Inhibition of Mammalian Target of Rapamycin Reverses Alveolar Epithelial Neoplasia Induced by Oncogenic K-ras


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Abstract

The serine/threonine kinase AKT and its downstream mediator mammalian target of rapamycin (mTOR) are activated in lung adenocarcinoma, and clinical trials are under way to test whether inhibition of mTOR is useful in treating lung cancer. Here, we report that mTOR inhibition blocked malignant progression in K-rasL1 mice, which undergo somatic activation of the K-ras oncogene and display morphologic changes in alveolar epithelial cells that recapitulate those of precursors of human lung adenocarcinoma. Levels of phospho-S6 Ser236/235, a downstream mediator of mTOR, increased with malignant progression (normal alveolar epithelial cells to adenocarcinoma) in K-rasL1 mice and in patients with lung adenocarcinoma. Atypical alveolar hyperplasia, an early neoplastic change, was prominently associated with macrophages and expressed high levels of phospho-S6 Ser236/235. mTOR inhibition in K-rasL1 mice by treatment with the rapamycin analogue CCI-779 reduced the size and number of early epithelial neoplastic lesions (atypical alveolar hyperplasia and adenomas) and induced apoptosis of intraepithelial macrophages. LKR-13, a lung adenocarcinoma cell line derived from K-rasL1 mice, was resistant to treatment with CCI-779 in vitro. However, LKR-13 cells grown as syngeneic tumors recruited macrophages, and those tumors regressed in response to treatment with CCI-779. Lastly, conditioned medium from primary cultures of alveolar macrophages stimulated the proliferation of LKR-13 cells. These findings provide evidence that the expansion of lung adenocarcinoma precursors induced by oncogenic K-ras requires mTOR-dependent signaling and that host factors derived from macrophages play a critical role in adenocarcinoma progression. (Cancer Res 2005; 65(8): 3226-35)

Introduction

Lung cancer is the most common cause of cancer-related death in the United States, and its incidence is increasing worldwide (1). The high mortality of lung cancer reflects its invasive nature and its resistance to current treatment modalities. Because the prognosis is grim once lung cancer has reached advanced stages, investigators have attempted to intervene earlier, before the development of overt disease (2). Efforts to develop effective lung cancer prevention strategies have stimulated interest in understanding the biology of lung neoplasia.

Lung adenocarcinoma, the most common subtype of non–small cell lung cancer (NSCLC), often occurs near sites of atypical alveolar hyperplasia (AAH). A subgroup of AAH cells contains activating mutations in K-ras, a genetic event found in 30% to 50% of lung adenocarcinomas (3). Because of their proximity and shared genetic changes, AAH is considered a precursor of lung adenocarcinoma. Another type of lung cancer, bronchioloalveolar cell carcinoma (BAC), is a noninvasive, slowly progressive tumor of alveolar epithelial cells that may evolve into invasive adenocarcinoma (4). Activating K-ras mutations are also common in BAC. Based on these observations, two models for lung adenocarcinoma progression have been proposed, one in which AAH evolves directly to adenocarcinoma and another in which AAH evolves to BAC, which can subsequently develop into adenocarcinoma.

The biochemical events required for malignant progression of the bronchial epithelium have been the focus of recent investigation. The serine/threonine kinase AKT is activated in NSCLC and in bronchial dysplasia, an early event in squamous bronchial neoplasia (5). NSCLC cells with activated AKT undergo proliferative arrest after treatment with inhibitors of phosphatidylinositol 3-kinase (PI3K), an upstream activator of AKT (6–8). Activation of AKT-dependent signaling contributes to the development of glioblastoma multiforme, endometrial cancer, prostate cancer, breast cancer, etc. (9–13).

Several biochemical events have been identified downstream of AKT that enhance cell survival, increase cell proliferation, and alter cell metabolism. AKT phosphorylates and inactivates downstream substrates, including BAD, FOXO proteins, GSK3, and tuberin, the protein product of Tsc2 (14). Phosphorylation of tuberin leads to activation of mammalian target of rapamycin (mTOR, encoded by the gene frap1 in mice), a critical mediator of protein translation (15–19). mTOR substrates required for protein translation include the serine/threonine kinase S6K1 (p70S6K/p85S6K) and the 4E binding protein 1 (20–22). Phosphorylation of 4E binding protein 1 causes it to dissociate from eukaryotic initiation factor (eIF) 4E, which then binds to the eIF4G scaffold protein, promoting the assembly of the eIF4F initiation complex. Of note, eIF4E is overexpressed in BAC and lung adenocarcinoma (23–25). These cumulative observations have led to derivatives of the mTOR inhibitor rapamycin being tested in clinical trials of lung cancer.

