Mice with Alterations in Both p53 and Ink4a/Arf Display a Striking Increase in Lung Tumor Multiplicity and Progression: Differential Chemopreventive Effect of Budesonide in Wild-type and Mutant A/J Mice1

Yian Wang,2 Zhongqiu Zhang,2 Elizabeth Kastens, Ronald A. Lubet, and Ming You3

Department of Surgery and The Alvin J. Siteman Cancer Center, Washington University School of Medicine, St. Louis, Missouri 63110 [Y. W., Z. Z., E. K., M. Y.], and Chemoprevention Agent Development Research Group, National Cancer Institute, Bethesda, Maryland 20892 [R. A. L.]

ABSTRACT

p53 transgenic mice carrying a dominant negative mutation were crossed with Ink4a/Arf heterozygous-deficient mice to investigate whether there is a synergy between these two germ-line mutations in promoting carcinogen-induced lung tumor progression in mice. Mice with a p53 dominant negative mutation and Ink4a/Arf heterozygous deficiency exhibited >20-fold increase in tumor volume compared with ~4-fold increase in Ink4a/Arf heterozygous-deficient mice and a 9-fold increase in mice with only the p53 dominant negative mutation. The effect of Ink4a/Arf heterozygous deficiency on lung tumor progression occurred late in the carcinogenesis process (> 30 weeks after carcinogen treatment). In addition, most of the lung tumors (~80%) from mice with a p53 mutation and deletion of Ink4a/Arf were lung adenocarcinomas. In contrast, lung adenocarcinomas were seen in <10% of the lung tumors from the wild-type mice and ~50% of the lung tumors from Ink4a/Arf heterozygous-deficient or p53 mutant mice. These results indicate a significant synergetic interaction between the presence of a mutant p53 transgene and the Ink4a/Arf deletion during lung tumor progression (P < 0.01). The usefulness of this new mouse model in lung cancer chemoprevention was examined. The chemopreventive efficacy of budesonide was examined in wild-type mice, mice with Ink4a/Arf heterozygous deficiency, mice with a mutation in the p53 gene, or mice with both a mutation in the p53 gene and deletion in the Ink4a/Arf locus. Mice treated with budesonide displayed an average of 90% inhibition of lung tumor progression in a standard 18-week chemoprevention assay, regardless of p53 and/or Ink4a/Arf status. However, the efficacy of budesonide against lung tumor progression decreased from 94 to 77% (P = 0.07) in mice with alterations in both p53 and Ink4a/Arf in a 46-week chemoprevention assay. Similarly, when mice bearing established lung adenomas were treated with budesonide, genotype-dependent differential effects of budesonide in wild-type and mutant mice were clearly revealed with a 82, 64, 45, and 33% decrease in tumor volume in wild-type mice, p53+/−/Ink4a/Arf+/− mice, p53−/−/Ink4a/Arf+/− mice, and p53−/−/Ink4a/Arf−/− mice, respectively. Thus, mutant mice with alterations in p53 and/or Ink4a/Arf exhibited a significant resistance to chemoprevention by budesonide. Because p53 and Ink4a/Arf mutations are the most prevalent mutations in human lung cancers, the effectiveness of chemopreventive agents on the mutant A/J mice containing alterations with p53 and Ink4a/Arf is the best preclinical estimate of their efficacy in humans. Thus, the mutant A/J mouse model should prove useful for chemoprevention studies.

INTRODUCTION

Several genetic changes, including genetic alterations in Kras, p53, and Ink4a/Arf, are commonly found in human lung cancers, especially in human lung adenocarcinomas (1, 2). Activating point mutations in the Kras2 gene have been detected in 30–50% of lung adenocarcinomas (1, 2). Activated Kras2 has also been detected in spontaneously occurring and chemical-induced mouse lung tumors (2, 3). The frequency of occurrence of activated Kras2 genes depends on the relative susceptibility of the mouse strain and carcinogen treatment protocol used to induce tumors (4–8). Recently, we performed a lung tumor bioassay in heterozygous Kras2-deficient mice to evaluate the effect of the presence of a wild-type Kras2 allele on lung tumorigenesis (9). Mice with a heterozygous K-ras deficiency had an increased susceptibility to the chemical induction of lung tumors when compared with wild-type mice, suggesting that loss of wild-type Kras2 allele promotes activating Kras2 mutation-driven lung carcinogenesis (9).

