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Preventive Effects of Bexarotene and Budesonide in a Genetically Engineered Mouse Model of Small Cell Lung Cancer

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Abstract In the present study, we examined the effect of bexarotene (Targretin) and budesonide in the chemoprevention of small cell lung carcinoma using a lung-specific knockout model of *Rb1* and *p53*. Upon treatment with bexarotene, tumor incidence, number, and load were significantly reduced ($P < 0.05$). Budesonide treatment trended to inhibition, but the effect was not statistically significant ($P > 0.05$). Immunohistochemical staining indicated that bexarotene treatment decreased cell proliferation and increased apoptosis in tumors. The *Rb1/p53* gene–targeted mouse seems to be a valuable model for chemopreventive studies on human small cell lung cancer. Our results indicate that the retinoid X receptor agonist bexarotene may be a potent chemopreventive agent in this cancer type.

Introduction

Small cell lung carcinoma (SCLC) is the most aggressive subtype of lung cancer, with a mortality rate as high as 95% (1). The high frequency of relapse after initial chemotherapy accounts for the poor prognosis of this cancer type with a 5-year survival rate of ~5% (2, 3). Cigarette smoking is associated with >90% of SCLCs (1). SCLC is believed to originate from cells residing in the epithelial lining of the bronchi, which have a neuroendocrine phenotype (4). Histopathologically, SCLC is formed by cancer cells small in size with highly pleomorphic involuted nuclei and a high nuclear/cytoplasmic ratio (4). They also express markers of neuroendocrine differentiation, such as chromogranin A, neuron-specific enolase, synaptophysin, or neural cell adhesion molecule (5). Alterations in the tumor suppressor genes *Rb1* and *p53* are found in 90% of SCLCs (6). Besides retinoblastoma, SCLC is the only other human neuroendocrine tumor that harbors RB1 mutations in almost all cases (7). Amplification of *L-myc* and *N-myc* oncogenes is exclusively present in SCLC compared with NSCLC (8). Increased BCL-2 and decreased BAX protein levels are often present coordinately with the loss of p53 (9).

Almost all mouse models of lung cancer produce adenomas and adenocarcinomas (10). Recently, Meuwissen et al. (5) generated mice with conditionally targeted alleles for both Rb1 and p53 that developed aggressive lung tumors with high incidence and with striking morphologic and immunophenotypic similarities to SCLC. *Rb1* and *p53* alleles were conditionally inactivated in the lung epithelium by using adenovirus-mediated somatic gene transfer of Cre recombinase (11). One potential strategy to prevent human SCLC in high-risk populations, such as smokers or ex-smokers, is to use chemopreventive agents to prevent the progression of preneoplastic lesions to late stage cancer and/or inhibit the development of new lesions. Bexarotene is a rexinoid and a retinoid X receptor (RXR) agonist. Budesonide is a synthetic glucocorticoid. Both of these agents had proven to be effective in blocking lung adenoma/adenocarcinoma formation in mouse models (12, 13). However, their effect on the development of SCLCs is not known. The present study used this mouse model of hSCLC to test the chemopreventive activities of bexarotene and budesonide. In addition, we examined the effect of these agents on cell proliferation and apoptosis.

Materials and Methods

Reagents

Adeno-Cre virus (Ad5-CMV-Cre virus) was purchased from the University of Iowa Gene Transfer Vector Core (Iowa City, IA). Ketamine (NDC 0856-2013-01, Ketaset III, Ketamine HCl INJ USP) and xylazine were obtained from the Washington University School of Medicine Veterinarian Pharmacy. Bexarotene was obtained from the National Cancer Institute Chemical Repository (Bethesda, MD). Budesonide (>99% pure) was purchased from Sigma Chemical, Co.