In this study, we hypothesized that mTOR signaling is activated in precursors of lung adenocarcinoma and that activation contributes to lung tumor progression. We addressed this question...
by testing human tissues and K-rasLA1 mice, which develop lung adenocarcinoma through somatic activation of a K-ras allele carrying an activating mutation in codon 12 (G12D; ref. 26). We chose K-rasLA1 as a model because K-ras is the most commonly mutated proto-oncogene in lung adenocarcinoma, because mutant K-ras activates PI3K/AKT-dependent signaling (27), and because tumors in K-rasLA1 mice recapitulate certain morphologic changes in alveolar epithelial cells that precede human lung adenocarcinoma, evolving through a series of morphologic stages from AAH to adenocarcinoma. The predominant epithelial changes observed in 4- to 8-week-old mice are AAH-like lesions and small adenomas followed by the appearance of adenosomas with papillary or atypical features and, at 6 to 8 months, well-differentiated adenocarcinomas that invade regional lymph nodes or, less often, metastasize to distant sites. The K-ras mutations, the growth patterns along alveolar surfaces (lepidic growth), and the infiltration of inflammatory cells (macrophages) observed in this model are all characteristic of human adenocarcinoma that evolves through BAC. Whereas the earliest precursor of lung adenocarcinoma in humans and K-rasLA1 mice is a hyperplastic phenotype (AAH), the subsequent morphologic changes that precede the appearance of adenocarcinoma are different in humans (BAC) and in K-rasLA1 mice (adenoma).

Materials and Methods

Animals, cells, and reagents. We studied K-rasLA1 mice, which carry a latent K-ras allele with two copies of exon 1, one wild-type and the other the G12D mutant (26). The latent allele is stochastically activated in cells through homologous recombination, which results in deletion of the wild-type copy of exon 1 and expression of an oncogenic form of the K-ras gene. Multifocal lung adenocarcinomas develop spontaneously in 100% of these mice.

The LKR-13 and LKR-10 cell lines were derived by serial passage of minced lung adenocarcinoma tissues from two tumors isolated from separate lobes of the same K-rasLA1 mouse. The cells were passaged in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Life Technologies, Gaithersburg, MD) and plated in 24-well tissue culture plates for 24 hours at 37°C and 5% CO₂. Macrophage conditioned medium was then recovered and frozen at −80°C until use. Macrophages (1 × 10^6 to 8 × 10^6) were present per milliliter of BAL and viability was ≥98% as assessed by trypan blue exclusion. Morphologic analysis of cytospin preparations stained with H&E revealed the cells to be 97.2 ± 0.5% macrophages, 1.9 ± 0.4% lymphocytes, and 0.8 ± 0.07% neutrophils.

We purchased rabbit polyclonal antibodies against phospho-S6 (Ser235/236) (p-S6), p66/68 (Thr421/422), p66, p-mTOR (Ser2448), mTOR, and cleaved caspase-3 (Cell Signaling Technology, Beverly, MA), rat monoclonal anti-mouse F4/80 (Serotec, Oxford, United Kingdom), murine monoclonal anti-human CD68 (DAKOcytomation, Carpinteria, CA), and murine monoclonal antibodies against proliferating cell nuclear antigen (PCNA; BioGenex Laboratories, San Ramon, CA). Blocking peptides for p-S6 antibodies were purchased from Cell Signaling Technology and mouse immunoglobulin was from DAKOCytomation. For fluorescence staining, we used rabbit secondary TRITC and rat secondary FITC (Immunology Consultants Laboratory, Inc., Newburg, OR) and 4′,6-diamidino-2-phenylindole (DAPI) II (Vysis, Inc., Downers Grove, IL) for nuclear counterstaining. The rapamycin analogue CCI-779 (Temsirolimus) was provided by Wyeth-Ayerst (Philadelphia, PA), Tween 80 and trypsin-EDTA were from Life Technologies, and glutaraldehyde, paraformaldehyde, formalin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bisbenzimide (Hoescht 33342), and polyethylene glycol-400 were from Sigma (St. Louis, MO).

K-rasLA1 mice experiments. Sixteen-week-old K-rasLA1 mice were given i.p. injections of CCI-779 at either 20 mg/kg/d (high-dose group, n = 12) or 0.1 mg/kg/d (low-dose group, n = 14) for 5 days/wk for 4 weeks; another 12 control mice were given vehicle (5% Tween 80/5% polyethylene glycol-400) on the same schedule. Mice were subjected to respiratory-gated microcomputed tomography (micro-CT) scans under general anesthesia as described elsewhere (28) at the beginning and at the end of the treatment; they were killed by cervical dislocation within 6 hours of the final micro-CT scan. During the autopsy procedure, visible lesions were counted on the surfaces of both lungs by investigators blinded as to treatment group (M.W. and D.J.). The lungs were then perfused with PBS and removed from the body. One lung was kept at −80°C for protein extraction and the other was fixed in 4% glutaraldehyde/paraformaldehyde for 30 minutes followed by 10% formalin overnight before being embedded in paraffin as described previously (29).