p53 mutations are common in human lung cancer (1, 2), p53 mutation carriers in LFS families are at a significantly increased risk for several cancer types, including lung cancer (10). Two primary mouse models for human LFS have been developed. The first involves knockout of one or both copies of the p53 gene (11–14), and these mice are viable and develop a variety of spontaneous malignancies, primarily lymphomas, and sarcomas. The second has involved transgenic expression of a dominant negative mutant p53 in animals still retaining copies of the wt of p53 (15). These mice developed a high incidence of spontaneous osteosarcoma, lymphoma, and lung adenocarcinoma (15). We have evaluated previously the effect of the p53 gene on mouse lung carcinogenesis using both p53 heterozygous knockout mice and p53 transgenic mice carrying a dominant negative mutation by crossing them with lung tumor susceptible A/J mice (16). Although there is no change in lung tumorigenesis in p53 heterozygous knockout mice comparing with wt mice, a 3-fold increase in lung tumor multiplicity was observed, suggesting that the mutant p53 transgene may have a dominant negative effect on the wt p53 (16).

The Ink4a/Arf locus encodes two functionally distinct tumor suppressors, p16INK4a and ARF (17, 18). The p16INK4a gene codes for an inhibitor of the cyclin D–cyclin-dependent kinase complex and thereby affects the RB/E2F pathway and cell cycle progression (19). Several studies indicated that the p16INK4a and Rb genes are reciprocally inactivated in lung cancer cells, although the RB and p16INK4a genes are preferentially altered in small cell lung carcinoma and NSCLC, respectively (20–22). Hypermethylation of the p16INK4a gene is frequently observed in NSCLC, and ~50% of losses of p16INK4a expression in NSCLC can be caused by hypermethylation of the 5′ regulatory region in the p16INK4a gene (22–24). Because of genetic alterations, the signaling pathway via RB and p16INK4a is estimated as being disturbed in most lung cancers. ARF is encoded by the Cdkn2a gene in which an alternative first exon is spliced into exon 2 in a reading frame different from that of p16INK4a (21, 22). p16INK4a and ARF are immunologically and functionally distinct proteins (21, 22). ARF is capable of inhibiting cell proliferation by blocking the ubiquitin E3 ligase activity of MDM2 (25) and by sequestering...
MDM2 into the nucleolus (26, 27). ARF can be induced by oncogenic signals and acts upstream of p53 to negatively regulate the effects of specific stresses, including oncogenic signaling, DNA damage, and microtubule disruption (28, 29). Decreased expression of ARF has been found in 60% of lung cancers (30). Recently, we reported that mice with an Ink4a/Arf heterozygous deficiency exhibited an increase in lung tumor progression (31). Thus, the Ink4a/Arf locus encodes two proteins that regulate two very important tumor suppressor pathways for lung cancer, RB and p53.

The first goal of the present study was to examine the effect of alterations of both p53 and Ink4a/Arf on lung tumorigenesis using A/J mice carrying germ-line alterations in both genes (p53<sup>+/−</sup>, Ink4a/Arf<sup>−/−</sup>). This was accomplished by backcrossing UL53-3 mice (FVB/J mice carrying three copies of the p53 transgene) to A/J mice for 10 generations and 129-B6 mixed mice (Ink4a/Arf KO) to A/J mice for 6 generations, respectively. A/J mice are highly susceptible to both spontaneously occurring and chemically induced lung tumors and routinely develop adenomas and adenocarcinomas with mutations in the K-ras proto-oncogene (3, 32). We saw a significant functional synergy among the presence of genetic alterations in Kras2, p53, and Ink4a/Arf in promoting lung carcinogenesis. Moreover, this study led to the development of an in situ mouse model with mutations in Kras2, p53, and/or Ink4a/Arf that could be used to identify chemopreventive regimens for lung cancer. The second goal of this study was to determine the efficacy in these mice of a potent chemopreventive agent, budesonide, which has been shown previously to be highly efficacious in the A/J mouse model (2, 3). This was used to systemically validate a p53-Ink4a/Arf transgenic mouse model for lung cancer chemoprevention. Budesonide is a synthetic glucocorticoid and has exhibited a strong preventative activity in BP-induced pulmonary adenoma formation in the female A/J mouse model. This inhibitory effect was achieved with budesonide administered either by diet and/or aerosol even at low levels (33–36). Budesonide was one of a few compounds with lung tumor inhibitory effects even when administered after carcinogen initiation (34). In addition, budesonide is currently undergoing human trials. Accordingly, budesonide is selected as a model compound for the present study.