Genotyping

Mice carrying conditional alleles for *Rb1* (floxed at exon 19) and *p53* (floxed at exons 2-10) were obtained from Dr. Anton Berns' laboratory (Division of Molecular Genetics, Netherlands Cancer Institute, Amsterdam, The Netherlands) (5). The original mice were on a mixed background. These mice were backcrossed to A/J mice (Jackson Laboratory) for five generations in our laboratory before use in the present study. For each generation, mouse-tail clippings were taken for

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genotyping of the Rb-Flox (RF) and p53-Flox (PF). Tail clips were incubated overnight at 37°C in lysis buffer [0.4 mg/mL Pronase, 10% (w/v) SDS, 10 mmol/L Tris, 400 mmol/L NaCl, and 2 mmol/L EDTA]. DNA isolation was then carried out with saturated NaCl and precipitation with ice-cold alcohol. Genotyping was done on DNA from each mouse for the presence of the transgenes by PCR. The PCR products were subjected to electrophoresis on a 2% agarose gel along with a DNA size marker and visualized by UV light after staining with ethidium bromide. For the *p53*-flox allele, PCR was carried out with primers p53-10F (5′-AAGGGGTATGAGGGACAAGG-3′) and *p53*-10R (5′-GAAGACAGAAAAGGGGAGGG-3′) to amplify a 460-bp product for the wild-type allele and a 584-bp product for the *p53*-floxed allele. DNA with both wild-type *p53* ($p53^{wt/wt}$) displayed only a single 460-bp fragment, DNA with wild-type *p53* and Floxed ($p53^{Flox/wt}$) alleles showed both 460-bp and 584-bp fragments, whereas DNA with both Floxed ($p53^{Flox/Flox}$) alleles showed a single 584-bp fragment. For the *Rb*-flox, the PCR was carried out with primers Rb19E (5′-CTCAAGAGCTCAGACTCATGG-3′) and Rb18 (5′-GGCGTGTGCCATCAATT-3′) to amplify a 200-bp product for the wild-type allele and a 283-bp product for the *Rb*-floxed allele. DNA with both wild-type *Rb* alleles ($Rb^{wt/wt}$) displayed only single 200-bp fragment, DNA with wild-type *Rb* allele and Floxed ($Rb^{Flox/wt}$) allele showed both 200-bp and 283-bp fragments, whereas DNA with both Floxed ($Rb^{Flox/Flox}$) alleles showed a single 283-bp fragment. Only the B5 (AJ × $Trp53^{F2\text{-}10/F2\text{-}10}$;$Rb1^{F19/F19}$) mice were selected for use in this study.

Intratracheal Adeno-Cre virus administration

Adeno-Cre virus was suspended in 3% sucrose in PBS at a concentration of 1×10^{12} particles/mL and stored at −80°C until use. Ad-Cre virus was administered via intratracheal injection to somatically inactivate *p53* and *Rb1* in pulmonary bronchial epithelial cells of B5 (AJ × $Trp53^{F2\text{-}10/F2\text{-}10}$;$Rb1^{F19/F19}$) mice. For each mouse, 5×10^{10} particles of virus were delivered through the trachea. A cocktail was made with 1 mL of ketamine, 0.15 mL of xylazine, and 4 mL of PBS. Mice were anesthetized with 100 μL of the cocktail per 20-g mouse through i.p. injection, placed in a supine position with a rubber "pillow" under its neck to ensure that the airway was straight. A catheter [26-gauge × 19 mm (3/4-in., Venisystems, Abbocath-T, Abbott Ireland)] was inserted slowly into the trachea, a 1 mL syringe was attached, and virus was delivered slowly. The mouse was held by the performer with two hands at its forearms and a soft rub was given to its chest gently for about 15 s for the virus to move down into the lung and to prevent death by bronchus blockage.