Micro-CT scan analysis. Mice were anesthetized and intubated by experienced veterinary personnel and connected to a SAR-830/P small animal ventilator (CWE, Ardmore, PA). A single respiratory-gated three-dimensional micro-CT image set was acquired for each mouse using 80 kVp, 405 μA, 100 ms per frame, 5 frames per view, 360 views, and 1° incrementation per view. This acquisition resulted in a set of contiguous axial DICOM formatted images through each mouse thorax with voxels of dimensions 91 × 91 × 91 μm. Lesions were characterized on the initial and final micro-CT scans by one investigator (M.W.) who was blinded to treatment group and autopsy results. Lesions visualized on the CT images included solid or “ground-glass” opacities or areas of consolidation resembling adenocarcinoma with bronchioalveolar features in humans (30). Lesion volume was measured with “Analyze” software (AnalyzeDirect, Inc., Lenexa, KS) as follows. The volumes of three to five lesions were measured in each mouse (10-30 or more axial images per lesion) on the final micro-CT scan, after which anatomic landmarks were used to locate the same lesions on the initial micro-CT scan and the volumes were measured again. Lesion growth was defined as the difference between the final and initial volumes. If a lesion from the final micro-CT scan could not be located on the initial micro-CT image set, it was considered a new lesion arising during the treatment.

Tissue microarrays and immunohistochemical analyses. Three types of tissue microarrays were constructed with cores from formalin-fixed, paraffin-embedded blocks (microarrays are summarized in Supplementary Table S1). The first array was made with specimens of human normal lung, AAH, BAC, and invasive adenocarcinoma. The first array was made with specimens of human normal lung (n = 30), AAH (n = 27), BAC (n = 41), and invasive adenocarcinoma (n = 97) obtained from surgical resections performed between 1994 and 2004 at the University of Texas MD Anderson Cancer Center (Houston, TX). The histologic groups (AAH, BAC, and adenocarcinoma) were balanced with respect to age gender and smoking history (data not shown). The adenocarcinomas included pathologic stages I, II, and IIIA. None of the patients received neoadjuvant therapy. The BAC lesions were not pure BAC but rather were considered “mixed adenocarcinoma with predominant BAC features” according to the 1999 WHO definitions (31). Three cores were obtained from each specimen, with core diameters of 1 mm for adenocarcinoma and 1.5 mm for BAC and AAH. The second array consisted of specimens of murine normal lung (n = 30), AAH (n = 40), adenoma (n = 206), and adenocarcinoma (n = 11; according to the histologic criteria established by Johnson et al. (26)). The third array comprised all lesions (identified by histologic analysis) from the mice in the current study treated with CCI-779 or vehicle (47 AAH, 153 adenomas, and 36 normal lung). Each murine lesion was sampled with a single core of 1 mm diameter; the lesions were too small to permit multiple cores to be obtained.

For immunohistochemical analyses, 4 μm sections were deparaffinized, rehydrated, and washed with PBS as described previously (29). Antigens were retrieved with 0.01 mol/L citrate buffer (pH 6, DAKOCytomation) for...
30 minutes in a steamer. To detect the macrophage antigen F4/80, slides were exposed to 0.025% trypsin-EDTA for 10 minutes. Samples were blocked for endogenous activity in 3% hydrogen peroxide/PBS, avidin/biotin solution (Zymed, South San Francisco, CA), and DAKO serum-free protein block (DAKOcytomegation) before incubation with the primary antibody overnight at 4°C. Standard avidin-biotin immunoperoxidase methods with diaminobenzine as the chromogen were used for detection. The Animal Research Kit (DAKOcytomegation) was used to reduce nonspecific binding of the murine PCNA antibody to murine tissues. For dual-fluorescence staining, we followed the same protocol, except for the detection step for which we used a secondary antibody coupled with a fluorochrome. Immunofluorescence-generated signals were visualized with a Zeiss Axiosplan epifluorescence microscope (Nikon, Inc., Melville, NY) equipped with oil immersion objective and single band pass filters for FITC, Texas red, and DAPI. Digitized images of each fluorochrome were captured individually with a high-resolution image analysis system (MetaMorph, Universal Imaging Corp., Downingtown, PA) with a cooled charge-coupled device camera (Hamamatsu C4742-95-12, Hamamatsu Photonics K.K., Hamamatsu City, Japan).

As negative controls for determining the specificity of the immunostaining results, we omitted the primary antibody (cleaved caspase-3), pretreated the samples with blocking peptides (p-S6) or isotype immunoglobulins (PCNA), and stained a paraffin-embedded pellet of H1607 human lung adenocarcinoma cells for the macrophage antigens F4/80 and CD68. As positive controls, we used paraffin-embedded pellets of H1607 cells (which are PTEN−/−, p-S6), normal lung tissue from mouse (F4/80) and human (CD68), murine lymphoma tissue (cleaved caspase-3), and normal human tonsil tissue (PCNA).