**MATERIALS AND METHODS**

**Reagents.** BP (99% pure), budesonide (>99% pure), and tricaprylin were obtained from Sigma Chemical Co. (St. Louis, MO). BP was prepared immediately before use in animal bioassays by dissolving in tricaprylin.

**Animals.** A/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). UL53-3 mice on FVB mouse background, carrying three copies of a transgene containing an A-la-135-Val p53 mutation, were obtained from Dr. Roger Wiseman’s group at the National Institute of Environmental Health Sciences, NIH (Research Triangle Park, NC). UL53-3 mice were backcrossed to A/J mice for 10 generations to create the N10 (A/J × UL53-3) mice before use in the present study. Ink4a/Arf<sup>−/−</sup> mice on a 129-B6 mixed background were obtained from Dr. Ronald A. DePinho’s group at Dana Faber Cancer Institute (Boston, MA). The development of the Ink4a/Arf<sup>−/−</sup> mice (knockout mice lacking both p16INK4a and p19ARF genes) was reported previously (37). Ink4a/Arf<sup>−/−</sup> mice were backcrossed to A/J mice for 6 generations to create the N6 (A/J × Ink4a/Arf<sup>−/−</sup>) mice. All of the bioassays in this study were performed on the F1 mice generated by crossing N10 (A/J × UL53-3) and N6 (A/J × Ink4a/Arf<sup>−/−</sup>) mice. Animals were housed in plastic cages with hardwood bedding and dust covers, in a high efficiency particulate air (HEPA)-filtered, environmentally controlled room (24 ± 1°C, 12/12 h light/dark cycle). Animals were given Rodent Lab Chow (5001; Purina) and water ad libitum. For all animal studies, mice were fed powdered AIN-76A Purified Diet (100000; Dyets, Inc., Bethlehem, PA). Body weights were monitored monthly for the duration of the studies.

**p53 and Ink4a/Arf<sup>−/−</sup> Genotype.** All of the animals were genotyped for the p53 mutation (A1aL35Val) and Ink4a/Arf<sup>−/−</sup> using the procedures reported previously (16, 31). Briefly, tail clippings from each N10 (A/J × UL53-3) mouse were homogenized and incubated overnight at 37°C in lysis solution [0.4 mg/ml Pronase, 10% SDS (w/v), 10 mM Tris, 400 mM NaCl, and 2 mM EDTA] followed by phenol-chloroform extraction and precipitation with ice-cold alcohol. The p53 transgene created a restriction fragment length polymorphism with a new HpaII restriction enzyme cleavage site (recognition site: GGGTA). This mutation was used to genotype (UL53-3 × A/J)F1 mice using the PCR-restriction fragment length polymorphism method. PCR primers were designed from the regions of mouse p53 exon 5 that contained the Ala-135-Val mutation. The primer sequences were as follows: (a) 5′-TAC TCT CCT CCC CTC AAT AAG-3′; and (b) 5′-CTC GGG TGG TGG CTA ATG TAC CAC-3′. These PCR primers generated a 190-bp amplified exon 5 fragment from both the wt p53 allele and transgene allele. After amplification, the fragment was incubated with the restriction endonuclease HpaII, which cleaves once within the amplified transgene and none within the wt allele. The cleaved fragments were then subjected to electrophoresis on an 8% polyacrylamide gel along with a DNA size marker and visualized by UV light after staining with ethidium bromide. This procedure was also repeated at least once for each mouse for confirmation.

