ checkpoint and blocked proliferation of both KLN-205 and A-549 cells but did not induce apoptosis. Cyclosporine did not prevent cell cycle progression and had a minimal antiproliferative effect on KLN-205 and A-549 cells.

Conclusions: The immunosuppressive macrolide rapamycin but not cyclosporine prevents the growth and metastatic progression of NSCLC. A rapamycin-based immunosuppressive regimen may be of value in recipients of allografts.

INTRODUCTION

Lung cancer is the leading cause of cancer mortality in the United States. More than 28% of all cancer-related deaths result from primary lung tumors. The majority of patients fail current treatment strategies. The 5-year-combined survival rate is a dismal 14% (1, 2).

The incidence of lung cancer in solid organ recipients such as cardiac allograft recipients is higher than that of the general population, and survival rates are lower (3, 4). The tumors are generally stage III or higher when diagnosed, with a median survival of only 1–3 months (5, 6).

Rapamycin, a macrolide antifungal agent, is a potent immunosuppressant (7–9). In some but not all post-transplant immunosuppression protocols, rapamycin is currently used as a component of a multidrug regimen (10). Rapamycin binds the immunophilin FKBP12, targets, and inactivates mammalian target of rapamycin, a serine threonine kinase (11–14). Mammalian target of rapamycin, activated after the ligation of a growth factor receptor (14), promotes translation by phosphorylating 4E-BP1 and forces the release of the initiation factor eIF-4E (15). In addition, ribosome formation is enhanced by phosphorylation of p70-S6-kinase. The net result is increased expression of cell cycle-related genes such as cyclin D1 and cell cycle progression from G₁-S phase. Thus, rapamycin blocks proliferation by inducing cell cycle arrest at the transition from G₁-S phase (16).

Earlier studies suggest that rapamycin has potent antitumor activity against certain tumor cell types (9). The important issue of whether rapamycin inhibits the growth of non-small cell lung cancer (NSCLC) cells has not been examined. In a series of interrelated in vitro and in vivo studies, we explored whether rapamycin blocks the growth and metastatic progression of NSCLC.

MATERIALS AND METHODS

Mice. Eight- to ten-week-old male DBA/2 mice were purchased from Taconic (Germantown, NY). Mice were kept in the Cornell Medical Center animal housing facility. Food and water were given ad libitum. The experiments were approved by the Institutional Animal Care and Use Committee.

Reagents and Cell Lines. Rapamycin was purchased from Wyeth (Philadelphia, PA). Cyclosporine (CsA) was purchased from Novartis Pharmaceuticals (Summit, NJ). Murine KLN-205 is a NSCLC line originally induced in a DBA/2 mouse (17). KLN-205 and Lewis lung carcinoma were generous gifts from Robert Korst (Division of Cardiothoracic Surgery, The New York Presbyterian Hospital, NY). Human A-549 is an adenocarcinoma NSCLC purchased from American Type Culture Collection. Both cell lines were cultured in MEM (Life...
Cell Cycle Analysis. NSCLC cells were plated on 100 \times 20 \text{ mm}^2 tissue culture plates (Corning, Corning, NY). The cells were collected at indicated times, fixed in 70% cold ethanol for 1 h, and resuspended in a hypotonic propidium iodide (PI) solution (Sigma, St. Louis, MO) containing RNase. Flow cytometry was performed with the use of Coulter epic flow cytometer. DNA histograms were analyzed using Modfit computer program (Verity Software House, Topsham, ME).

Apoptosis. The cells were collected 24 h after the incubation without or with rapamycin, CsA, or rapamycin plus CsA. Cells were then treated with 5 \mu l of Annexin V-FITC solution and PI (1 \mu g/ml; BD PharMingen, San Jose, CA). Dual parameter flow cytometric analysis was performed to determine the percentage of apoptotic cells (Annexin V alone-positive cells), necrotic cells (PI-positive cells), or viable cells (staining negative for Annexin V and PI).

Proliferation. NSCLC lines (1000 cells/well) were plated in 96-well flat-bottomed tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) and incubated at 37°C in a 5% CO_2/95% air atmosphere. One \mu Ci of [\text{^3}H]thymidine was added 6 h before cell harvest. Proliferation was quantified by measuring [\text{^3}H]thymidine incorporation into DNA with the use of TopCount liquid scintillation counter (Packard, Meriden, CT).