Staining in normal lung and lung lesions was quantitated by one investigator (L.S.), blinded as to treatment group, using two approaches: frequency of staining (defined as the presence of any positive intralesional cells in a single tissue section) and degree of staining [a combined score based on staining intensity and extension in a single tissue section (intensity × extension)]. Staining intensity for p-S6 was recorded as undetectable (0), weak (1), medium (2), or strong (3). For F4/80, CD68, and cleaved caspase-3, staining intensity was defined as undetectable (0) or detectable (1). Staining extension was evaluated for the whole of the tumor and defined as the percentage of positive cells per 20× square (for p-S6, F4/80, and CD68), per 40× square (for PCNA), or per 100× square (for cleaved caspase-3) magnification. The degree of staining was compared between each histologic category and treatment group and expressed as the mean ± SE.

**Western blot analysis.** Lysates from cell lines or mouse tissue containing the same amounts of protein were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were immunoblotted overnight at 4°C with primary antibodies in TBS containing 5% nonfat dry milk. Antibody binding was detected with an enhanced chemiluminescence kit according to the manufacturer’s directions (Amersham, Inc., Arlington Heights, IL).

**Apoptosis analysis.** Nuclei from alveolar macrophages obtained from BAL fluid were stained with bisbenzimide (Hoechst 33342) as follows. Cells were fixed in 4% formaldehyde, washed with PBS, incubated with Hoechst 33342 (10 µg/mL) for 15 minutes at room temperature, and counted with a fluorescence microscope (with a UV filter) at a magnification of ×63 with oil immersion objective. Results were expressed as the percentage of 200 counted cells with the morphologic characteristics of apoptosis (chromatin condensation and nuclear fragmentation).

**Proliferation assay.** To measure sensitivity to the rapamycin analogue CCI-779 in vitro, LKR-13 cells were plated (1,000 cells/well) in 96-well tissue culture plates and incubated for 24 hours before being exposed to different concentrations of CCI-779 or conditioned medium from macrophages. Proliferation was quantified after 3 days by MTT assays.

**Syngeneic tumors.** To measure the sensitivity of LKR-13 cells to CCI-779 in vivo, 10^4 LKR-13 cells (in 100 µL PBS) were injected s.c. into 129/Sv mice. Five days later, when tumor volume had reached 50 mm^3, mice were randomly assigned to one of two experimental groups [CCI-779 20 mg/kg/d (n = 10) or vehicle (n = 10) given i.p.]. Tumor diameters were measured daily by an investigator (M.W.) who was blinded to treatment group. Tumor volumes were calculated as follows: 0.5 (greatest diameter) × (shortest diameter)^2. Treatment was continued until one tumor reached 1.5 cm^3 (8 days), at which time all mice were killed. Tumors (n = 36) were then removed and either frozen (−80°C) for protein extraction or fixed in 10% formalin overnight before being embedded in paraffin.

**Statistical analysis.** For statistical analysis, the immunostaining scores, tumor numbers, and tumor volumes were considered continuous variables, whereas new tumor formation was considered a categorical variable. The Mann-Whitney nonparametric test was used to compare the continuous variables with Bonferroni correction. P = 0.05 was considered significant for two pair-wise comparisons (experiments with syngenic tumors), 0.017 for three pair-wise comparisons (mouse experiments with three treatment arms), and 0.012 for four pair-wise comparisons (comparison of expression in lung lesions). Fisher’s exact test was used to compare categorical variables. Spearman’s ρ coefficient was used for correlative studies between quantitative variables and P = 0.05 was considered significant. Data were processed with StatView and Survival Tools 5.0 (Abacus Concepts, Berkeley, CA).

**Results**

**Activation of AKT-dependent signaling with malignant progression.** We examined p-S6, which increases with mTOR activation, in lung tissues from K-rasLA1 mice (relevant signaling pathways are summarized in Supplementary Fig. S1). We assessed

![Image](https://example.com/image.png)

Figure 1. Typical histologic features of normal lung (A), AAH (B), adenoma (C), and adenocarcinoma (D) from 16- to 20-week-old K-rasLA1 mice stained with H&E and visualized at ×20 magnification. Columns, mean PCNA score of positive cells per square at ×40 magnification for each histologic subtype; bars, SE (E).

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p-S6 by immunohistochemical analysis of a tissue microarray containing samples of murine normal lung, AAH, adenoma, and adenocarcinoma and determined whether p-S6 levels changed during malignant progression. To compare the K-ras^LA1 mice to patients with lung adenocarcinoma, we also examined p-S6 in a tissue microarray containing human normal lung, AAH, BAC and adenocarcinoma. Typical histologic features of precursor lesions and adenocarcinoma from the K-ras^LA1 mice are illustrated in Fig. IA to D. Phenotypically, the precursors were low-grade, noninvasive epithelial cells, demonstrating no evidence of invasion through the basement membrane and low proliferative activity, as shown by PCNA staining (Fig. 1E).