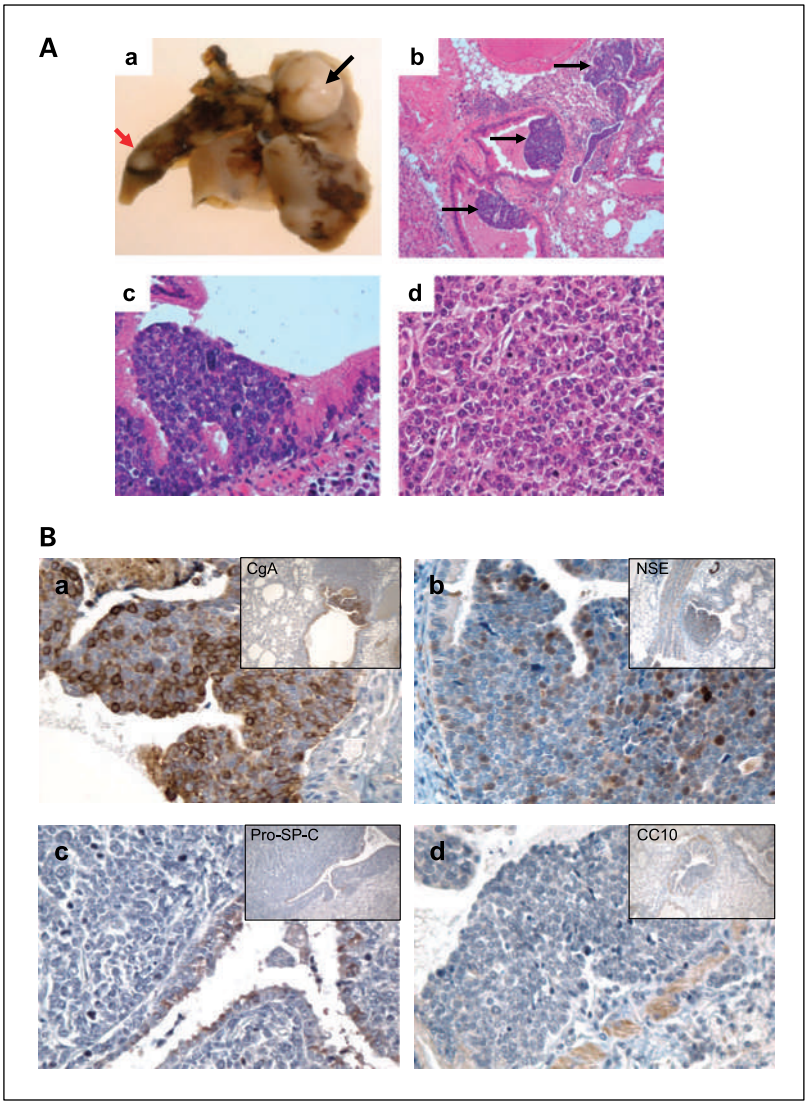

Fig. 1. Development of SCLCs in B5 (AJ × $Trp53^{F2\text{-}10/F2\text{-}10}$;$Rb1^{F19/F19}$) mice. *A*, gross and histopathologic appearance of mSCLCs. *a*, gross appearance of mouse SCLC (*black arrow*). *b*, multiple dysplastic lesions (*arrows*) stained with H&E from a mouse 21 wk after intratracheal Adeno-Cre (×40). *c*, dysplastic lesion stained with H&E from a mouse 21 wk after intratracheal Adeno-Cre (×400). *d*, H&E stain of mSCLC from mouse 36 wk after intratracheal Adeno-Cre (×400). *B*, immunohistochemical staining on lung mSCLCs in B5 (AJ × *Trp53F2-10/F2-10;Rb1F19/F19*) mice with markers of neuroendocrine and epithelial differentiation. *a*, anti–chromogranin A antibody; *b*, anti–neuron-specific enolase antibody; *c*, anti–pro-surfactant protein-C antibody; *d*, anti-CC 10 antibody.