ELISA. KLN-205 cells were incubated in the absence or presence of rapamycin, and cell-free culture supernatants were collected and stored at −20°C until measurement of cytokine levels by ELISA. Blood specimens were obtained from mice after termination of \textit{in vivo} experiments, and the serum was separated and stored at −20°C until measurement of cytokine levels by ELISA. A vascular endothelial growth factor (VEGF)-A ELISA kit was used to measure VEGF-A protein levels in the mouse serum and in cell culture supernatants.

In Vivo Tumor Models. \textit{In vivo} tumor progression was determined with the use of s.c. tumor formation model and pulmonary metastasis model. The s.c. tumor growth model was established by injecting 2.0 \times 10^5 KLN-205 cells in 0.1 cc of PBS s.c. into syngeneic DBA/2 mice. Mice were randomly assigned to four experimental groups once tumor volume had reached 50 mm^3 (4–6 days after tumor injection). The four experimental groups were as follows: untreated control group; rapamycin treatment group; CsA treatment group; and rapamycin plus CsA treatment group. Rapamycin (2 mg/kg) was given p.o. by gavage every day. The dosage of rapamycin is based on dosages commonly used in murine models of allograft transplantation (18–20). The mice randomized to CsA treatment group were given s.c. injections of 20 mg/kg every other day. The mice treated with both rapamycin and CsA received the drugs as described above. Tumor volume was calculated with the use of the following equation: tumor volume = 0.5 (greatest diameter) \times (shortest diameter)^2.

KLN-205 cells are highly metastatic in nature (21). The s.c. tumor implantation procedure described above was used to investigate the effect of rapamycin on the formation of distant pulmonary metastases. Mice were randomly assigned to one of the following four experimental groups: untreated control group; rapamycin treatment group; CsA treatment group; and rapamycin plus CsA treatment group. Rapamycin and CsA were administered as described. The mice were sacrificed 5 to 6 weeks after receiving s.c. injection of KLN-205 cells, and the presence or absence of pulmonary metastases was determined after endotracheal insufflation of lungs with 15% India ink, as described previously (22).

If no lesions were found on the surface of the intact lungs, further dissection was performed. A lung was considered negative only after all five anatomical lobes had been examined and found to be free of tumors.

Statistics. One-way ANOVA and Bonferroni correction were performed to identify differences among multiple groups. Independent or paired \textit{t} test was performed to identify differences between two groups. Categorical variables were tested with the use of Fisher’s exact test.

RESULTS

Rapamycin Prevents \textit{in Vivo} Growth of KLN-205 NSCLC. Murine KLN-205 NSCLC cells were injected into the s.c. space of syngeneic DBA/2 mice, and treatment with
rapamycin, CsA, or rapamycin plus CsA was begun once tumor volume had reached 50 mm$^\text{3}$ (4–6 days after tumor injection).

Fig. 1 demonstrates that KLN-205 NSCLC cells exhibit robust tumor growth in untreated mice but not in the mice treated with rapamycin. Fig. 1 also shows that CsA does not prevent tumor formation and that rapamycin inhibits tumor growth in mice treated with CsA.

Comparison of tumor volumes in the four experimental groups showed that the group means were similar at the time of initiation of treatment ($P = 0.48$) and that the tumor volumes among the experimental groups were significantly different at each of the subsequent time points: $P = 0.005$ at 13 days; $P = 0.0002$ at 17 days; $P = 0.0001$ at 22 days; and $P < 0.0001$ at 29 days after implantation (Fig. 1). The experiment was terminated on day 29 after tumor implantation, and all pair-wise comparisons of tumor volumes showed that the tumor volume of 1290 mm$^3$ in the control mice ($n = 7$ mice) is significantly higher compared with the tumor volume of 246 ± 80 mm$^3$ in mice treated with rapamycin ($n = 7$; Bonferroni’s $t$ test, $P < 0.001$) or the tumor volume of 136 ± 48 mm$^3$ in mice treated with rapamycin plus CsA ($P < 0.001$). Additional comparisons showed that the tumor volume of 1203 ± 227 mm$^3$ in mice treated with CsA ($n = 8$) is not different from that of untreated mice ($P > 0.05$) and that the tumor volume in mice treated with rapamycin alone is not different from that in mice treated with both rapamycin plus CsA ($P > 0.05$).

**Rapamycin Constrains KLN-205 NSCLC Cell Pulmonary Metastasis.** KLN-205 NSCLC is a highly metastatic tumor (21). Having demonstrated that rapamycin prevents s.c. tumor formation, we investigated whether rapamycin prevents metastatic tumor progression. Lungs of mice were examined for pulmonary metastases after implantation of KLN-205 cells in the s.c. space. The mice were either untreated or treated with rapamycin, CsA, or rapamycin plus CsA.