For Ink4a/Arf<sup>−/−</sup> genotype, tail clippings from each mouse were processed for DNA isolation similarly as those described above. PCR primers were derived from the regions of mouse p16<sup>INK4a</sup> exons 2 and the neo cassette. The primer sequences were as follows: (a) p16<sup>INK4a</sup> exon2F: 5′ TTTA ACA GCC GAG CCT GTGT AC-3′; and (b) p16<sup>INK4a</sup> exon2R: 5′ GAA TCT GCA CCG TAG TTG AG-3′. Together, p16<sup>INK4a</sup> exon2F and p16<sup>INK4a</sup> exon2R amplify a 159-bp product from the exon 2 of p16<sup>INK4a</sup>. The other pair of primers are: neoF: 5′ CTTG GGG TGG AGA GGC TAT TC-3′ and neoR: 5′ AAG TGA GAT CAC GAG AGA TC-3′. Together, neoF and neoR amplify a 280-bp product from the neo insert. The PCR products were then subjected to electrophoresis on a 1% agarose gel, along with a DNA size marker, and visualized by UV light after staining with ethidium bromide. The PCR having both wt p16<sup>INK4a</sup>/ARF alleles (Ink4a/Arf<sup>—/—</sup>) displayed only a single 159-bp fragment; DNA with a wt p16<sup>INK4a</sup>/ARF and target mutation allele (Ink4a/Arf<sup>+/−</sup>) showed 159- and 280-bp bands, whereas DNA with both target mutation alleles (Ink4a/Arf<sup>+/−</sup>) showed only a single 280-bp fragment. This procedure was also repeated at least once for confirmation.

**Lung Tumorigenesis Studies.** Three lung tumor bioassays were conducted simultaneously. For each bioassay, 6-week-old A/J, p53<sup>−/−</sup>/Ink4a/Arf<sup>−/−</sup>, p53<sup>−/−</sup>/Ink4a/Arf<sup>+/−</sup>, and p53<sup>−/−</sup>/Ink4a/Arf<sup>+/+</sup> mice were randomized into eight groups according to the p53 and Ink4a/Arf genotypes and treatments. As seen in Fig. 1A and Table 1, mice in groups 1–4 were given a single i.p. injection of 0.1 ml of tricaprylin as vehicle controls. Mice in groups 4–8 were given a single i.p. injection of BP (100 mg/kg body weight) in 0.1 ml of tricaprylin. Animals were terminated 18 weeks (lung tumor bioassay I), 30 weeks (lung tumor bioassay II), and 40 weeks (lung tumor bioassay III) after exposure to the carcinogen. During the bioassay, all of the animals were observed daily for classical signs of ill health and weighed individually twice a month for the duration of the study. At termination, all of the animals were killed by CO2 asphyxiation. The livers were fixed in formalin and Finkellyszniczky’s (90% ethanol (70% volume for the duration of the study), 5% glacial acetic acid, and 5% formalin (10% volume for volume buffered formalin) overnight and then kept in 70% ethanol for evaluation before paraffin embedding. Lung tumor development was then evaluated by counting tumor number (N), calculating tumor volume (V) and the total tumor load (total tumor V per mouse). The tumor volumes were determined by measuring the diameter of each tumor, which is generally in a round shape. The radius (r = diameter/2) was determined, and the total tumor volume was calculated by: V = 4/3πr<sup>3</sup>. All available lung tumors were subjected to histopathological examination.