Although the frequency of p-S6 staining was not different in cells along the progression to lung adenocarcinoma in humans or in K-ras^LA1 mice (p-S6 was detected in 60-100% of the lesions in each category), the degree of staining (intensity × extension) increased with progression from normal lung to adenocarcinoma in K-ras^LA1 mice (Fig. 2A-D) and from normal lung to AAH in human tissues (Fig. 2E-H). Thus, mTOR signaling was activated in early precursors of lung adenocarcinoma in K-ras^LA1 mice and humans.

**Macrophages infiltrate AAH and express p-S6.** A subset of the cells within AAH and adenomas that expressed p-S6 had typical epithelial morphology. In addition, a morphologically distinct population of cells in K-ras^LA1 mice also stained positively for p-S6. This population exhibited more intense cytoplasmic staining and morphologic characteristics of macrophages. To better characterize this population, we did immunohistochemical analysis of F4/80, a murine macrophage marker, and observed a population of intracellular cells that stained positively (Fig. 3A-D). Immunofluorescence staining for F4/80 and p-S6 revealed a population of cells in which p-S6 colocalized with F4/80 (Fig. 3E-H), supporting the immunohistochemical evidence that macrophages were expressing p-S6. We next examined whether the prevalence of macrophages changed with malignant progression. In samples from both humans and K-ras^LA1 mice, the numbers of macrophages increased with malignant progression (from normal lung to adenocarcinoma in human and from normal lung to adenoma in mouse; Fig. 4). The prevalence of macrophages in K-ras^LA1 mice dropped with the progression of adenoma to adenocarcinoma, indicating that the association of macrophages with epithelial precursors was stage specific (Fig. 3).

**CCI-779 inhibited malignant progression in K-ras^LA1 mice.** Based on these findings, we hypothesized that S6 phosphorylation is required for malignant progression. Because S6^Ser236/235 is phosphorylated by S6K through mTOR-dependent mechanisms, we investigated the effect of treatment with CCI-779, a mTOR inhibitor, in K-ras^LA1 mice, hypothesizing that loss of p-S6 would block progression of AAH to adenoma. CCI-779 treatment was begun at 16 weeks of age, at which time AAH and adenomas were identifiable but adenocarcinomas were not yet evident. The doses of CCI-779 (0.1 or 20 mg/kg daily for 4 weeks) were chosen based on a previous study that showed dose-dependent growth inhibition of phosphatase and tensin homologue on chromosome 10 (PTEN) wild-type xenografts in severe-combined immunodeficient mice (13). The only appreciable toxicity we observed with CCI-779 treatment was weight loss, which occurred in the high-dose group. Mean changes in body weight (expressed as the percentage of total body weight ± SD) were 2.64 ± 3.27% in the control group, 1.80 ± 3.03% in the low-dose group, and -3.97 ± 4.74% in the high-dose group. Weight loss in the high-dose group did not correlate with treatment-induced changes in mean number of tumors (P = 0.44, ρ = 0.21).

![Figure 2](image)

**Figure 2.** p-S6 increased with malignant progression in lung tissue from K-ras^LA1 mice and patients with adenocarcinoma. Murine (A-D) and human (E-H) lung tissues illustrated include representative normal lung (A and E), AAH (B and F), adenoma (C), BAC (G), and adenocarcinoma (D and H) at ×20 magnification. Degree of p-S6 staining for each histologic category, including normal (NI), AAH, adenoma (Ad), BAC, and adenocarcinoma (ADC) in K-ras^LA1 mice (top) and patients with lung adenocarcinoma (bottom).
Response to treatment was documented by using three approaches: by counting lesions on the surfaces of both lungs at autopsy, by imaging lesions in vivo with micro-CT at the beginning and end of treatment (an example is illustrated in Fig. 5A), and by counting sites of AAH and adenomas in one lung from each mouse by histologic analysis of one tissue section stained with H&E. AAH could be distinguished from adenoma by histologic assessment but not by visual inspection or micro-CT scanning; the limit of detection by visual inspection and micro-CT was ~0.5 mm in diameter (28).

After the completion of treatment, the mice were killed by cervical dislocation and subjected to necropsy. Counting visible lesions on the surfaces of both lungs at autopsy showed a reduction in lesion number only in the high-dose treatment group (Fig. 5B). Micro-CT scanning indicated an increase in the average number of lung lesions over time in the vehicle and low-dose CCI-779 group but no change in the number of lesions in the high-dose CCI-779 group (Fig. 5C). Volumetric analysis of lung lesions (three to five lesions per mouse) showed that average lesion size decreased in mice treated with high-dose CCI-779 but not with low dose or vehicle (Fig. 5D). Further, more new lesions (defined as a lesion present on the final micro-CT scan but not the initial one) formed in the control and low-dose groups than in the high-dose group (Fig. 5E). Histologic analysis of lung tissue sections revealed a reduction in the number of adenomas per mouse, but no change in number of AAH lesions, in the high-dose CCI-779 group (Fig. 5F).

However, this change in adenoma numbers was not statistically significant. Together, these data indicate that high-dose CCI-779 inhibited the expansion of lung adenocarcinoma precursors and may also have blocked the progression of AAH into more advanced epithelial changes.