Fig. 2A shows the histological features of the KLN-205 NSCLC at the primary s.c. tumor site, and Fig. 2B shows a representative pulmonary metastasis observed in the untreated control mouse. Rapamycin was highly effective in preventing the formation of pulmonary metastases. Although eight of eight untreated mice developed pulmonary metastases from a distant primary site, only one of six mice treated with rapamycin developed pulmonary metastases ($P = 0.003$, Fisher’s exact test; Fig. 2C).

Rapamycin was also effective in preventing the development of pulmonary metastases in mice treated with CsA; although four of six mice treated with CsA developed metastases, only one of five mice treated with both rapamycin and CsA showed pulmonary metastases (Fig. 2C). Thus, 12 of 14 untreated or CsA-treated mice developed pulmonary metastases whereas only 2 of 11 mice treated with rapamycin or rapamycin plus CsA developed metastases ($P = 0.001$).

The liver and the peritoneal cavity were found to be free of visible metastasis. Our observation that pulmonary metastasis develops after s.c. implantation of KLN-205 NSCLC cells confirms and extends an earlier report of the propensity of KLN-205 NSCLC cells to form pulmonary metastasis not only after i.v. administration but also after s.c. implantation (21).

Angiogenesis is considered important for both tumor formation and metastasis (25). The fact that rapamycin prevented pulmonary metastasis in the current study may be a consequence of its antiangiogenic properties (26). In support of this notion, we have shown that rapamycin inhibits the proliferation of the endothelial cell line HMEC-1 (27). Further study is required to establish the mechanism by which rapamycin prevents pulmonary metastasis.

**Fig. 2** Rapamycin prevents the development of pulmonary metastases from a s.c. tumor implant. Murine KLN-205 non-small cell lung cancer (NSCLC) cells ($2 \times 10^5$ cells) were injected into the s.c. space of syngeneic DBA/2 mice. The mice were untreated, treated with rapamycin (2 mg/kg/day), cyclosporine (CsA; 20 mg/kg/every other day), or rapamycin (2 mg/kg/day) plus CsA (20 mg/kg/every other day), once the tumor volume was 50 mm$^3$. The mice were sacrificed 37 days after tumor injection into the s.c. space. Pulmonary metastases were visualized as filling defects after intratracheal infusion of India ink. A shows H&E staining of s.c. primary tumor. B shows a representative pulmonary metastasis arising from the s.c. primary source (magnification at 200×). The lesion is seen surrounded by India ink-stained alveoli. C shows treatment-dependent differences in the development of pulmonary metastases. Rapamycin remained effective at limiting the growth of individual lesions and was able to reduce overall tumor burden in the combination group.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Mice bearing pulmonary metastasis/total mice</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>8/9</td>
<td>100%</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>1/6</td>
<td>16.7%</td>
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<tr>
<td>Rapamycin/CsA</td>
<td>1/5</td>
<td>20%</td>
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<td>CsA</td>
<td>4/6</td>
<td>67.7%</td>
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growth and metastasis formation, and VEGF may play a role in these processes. As a potential mechanism for the antitumor efficacy of rapamycin, we determined the effect of rapamycin on VEGF expression. We investigated the effect of rapamycin on VEGF expression both in vitro and in vivo. We examined whether in vitro conditioning of KLN-205 NSCLC cells with rapamycin inhibits VEGF protein production. We also investigated whether rapamycin reduced circulating levels of VEGF in tumor-bearing mice.

Rapamycin treatment resulted in a marked inhibition of VEGF production. The concentration of VEGF protein in cell-free supernatants obtained from KLN-205 NSCLC cells treated with rapamycin for 72 h was 631 ± 4 pg/ml, whereas it was 1224 ± 11 pg/ml with untreated cells.

In vivo effects of rapamycin on VEGF production were investigated by measurement of the circulating levels of VEGF in DBA/2 mice after a s.c. injection of $2 \times 10^5$ KLN-205 NSCLC cells. Treatment with rapamycin resulted in a reduction in serum VEGF levels. The mean ± serum VEGF levels, measured 29 days after tumor implantation, was $47 ± 3$ pg/ml in rapamycin treated mice ($n = 4$) and $63 ± 6$ pg/ml in the untreated control mice ($n = 4$; two-tailed t test, $P = 0.0014$).