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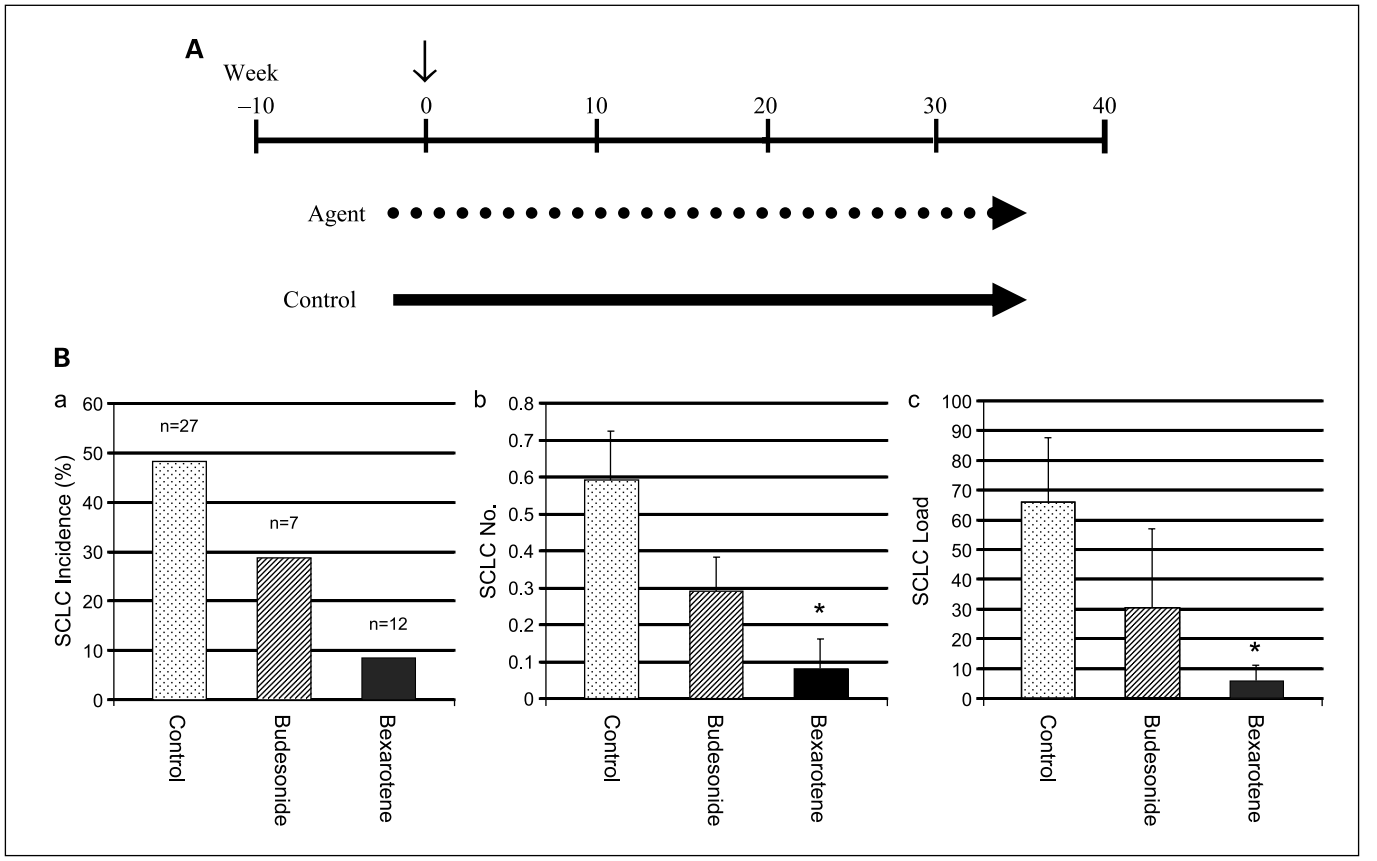

Fig. 2. Chemopreventive effect of bexarotene and budesonide on mouse SCLC. *A*, experimental design of chemoprevention. The time (*vertical arrow*) of Adeno-Cre virus administration was counted as week 0 (~9 week-old). Chemopreventive agent (*dotted line*) or control (*solid line*) groups were administered 2 wk before Adeno-Cre (~7 week-old). Mice were given the agent continuously until the end of the experiment (36 wk after Adeno-Cre virus; ~45 week-old). *B*, chemopreventive effect of bexarotene and budesonide on mouse SCLC. Effect of bexarotene and budesonide treatments on mSCLC tumor incidence (*a*), SCLC tumor multiplicity (*b*), and SCLC tumor load (*c*). *Columns*, mean; *bar*, SE (*, $P < 0.05$).

Histopathologic analysis

Lung tissue was fixed in Tellyesniczky's solution (90% ethanol, 5% glacial acetic acid, and 5% formalin) for 24 to 48 h and stored in 70% ethanol. Tissue sections (5 μm each) were cut from each lung and stained with H&E for histologic examination (Fig. 1A) or unstained for future immunohistochemical analysis.

Immunohistochemical study

Three lungs from each group of B5 (AJ × $Trp53^{F2\text{-}10/F2\text{-}10}$;$Rb1^{F19/F19}$) mice were analyzed. All slides were deparaffinized in xylene and rehydrated in gradient ethanol. Microwave antigen retrieval was carried out for 20 min in citrate buffer (pH 6.0). After blocking in 10% normal goat serum in PBS, primary antibody was diluted in 10% normal goat serum and incubated at 4°C overnight. Neuroendocrine markers, including chromogranin A (14) and neuron-specific enolase (15), and epithelial markers, including pulmonary surfactant protein C (SP-/C) and Clara cell secretory protein (10 = 10 kDa) were used to evaluate mSCLC tumors. Cell proliferation was assessed using primary monoclonal antibody against Ki-67 (1:200 dilution; Novo Castra). Cells undergoing apoptotic changes were detected using a terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay according to the instructions of the manufacturer (ApopTag, *In situ* Apoptosis Detection Kit; Intergen). Negative control slides were processed at the same time. Manual counting of labeled and total cells in high-powered (×400) fields of tumor tissue was conducted.