**Effect of CCI-779 on p-S6 expression and cell viability.** Immunohistochemical and Western blot analysis of lung tissues revealed that treatment with CCI-779 decreased p-S6 in a dose-dependent manner (Fig. 6A), and inhibition of mTOR was maximal in the high-dose group. Thus, mTOR inhibition correlated in a dose-dependent fashion with reversal of neoplasia.

We also examined the lungs for evidence of apoptosis by immunohistochemical analysis with an antibody for cleaved caspase-3. We observed focal staining in adenomas from mice treated with high-dose CCI-779 (Fig. 6B). The focal nature of the apoptosis raised the possibility that the cells undergoing apoptosis were infiltrating macrophages rather than epithelial cells. In support of this possibility were the dose-dependent decrease in F4/80 expression observed with CCI-779 treatment and the colocalization noted on immunofluorescent staining for F4/80 and cleaved caspase-3 (Fig. 6B and C). Of note, we observed no F4/80-negative cells that were cleaved caspase-3 positive, suggesting that the apoptotic rate among epithelial cells was quite low. To further evaluate the effect of CCI-779 on alveolar macrophages,
we treated primary cultures of alveolar macrophages derived from K-rasLA1 mice (n = 4) with CCI-779 using doses comparable with the 15 μmol/L serum levels achieved in mice treated with a single dose of CCI-779 at 20 mg/kg. Staining with bisbenzimide (Hoechst 33342) revealed that treatment with CCI-779 induced chromatin condensation and nuclear fragmentation, morphologic changes consistent with apoptosis (Fig. 6D).

Sensitivity of K-rasLA1-derived tumor cells to CCI-779 differed in vitro and in vivo. Several host factors are important in lung tumor progression, including the intratumoral migration of endothelial cell precursors and inflammatory cells (32–34). Given the prominence of macrophages in this model and their sensitivity to CCI-779, we hypothesized that macrophages promote lung tumorigenesis and that macrophage loss is required for the antitumor effect of CCI-779 in K-rasLA1 mice. We examined the effect of CCI-779 treatment on LKR-13 cells, an adenocarcinoma cell line derived from K-rasLA1 mice. Treatment of LKR-13 cells with 20 μmol/L CCI-779 in vitro inhibited p-S6 levels (Fig. 7A) but had no detectable effect on LKR-13 cell proliferation (data not shown), indicating that p-S6 inhibition was not sufficient to inhibit growth. To examine LKR-13 cells in an in vivo context, we established LKR-13 cells as syngeneic tumors in K-rasLA1 wild-type littermates. LKR-13 cells were highly tumorigenic; tumors grew in all 20 of the injected mice and showed central necrosis at necropsy. Daily treatment of the mice with CCI-779 (20 mg/kg) decreased tumor volume relative to that of controls (P < 0.05; Fig. 7B). Immunofluorescence staining revealed a population of intra-tumoral cells that coexpressed F4/80 and p-S6, indicating that macrophages infiltrated the tumor and expressed p-S6 (Fig. 7C). Treatment with CCI-779 decreased intratumoral p-S6 levels (Fig. 7D), demonstrating mTOR inhibition, and F4/80 levels (Fig. 7E), indicating that CCI-779 decreased intratumoral macrophages. Taken together, treatment with CCI-779 inhibited the growth of LKR-13 cells in vivo but not in vitro, raising the possibility that CCI-779 mediated its antitumor effect by inhibiting host factors required for the growth of LKR-13 cells in the in vivo setting.

Finally, we examined whether factors secreted from alveolar macrophages have a direct effect on tumor cells from K-rasLA1 mice. We isolated alveolar macrophages from wild-type littermates (as opposed to K-rasLA1 mice) to avoid contaminating neutrophils.

\(^5\) J. Gibbons, personal communication.

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Figure 5. Treatment with high-dose CCI-779 decreased the size and number of precursor lesions. A, representative micro-CT scan axial images of one mouse at the beginning (left) and end (right) of treatment with CCI-779. Lesions that appear as consolidations in the right lower lobe and left lower lobe (arrows) decreased markedly with treatment. B, lesion numbers were determined for the three treatment groups by autopsy. Total lesions (C) and new lesions (E) were quantified for the three treatment groups by micro-CT scanning done at the beginning and end of treatment. Mean changes in lesion volume (D) were determined for the three treatment groups by volumetric analysis of three to five lesions per mouse at the beginning and end of treatment. F, numbers of AAH and adenomas were counted in a single tissue section from each mouse in the three treatment groups (mean (SE)).