Rapamycin Induces Cell Cycle Arrest Cells at the $G_1$ Checkpoint. In some cell types, rapamycin blocks cell cycle progression by inhibiting progression from $G_1$ to $S$ phase. We determined whether treatment of KLN-205 NSCLC cells with rapamycin led to an accumulation of the cells in $G_0$-$G_1$ phase. Fig. 3A, a representative flow cytometry histogram, shows that rapamycin blocks cell cycle progression of KLN-205 NSCLC cells at the $G_1$ checkpoint; the percentage of $G_0$-$G_1$ phase cells was $49\%$ with the untreated cells, and the percentage of $G_0$-$G_1$ cells increased to $60\%$ with rapamycin treatment; the percentage...
of S phase cells was 43% with untreated cells, and the percentage was reduced to 32% with treatment. Single cell analysis of KLN-205 NSCLC cells also demonstrated that CsA did not induce cell cycle arrest and that rapamycin blocks cell cycle progression of CsA-treated KLN-205 cells at the G1-S phase (Fig. 3A).

We next determined whether cell cycle inhibition by rapamycin altered NSCLC cell proliferation. Cellular proliferation was evaluated by quantifying the uptake of [3H]thymidine by KLN-205 NSCLC cells and by quantifying cell number. Proliferation was markedly inhibited by rapamycin at each of the time points tested (control versus rapamycin; Bonferroni’s t test, \( P < 0.001 \) at 24, 48, and 72 h), and, as found with cell cycle analysis, rapamycin was also inhibitory in the presence of CsA (Fig. 3B). The inhibitory effect, demonstrated by quantifying [3H]thymidine uptake by KLN-205 NSCLC cells, was confirmed and extended by measurement of cell yield after incubation of KLN-205 NSCLC cells without or with rapamycin, CsA, or rapamycin plus CsA. The cell yields after 24 h of incubation were 91 ± 2.2 × 10^4 cells with untreated cells, 48 ± 1.8 × 10^4 cells after incubation with 10 ng/ml rapamycin, 94 ± 4.4 × 10^4 cells after incubation with 500 ng/ml CsA, and 65 ± 4.4 × 10^4 cells after incubation with 10 ng/ml rapamycin and 500 ng/ml CsA (\( P < 0.0001 \), ANOVA; Fig. 3C). Pair-wise comparisons of cell yields showed that the reduction in cell yield after incubation of the cells with rapamycin (Bonferroni’s t test, \( P < 0.001 \)) or with rapamycin plus CsA (\( P < 0.001 \)) was significant. In contrast, the difference in cell yields between untreated cells and cells incubated with CsA was not significant (\( P > 0.05 \)).

In addition to our evaluation of the effects of rapamycin on KLN-205 NSCLC cells, we have examined the effect of rapamycin on the proliferation of Lewis lung carcinoma cells, a lung carcinoma cell line of murine origin (17). Rapamycin inhibited the proliferation of Lewis lung carcinoma cells, albeit to a lesser extent than that observed with KLN-205 NSCLC cells. Proliferation of Lewis lung carcinoma cells, as assessed by [3H]thymidine incorporation into DNA during 48–64 h of culture, was reduced from 79,330 ± 774 to 64,958 ± 584 cpm/culture after incubation of Lewis lung carcinoma cells with 10 ng/ml rapamycin (\( P < 0.001 \)). Rapamycin associated inhibitory effect was considerably lesser with the Lewis lung carcinoma cells as compared with KLN-205 NSCLC cells (18% inhibition with Lewis lung carcinoma cells versus 76% inhibition with KLN-205 NSCLC cells).

**Rapamycin Does Not Induce Apoptosis of KLN-205 NSCLC Cells.** We explored whether induction of apoptosis by rapamycin was a mechanism for the inhibitory effect of rapamycin. Two-color flow cytometric analysis of KLN-205 NSCLC cells stained with Annexin V-FITC and PI demonstrated that rapamycin treatment, as determined by the binding of Annexin V to externalized phosphatidylserine, does not in-
A-549 cells from G1 to S phase and without inducing apoptosis. The murine cell line, rapamycin blocked cell cycle progression of 10^4 untreated cells, 6.1% with rapamycin-treated cells, 5.6% with Annexin V to externalized phosphatidylserine, was 6.9% with the percentage of apoptotic cells, as determined by the binding of some tumor cells, in part by blocking insulin-like growth factor-I-mediated cell growth (23, 24). Our evaluation, with the use of Annexin V binding to externalized phosphatidylserine as a marker of apoptotic cells, failed to implicate apoptosis of NSCLC cells as a mechanism for the rapamycin-associated reduction in the tumor progression. Despite our observation that rapamycin does not promote apoptosis of either KLN-205 or A-549 cells in vitro, the possibility remains that tumor cells undergo apoptosis in vivo after treatment with rapamycin.