Chemoprevention study

Seven-week-old mice were randomized into three groups as shown in Fig. 2. Chemopreventive agents (*dotted line,* bexarotene and budesonide) or control diet (*solid line*) were given to mice 2 wk before intratracheal administration of Adeno-Cre virus (counted as week 0; indicated by arrow in Fig. 2). Nine-week-old B5 (AJ × $Trp53^{F2\text{-}10/F2\text{-}10}$;$Rb1^{F19/F19}$) mice were given AIN-76A Purified Diet no. 100,000 (Dyets, Inc.) with or without chemopreventive agent continuously until the end of experiment. Mice in group 1 were fed AIN-76A–purified diet as controls. Mice in group 2 were fed AIN-76A–purified diet containing budesonide (1.5 mg/kg diet), which was freshly made every week. Mice in group 3 were fed AIN-76A–purified diet and received bexarotene treatment. Bexarotene, 180 mg/kg body weight, was suspended in corn oil and delivered by oral gavage once a day and 5 d/wk. Bexarotene suspension was made fresh daily. Food and water were available *ad libitum*. Lung tissue was fixed in Tellyesniczky's solution and stored in 70% ethanol. Lung tumor number was counted and the tumor diameter measured. For spherical tumors, the radius was used to calculate volume using the formula $V = (4/3)\ \pi r^3$ (16). For irregular tumors, three measurements were taken at height (H), width (W), and length (L). The volume was calculated using the formula $V = (4/3)\ \pi \times L/2 \times W/2 \times H/2$ (17).

Results

In B5 (AJ × $Trp53^{F2\text{-}10/F2\text{-}10}$;$Rb1^{F19/F19}$) mice that received intratracheal Adeno-Cre virus, multiple foci of dysplastic cells

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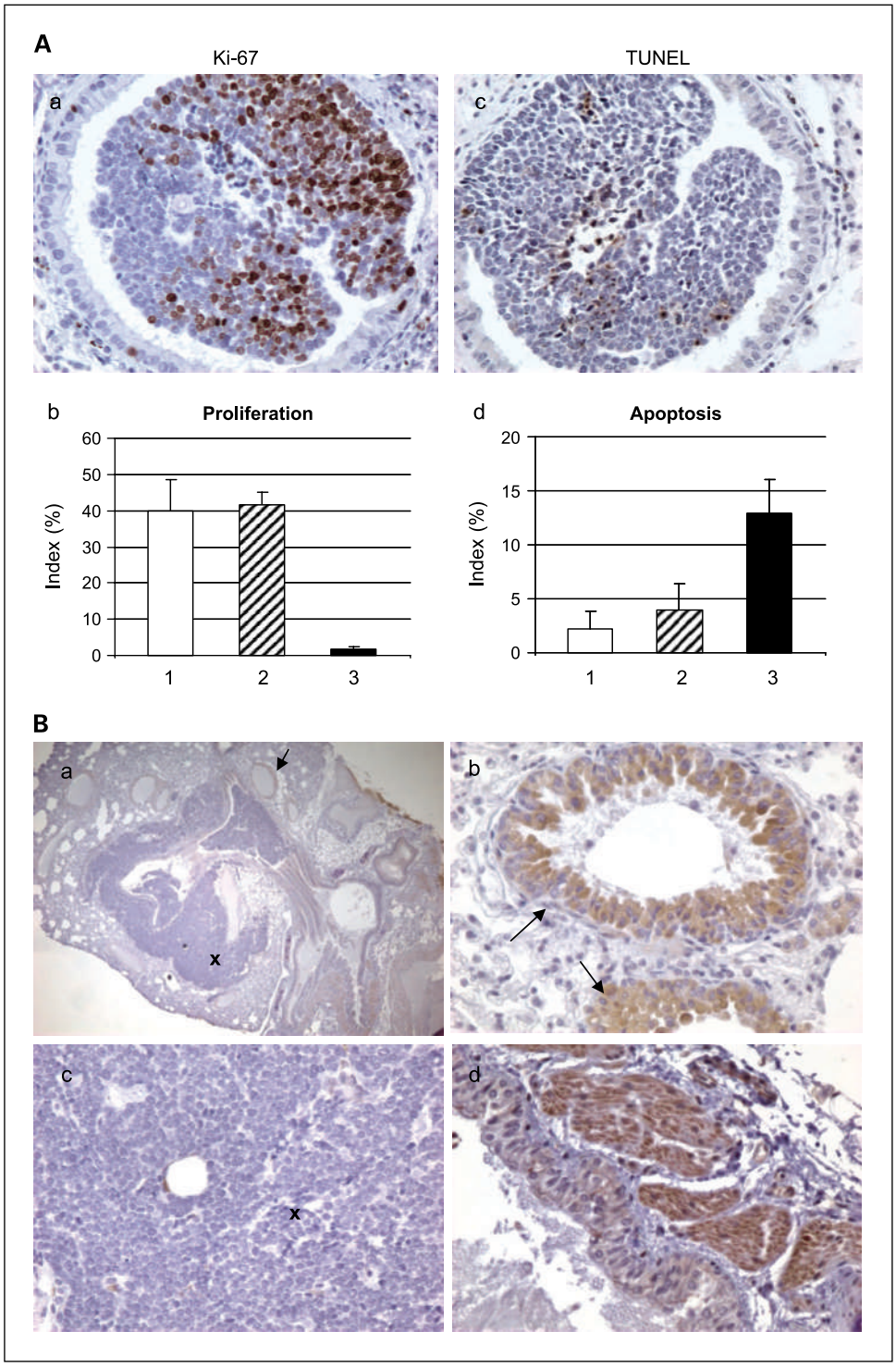