**Chemoprevention Studies with Budesonide.** Three separate lung tumor chemoprevention studies were performed. For each study, 6-week-old A/J, p53<sup>−/−</sup>/Ink4a/Arf<sup>−/−</sup>, p53<sup>−/−</sup>/Ink4a/Arf<sup>+/−</sup>, and p53<sup>−/−</sup>/Ink4a/Arf<sup>+/+</sup> mice were randomized into eight groups according to the p53 and Ink4a/Arf genotypes and treatments. As seen in Table 2, mice in groups 1–4 were given a single i.p. injection of BP (100 mg/kg body weight) in 0.1 ml of tricaprylin and fed AIN-76A-purified diet (Diets, Inc.). Mice in groups 4–8 were given a single i.p. injection of BP (100 mg/kg body weight) in 0.1 ml of tricaprylin and fed AIN-76A-purified diet containing budesonide (1.5 mg/kg diet). As shown in Fig. 1B, three study designs were used: (a) budesonide was given 2 weeks previous BP treatment and continued for an additional 18 weeks; (b) budesonide was given 2 weeks previous to BP treatment; and (c) mice were fed AIN-76A-purified diet for 18 weeks after initiation of BP treatment.
At 6 weeks of age, mice were given single i.p. injections of BP at a dose of 100 mg/kg in tricaprylin, which was counted as week 0. Mice in all experiments (1, 2, and 3; Fig. 1A and Table 1, mice in groups 1–4 were given a single i.p. injection of 0.1 ml of tricaprylin as vehicle controls. Mice in groups 4–8 were given a single i.p. injection of BP (100 mg/kg body weight) in 0.1 ml of tricaprylin. Animals were terminated 18 weeks after exposure to the carcinogen. At this time, all of lung tumors were diagnosed as lung adenomas. As shown in Table 1, p53−/− Ink4a/Arf−/− and p53−/−/Ink4a/Arf−/− mice carrying a mutant p53 transgene (Val135) with or without Ink4a/Arf heterozygous deletion developed a higher number of lung tumors (an average of 12.5 tumors/mouse) with larger size (~7.5 mm³) after treatment with BP than wt and p53−/−/Ink4a/Arf−/− mice (an average of 7.5 tumors/mouse; ~3 mm³), P < 0.001; Table 1). In the vehicle control groups, a low incidence of lung tumors was observed, and there was no significant difference in tumor multiplicity among the four groups. In general, p53 mutation caused an increase in both tumor multiplicity and size, whereas Ink4a/Arf heterozygous deficiency did not affect tumor multiplicity and size.
have any effect on either tumor multiplicity or size at this stage of lung tumorigenesis, which is consistent with our previous observations (16). The designs of the second and third bioassays were similar to the first bioassay with the exception of the termination time point. Animals were terminated 40 weeks after exposure to BP for the second bioassay and 30 weeks for the third bioassay. Both of these bioassays were designed to determine the effect of the p53 and Ink4a/Arf on lung tumor progression (Fig. 1A and Table 1). The incidence of lung tumors in all four groups of treated mice was 100%. Similar to the first lung tumor bioassay, p53<sup>+/−</sup>/Ink4a/Arf<sup>−/+</sup> and p53<sup>−/+</sup>/Ink4a/Arf<sup>−/+</sup> mice developed a higher number of lung tumors (an average of 21 tumors/mouse for bioassay 3 and an average of 25 tumors/mouse for bioassay 2) after treatment with BP than wt and p53<sup>−/+</sup>/Ink4a/Arf<sup>−/+</sup> mice (an average of 12.5 tumors/mouse for bioassay 3 and an average of 12 tumors/mouse for bioassay 2). More interestingly, p53<sup>−/+</sup>/Ink4a/Arf<sup>−/+</sup> mice exhibited a striking increase in tumor volume (~24-fold in bioassay 3 and ~23-fold in bioassay 2) compared with a 9-fold increase in tumor volume in mice with only the p53 dominant negative mutation (p53<sup>−/+</sup>/Ink4a/Arf<sup>−/−</sup>). There was also a ~50% and a ~4-fold increase in tumor volume in Ink4a/Arf heterozygous-deficient mice (p53<sup>−/+</sup>/Ink4a/Arf<sup>−/−</sup>) in bioassays 3 and 2, respectively, indicating that the effect of Ink4a/Arf heterozygous deficiency is mostly on late stage lung tumor progression.

In addition, most of the lung tumors (~80%) from mice with a p53 mutation and deletion of Ink4a/Arf (p53<sup>−/−</sup>/Ink4a/Arf<sup>−/−</sup>) were lung adenocarcinomas. In contrast, lung adenocarcinomas were seen in <10% of the lung tumors from the wt mice and ~50% of the lung tumors from either p53 transgenic mice or Ink4a/Arf heterozygous deficient mice. Fig. 2 shows the gross photomicrographs of lung tumors from mice with various genotypes. Lung tumors were significantly larger in mice treated with BP alone (Fig. 2, A–D) than those treated with BP and budesonide (Fig. 2, E–H). Fig. 3 shows the light
Of human adenocarcinoma, namely p53 transgenic and Ink4a/Arf
heterozygous deficient mice on an A/J background, includes alter-
ations in Kras2, p53, and Ink4a/Arf type-specific variations. However, the degree of budesonide chemopreventive effect on tumor size was profoundly dependent on mouse genotypes. Thus, mice with wt, p53+/−/Ink4a/Arf+/−, p53+/−/Ink4a/Arf+/−, and p53+/−/Ink4a/Arf+/− showed 82, 64, 45, and 33% decrease in tumor volume, respectively. The difference between 82% inhibition and 33% inhibition is statistically significant (P < 0.01; Table 2). The mutant mice with alterations in p53 and/or Ink4A/Arf seemed to exhibit a significant resistance to chemoprevention by budesonide. This observation has immediate clinical implications in at least two aspects: (a) the third chemoprevention study may more closely parallel potential clinical trials by exposing individuals with established precancerous lesions; and (b) budesonide may be much less efficacious in lesions that contain alterations in p53 and Ink4A/Arf.