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and tumor cells. F4/80 staining of cytospin preparations confirmed that >97% of the cells were macrophages (data not shown). We collected conditioned medium from alveolar macrophage samples, transferred conditioned medium (or control medium) to LKR-13 cells or a second cell line derived from a K-ras<sup>LA1</sup> lung adenocarcinoma (LKR-10) for 24 hours, and examined changes in proliferation by MTT assay. We did seven experiments with LKR-13 cells and seven experiments with LKR-10 cells, each with

![Figure 6.](cancerres.aacrjournals.org) CCI-779 decreased p-S6 in a dose-dependent manner and induced apoptosis of intralesional macrophages. Western blot analysis of p-S6 and total S6 (A) and CD68 and actin (C) in whole lung extracts from 12 mice and representative immunohistochemical stains of p-S6 (A), cleaved caspase-3 (CC-3; B), and F4/80 (C) in K-ras<sup>LA1</sup> mice treated with vehicle, low-dose CCI-779 (Low), or high-dose CCI-779 (High). Columns, mean degree of immunohistochemical staining in the three treatment groups; bars, SE. B, representative immunofluorescent staining (~63 magnification under oil immersion objective) for DAPI (a), F4/80 (b), cleaved caspase-3 (c), and merging of the images (d) of a lung section from a mouse treated with high-dose CCI-779. Arrowheads, cells that coexpress F4/80 and cleaved caspase-3. D, alveolar macrophages isolated from 129/Sv mice were subjected to treatment with different doses of CCI-779 for 24 hours and stained with Hoechst 33342 dye. Typical morphologic characteristics of cells examined by fluorescence microscopy (~63 under oil immersion objective) treated with CCI-779 or medium alone. Columns, mean percentages of cells with morphologic characteristics of apoptotic cells (chromatin condensation and nuclear fragmentation); bars, SE.
a separate conditioned medium sample. Relative to control, conditioned medium increased cell proliferation by a factor of 1.26 ± 0.15 (mean ± SD for the 14 experiments; P = 0.01; results of representative experiments are in Supplementary Fig. S2).

**Discussion**

Genetic mouse models of cancer are tools for investigating the sufficiency of specific genetic events in tumorigenesis. In K-rasLA1 mice, the rapidity with which AAH develops and progresses to adenoma supports the concept that K-ras mutation is an initiating event in lung adenocarcinoma. Here, we report that mTOR was activated in AAH, and mTOR inhibition was sufficient to decrease the size and number of AAH and adenomas and may reverse progression of AAH to adenoma, suggesting that the expansion of lung adenocarcinoma precursors induced by oncogenic K-ras requires mTOR-dependent signaling. Human AAH and invasive lung adenocarcinoma share a variety of genetic and biochemical features other than K-ras mutations (35–40). Certain genetic changes described in NSCLC are sufficient to induce AAH when modeled in mice (41). Thus, AAH is a histologic change caused by a variety of genetic events that can initiate the development of lung adenocarcinoma.

Several genetic events described previously in human NSCLC can activate mTOR, including deletion of the tumor suppressors PTEN and LKB1/STK11, amplification of the p110 catalytic subunit of PI3K (PI3KCA), and activating mutations in PI3KCA. epidermal growth factor receptor (EGFR), and human epidermal growth factor receptor 2 (HER2).

**Figure 7.** Sensitivity of K-rasLA1-derived tumor cells to CCI-779 differed in vitro and in vivo. A, Western analysis was done on LKR-13 cells (40 μg/sample) treated for 24 hours with 20 μmol/L CCI-779 (+) or medium alone (-). B-E, LKR-13 cells were established as syngenic tumors in 129/Sv mice, and the mice were treated with CCI-779 or vehicle alone. B, tumors were measured daily. Points, mean tumor volumes; bars, SE. C, the mice were killed, and the tumors were subjected to immunofluorescence staining with DAPI (a), F4/80 (b), and p-S6R (c), and merging of the images (d). Magnification, ×63. Arrowheads, cells that coexpress p-S6 and F4/80. D and E, effect of treatment with CCI-779 on intratumoral p-S6 (D) was examined by Western blotting (40 μg/sample) and immunohistochemical analysis, and changes in F4/80 (E) were examined by immunohistochemical analysis. Columns, mean degree of immunohistochemical staining in the treatment groups; bars, SE.
factor receptor, or K-ras (42–46). The increase in p-S6 we observed with progression from normal lung to AAH in K-ras\textsuperscript{LA1} mice supports a role for oncogenic K-ras in S6 activation. However, two other models of lung tumorigenesis that conditionally express mutant K-ras (K-ras\textsuperscript{G12V-IRES-Bgeo} and Lox-K-ras\textsuperscript{G12D}) differ with respect to activation of AKT-dependent signaling: AKT is activated in K-ras\textsuperscript{G12V-IRES-Bgeo} but not Lox-K-ras\textsuperscript{G12D} mice. Given the presence of mutant K-ras in all three models, it is not clear why they differ with respect to evidence for AKT activation. One potential contributing factor may be ongoing genetic damage, which was evident in K-ras\textsuperscript{G12V-IRES-Bgeo} but not Lox-K-ras\textsuperscript{G12D}. In fact, stochastic genetic events contribute to the phenotypes of other mouse models of human cancer (49, 50). Interestingly, we observed that p-S6 increased with the progression of AAH to adenocarcinoma, supporting the possibility that genetic events other than K-ras mutations contributed to S6 activation in K-ras\textsuperscript{LA1} mice.