Our in vivo experiments show that treatment of mice with established s.c. tumor prevents further growth of implanted s.c. tumor, a finding consistent with a local antitumor effect. Clinically, however, NSCLC is both a local and systemic disease. The metastatic progression of a primary tumor reflects multiple stages, including dissociation from neighboring primary tumor cells, survival in the circulation, and growth in a secondary location. In our studies, treatment with rapamycin reduced the formation of pulmonary metastases. To the best of our knowledge, our study represents the first demonstration of rapamycin controlling the metastatic progression of NSCLC from a remote primary lesion.

The mechanisms responsible for the efficacy of rapamycin against NSCLC may go beyond its antiproliferative effect. Rapamycin has been shown to inhibit angiogenesis by reducing the level of expression of VEGF-A (24). Our observations that rapamycin treatment reduced VEGF production by NSCLC cells in vitro and that circulating levels of VEGF are also lower in mice treated with rapamycin, are in accord with the hypothesis that impaired angiogenesis contributes to the efficacy of rapamycin. We have shown previously that an excess of transforming growth factor β1 contributes to metastatic progression of murine renal cancer (25); we have also shown that treatment of tumor-bearing mice with rapamycin is associated with a reduction not only in the circulating levels of VEGF but also in a reduction of transforming growth factor β1 (26). Thus, targeted reduction of VEGF as well as other tumor promoting factors such as transforming growth factor β1 may contribute to the antitumor effect of rapamycin on NSCLC cells.

The differential sensitivity of Lewis lung carcinoma (18% inhibition), A549 (52%), and KLN-205 (76%) to rapamycin is CsA-treated cells, and 8.8% with rapamycin plus CsA-treated A-549 NSCLC cells.

**DISCUSSION**

The new observation that has emerged from our investigation is that rapamycin is effective in reducing the growth of murine as well as human NSCLC cells. Our study, with the use of KLN-205 as the model tumor, demonstrates further that rapamycin is highly efficacious in preventing the development of metastases.

Rapamycin prevented cell cycle progression of NSCLC cells from G1 to S phase and decreased their proliferation. Our observation extends prior observations that cell cycle arrest by rapamycin occurs at the G1 checkpoint (22). It is worth noting that the drug concentration we used to achieve cell cycle arrest is within the recommended therapeutic concentrations (5–20 ng/ml) of rapamycin for transplant recipients (23).

Rapamycin has been shown to induce apoptotic cell death of some tumor cells, in part by blocking insulin-like growth factor-I-mediated cell growth (23, 24). Our evaluation, with the use of Annexin V binding to externalized phosphatidylserine as a marker of apoptotic cells, failed to implicate apoptosis of NSCLC cells as a mechanism for the rapamycin-associated reduction in the tumor progression. Despite our observation that rapamycin does not promote apoptosis of either KLN-205 or A-549 cells in vitro, the possibility remains that tumor cells undergo apoptosis in vivo after treatment with rapamycin.

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The differential sensitivity of Lewis lung carcinoma (18% inhibition), A549 (52%), and KLN-205 (76%) to rapamycin is
of interest and may have mechanistic and therapeutic implications. Recently, rapamycin sensitivity has been linked to a reduction in functional tumor suppressor phosphatase PTEN, an inhibitor of the phosphatidylinositol 3′-kinase/AKT signaling pathway (27, 28). The hypothesis that tumors in which the pathway(s) leading to uncontrolled cell division converge(s) on mammalian target of rapamycin would be sensitive to rapamycin is an attractive one, but other mechanisms for sensitivity/resistance may be operative as well. For example, mechanisms of resistance may also involve aberrant binding of rapamycin or additional mediators of signal transduction (29).

Lung cancer is responsible for 39% of the solid malignancies in heart transplant recipients (30). Treatment of NSCLC has yielded limited success both in transplant recipients and in the general population (31, 32). Currently, rapamycin is used as a component of a multidrug regimen in some of the transplant recipients (10). Our study supports the idea that rapamycin may be useful for constraining tumor growth and metastatic progression. That rapamycin is also effective in conjunction with CsA raises the intriguing possibility that the drug combination of CsA and rapamycin, currently used for the prevention of allograft rejection, might also be useful for the management of certain post-transplant neoplasms. The calcineurin inhibitors are of particular interest because CsA as well as tacrolimus promote tumor progression in preclinical models (25, 33). Moreover, rapamycin and tacrolimus bind the same intracellular immunosuphiblin, FKBP12 (34).

Additional research to evaluate whether rapamycin is useful in the treatment of NSCLC, a common malignancy with dismal outcome, appears warranted.

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