DISCUSSION

In this study, we described two major findings: (a) we demonstrated a significant synergistic effect of germ-line alterations in p53 and Ink4A/Arf on lung tumor progression; and (b) we observed differential chemopreventive effects of budesonide on BP-induced lung tumorigenesis in wt and mutant mice with alterations in p53 and/or Ink4A/Arf, which was particularly striking when budesonide treatment was initiated later. We have clearly demonstrated the functional role of p53 and Ink4A/Arf on lung tumorigenesis. The resulting tumor model for human adenocarcinoma, namely p53 transgenic and Ink4A/Arf heterozygous deficient mice on an A/J background, includes alterations in commonly altered genes, e.g., Kras2, p53, and Ink4A/Arf when treated with lung-specific chemical carcinogens. Clearly, this
Differential Effect of Budesonide on Lung Tumorigenesis

Mouse model is superior over the commonly used A/J mice in examining lung adenocarcinomas. Mutation or deletions of the Kras2, p53, and Ink4a/Arf tumor suppressor genes are the most common genetic defect detected in human lung cancer. p53 mutations have been found in 50–80% of sporadic lung cancers, indicating that p53 plays a crucial role in the development of human lung cancer (38). Thus, mouse lung tumor models with germ-line p53 mutations would be important for determining the role of p53 in lung tumorigenesis, e.g., our model is directly related to patients with LFS who develop lung cancer at a remarkably high rate.5 Lung cancer was found to be the most frequently observed cancer type in adult male p53 mutation carriers with a 50% risk of developing lung cancer by age 60.6 Although 10% of smokers develop lung cancer, ~50% of males with LFS develop lung cancer. In a previous study, we found that (UL53-3 × A/J) F1 p53 transgenic mice carrying a p53 transgene (Val135) exhibit an increased susceptibility to early stage lung tumor development with animals terminated 16 weeks after carcinogen treatment, suggesting that inactivation of p53 is involved in the early stage of lung tumorigenesis (16). The present study demonstrated that mice with a germ-line p53 mutation also developed a significant increase in lung tumor progression, indicating that the p53 mutation not only plays a role in early stage but also in late stage of the carcinogenic process.

p16 or INK4A is another tumor suppressor that is frequently inactivated in human lung cancer. Ng et al. (39) investigated methylated p16 in patients with NSCLC and found that the patients with plasma and preresection pleural lavage methylated p16 have shorter survival. Patients with p16INK4A-negative tumors had a significantly shorter survival (4 months) than those with p16INK4A protein expression (15 months). Jin et al. (40) have studied the association of the immunohistochemical expression of cyclin D1, p16, and pRB with the prognoses of 106 patients with NSCLC at stages I and II after complete resection was investigated. Their results indicate that cyclin D1 and p16, especially a combination of cyclin D1 and p16, are very useful in predicting the prognosis of a patient with NSCLC after curative resection, independent of pathological stages I and II (40). Similarly, mice with a heterozygous deficiency for Ink4a/Arf were significantly more susceptible to mouse lung tumor progression (31). The results from the present study extend our previous observations that mice with a heterozygous deficiency of Ink4a/Arf exhibited a striking enhancement in lung tumor progression at 30 and 40 weeks after carcinogen treatment when compared with their respective wt mice, whereas mice with Ink4a/Arf heterozygous deficiency showed little effect on early stages of lung tumor development.