In the setting of PTEN gene loss or AKT transgene expression, inhibition of mTOR with low doses of CCI-779 (1 nmol/L) or rapamycin induces proliferative arrest or apoptosis (51, 52). In cells without these genetic changes, treatment with low doses of these agents inhibits mTOR but has no effect on proliferation or survival, indicating that mTOR inhibition is not sufficient to inhibit growth; high doses, on the other hand, are cytostatic (13). Our findings in K-ras\textsuperscript{LA1} mice are partly consistent with the latter study in that a high dose of CCI-779 was required to inhibit tumor progression but, unlike the latter group, low-dose treatment was not sufficient to completely suppress p-S6, and the cytostatic effect correlated in a dose-dependent manner with complete suppression of p-S6. Although we do not know the minimal dose of CCI-779 required for complete suppression of p-S6 in K-ras\textsuperscript{LA1} mice, this minimal dose may have targets other than mTOR.

Cells derived from genetic mouse models of cancer are tools for investigating survival pathways in the setting of specific oncogenic events. For example, in PTEN heterozygous cells, mTOR inhibition is primarily cytostatic, whereas selective activation of AKT sensitizes cells to apoptosis in response to mTOR inhibition, suggesting that cells transformed by PTEN loss may have survival pathways not available to cells transformed by AKT activation (11, 51–53). In this study, treatment of LKR-13 cells with CCI-779 \textit{in vitro} inhibited p-S6 but had no effect on LKR-13 cell proliferation, suggesting that cells transformed by mutant K-ras have survival pathways other than those activated by mTOR. In contrast to the resistance we observed in LKR-13 cells, CCI-779 induced apoptosis of macrophages, indicating a cell type-specific role of mTOR in cell survival. Intraepithelial macrophages in K-ras\textsuperscript{LA1} mice expressed p-S6 at levels higher than those of adjacent epithelial cells, raising the possibility that high levels of mTOR sensitized alveolar macrophages to mTOR inhibition. The mechanisms responsible for S6 phosphorylation in macrophages are unclear. Macrophages from both wild-type and K-ras\textsuperscript{LA1} mice showed high levels of p-S6 in tissue section (Figs. 3E–F and 7C) and after isolation by BAL (data not shown), indicating that the presence of K-ras mutations is not required. One possibility is S6 phosphorylation by cytokines released from Ras-transformed epithelial cells.

Interactions between lung cancer cells and inflammatory cells can affect lung tumor growth positively or negatively. However, a growing body of experimental and clinical data has led to the proposal of a model in which lung cancer cells secrete CC chemokines, recruiting intratumoral macrophages, which interact with tumor cells to enhance tumor cell proliferation and invasion through a variety of mediators (54–58). Several lines of evidence presented here suggest that alveolar macrophages may also contribute to the transformation of alveolar epithelial cells. In human lung tissues, numbers of intraepithelial and airspace macrophages increased with malignant progression. In K-ras\textsuperscript{LA1} mice, we observed intraepithelial macrophage infiltration in AAH, indicating that alveolar epithelial cells recruited macrophages in the early stages of neoplasia induced by oncogenic K-ras. The inhibition of AAH progression to adenoma induced by treatment with CCI-779 was accompanied by macrophage loss. LKR-13 cells were resistant to CCI-779 \textit{in vitro}, but when they were established as syngeneic tumors LKR-13 cells recruited intratumoral macrophages and were sensitive to treatment with CCI-779. Lastly, conditioned medium from primary cultures of alveolar macrophages stimulated the proliferation of LKR-13 cells, which is consistent with previous reports demonstrating a stimulatory effect of alveolar macrophages on the proliferation of normal proximal and distal bronchial epithelial cells in rat and bovine models (59, 60). Together, these findings provide substantial evidence that macrophages may be important in the expansion of early adenocarcinoma precursors and in maintaining the survival of established lung cancer cells induced by oncogenic K-ras.

Finally, the similarities we observed between lung tissues from K-ras\textsuperscript{LA1} mice and patients with lung adenocarcinoma suggest that findings from this study may have clinical applications. Findings presented here suggest that host factors are required for the antitumor effect of CCI-779 in K-ras\textsuperscript{LA1} mice. We have shown that one of these host factors may be intraepithelial macrophages, which secrete several cytokines that stimulate epithelial cell proliferation and tumor angiogenesis (54–58). However, our findings have not excluded the possibility that CCI-779 also had direct effects on intratumoral epithelial cells or vascular endothelial cells, inhibiting tumor growth directly or indirectly. Although the current clinical application of mTOR inhibition is targeted at late-stage cancers, the response of this and other early neoplastic disease models to mTOR inhibition (53) raises the possibility that these agents may find application in treating early lung cancers in humans.

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**References**

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