One of the most interesting results is the observation that mutant p53 acts synergistically with deletion of Ink4a/Arf to accelerate the development of undifferentiated malignant lung tumors. In fact, mice with p53 dominant negative mutation and Ink4a/Arf heterozygous deficiency exhibited a >20-fold increase in tumor volume compared with a 4-fold increase in Ink4a/Arf heterozygous deficient mice and a 9-fold increase in mice with only the p53 dominant negative mutation. This synergistic effect occurs at the stage of tumor progression because most of lung tumors (~80%) from mice with p53 mutation and deletion of Ink4a/Arf (p53+/−/Ink4a/Arf+/−) were lung adenocarcinomas compared with 10% in wt mice and 50% in Ink4a/Arf heterozygous deficient mice. The synergistic relationship between alterations of the p16INK4A and p53 genes in tumor development has recently been reported (41, 42), e.g., Kinoshita et al. (41) while examining NSCLC in humans have shown that proliferative activity was considerably higher in p53 mutant than normal tumors, and loss of p16 expression was associated with a further increase in proliferative activity in the p53 mutant tumors but not with proliferative activity in the p53-negative tumors. Bardeesy et al. (42) created compound mutants of inactivated p16 and mutant p53 in transforming growth factor-α transgenic mice and demonstrated a synergistic interaction between inactivated p16 and mutant p53 as the transforming growth factor-α animals heterozygous for both the Ink4a/Arf and p53 mutation showed a dramatically increased incidence of serous cystadenoma than those with heterozygosity at either locus alone.

In the present study, we have examined the use of these models in the chemoprevention studies using budesonide. The efficacy of budesonide in these models was examined because the altered p53 and Ink4a/Arf appear particularly relevant to human lung cancer but are not routinely altered in carcinogen-induced lung tumors in A/J mice until late in tumor development, if at all. These models are particularly relevant because many early preinvasive lesions of the human aerodigestive tract have alterations in both p53 and p16. On the other hand, our mouse models closely resemble the A/J mouse lung tumor model, because our mice have a pure A/J background with either p53 dominant negative mutation p53val135/−/wt, heterozygous deletion of Ink4a/Arf, or both. Early and continual budesonide administration in the diet exhibited a potent protective effect against BP-induced lung tumorigenesis in mice of different genotypes particularly at the 18-week time point. Budesonide produced a striking 70–80% reduction in tumor multiplicity and an 88–95% in total tumor volume in the short-term (18 week) study irrespective of the genotype. This is consistent with previous studies using either A/J mice or mice with a p53 mutation (2, 3). Similarly, in mice exposed to budesonide continually and sacrificed at 40 weeks, we found a consistent decrease in tumor multiplicity (66–76%) and a decrease in tumor volume of 94–98% in wt, p53 mutant, or Ink4a/Arf-deficient mice. In contrast, mice with both a p53 mutation and Ink4a/Arf deficiency showed only a 77% decrease in tumor volume. Most strikingly, however, in the late treatment experiment in which mice were treated with budesonide for 12 weeks after they had developed small adenomas, one observed a ~40–50% decrease in tumor multiplicity in all groups of animals. However, when examining effects on tumor volume, we observed a striking genotype-dependent effect with wt mice, p53+/−/Ink4a/Arf+/− mice, p53+/−/Ink4a/Arf+/− mice, and p53+/−/Ink4a/Arf+/− mice, showing 82, 64, 45, and 33% decreases in tumor volume, respectively. Thus, mice with genetic alterations of p53 and Ink4a/Arf are highly resistant to budesonide compared with wt mice during late intervention.

The mechanism for the observed differential chemopreventive effect of budesonide during late intervention is not clear at present. Two general mechanisms for the inhibitory effect of budesonide on cancer have been proposed, namely, induction of p27/p21 or induction of apoptosis (43–47). A recent study has shown that cells treated with corticosterone have an increased binding of p27 with CDK4, with a strong decrease in the kinase activity of the CDK4–cyclin D1 complex, suggesting that induction of p27/p21 may play a role in growth inhibition by corticosterone (43, 47). Glucocorticoids have also been shown to induce G1 arrest and apoptosis of several leukemia cell lines (44–46). It is likely that some of the budesonide-mediated chemopreventive effects would require the presence of either wt p53, wt Ink4a/Arf, or both. Because mutant A/J mice are the most likely to closely resemble the human counterpart than wt A/J mice, our study indicates that budesonide will be less effective in inhibiting or regressing human lung precancer lesions than might be predicted through the use of A/J mice (2, 3). Thus, the models described here would appear particularly relevant to examining potential chemopreventive and therapeutic agents using an in situ model with a number of the known mutations relevant to lung adenocarcinomas in humans.
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