Fig. 3. Immunohistochemical staining. *A,* effect of bexarotene on cell proliferation and apoptosis. Representative photo images of Ki-67 stains (*a*) and TUNEL stains (*c*). Bexarotene decreased cell proliferation (*b*; *1,* control; *2,* budesonide; *3,* bexarotene) and increased apoptosis in mSCLC (*d: 1,* control; *2,* budesonide; *3,* bexarotene). *B,* immunohistochemical staining on mSCLC in B5 (AJ × *Trp53*$^{F2\text{-}10/F2\text{-}10}$;*Rb1*$^{F19/F19}$) mice with anti-GR antibody. *a,* positive staining of GR in bronchial epithelial cells (*arrow*) and negative staining in SCLC cells (*cross*); *b,* enlarged bronchial epithelial cells; *c,* enlarged SCLC cells; and *d,* the positive staining in muscle cells. Original magnifications, ×100 (*a*); ×400 (*b-d*).

were observed inside the bronchial and bronchiolar lumen 10 weeks after intratracheal Adeno-Cre administration (Fig. 1A, *b*). Dysplastic cells were small and tightly clustered (Fig. 1A, *c*). Visible tumors were apparent 36 weeks post-intratracheal Adeno-Cre administration (Fig. 1A, *a*). These tumors lacked the typical glandular and papillary features (Fig. 1A, *d*) seen in mouse lung adenocarcinomas. In general, the cancer cells were small with a high nuclear/cytoplasmic ratio. As shown in Fig. 1A, *d*, additional features of the cancer cells included hyperchromatic nuclei and a diffuse chromatin pattern that obscured the nucleolus. Cytoplasm was poorly developed and nuclear molding was commonly present. The cancer cells grew mostly in sheets and spread diffusely through the pulmonary tissues and air spaces, eliciting little stromal response. The mSCLC features were also determined by immunohistochemical staining. Neuroendocrine markers neuron-specific enolase and chromogranin A were detectable in mSCLC tumors (Fig. 2B, *a* and *b*), although the epithelial markers surfactant protein C and Clara cell secretory protein

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stained only in epithelial cells (Fig. 1B, *c* and *d*). Average tumor multiplicity was 0.59 ± 0.13 tumors per mouse and average tumor load was 65.6 ± 21.9 mm^3 (Fig. 2B). No significant difference was observed between female and male mice in the occurrence or phenotype of SCLC.

B5 (AJ × $Trp53^{F2\text{-}10/F2\text{-}10}$;$Rb1^{F19/F19}$) mice were used to determine the chemopreventive efficacy of bexarotene and budesonide. As shown in Fig. 2B, 48% (13 of 27) of control mice developed SCLC compared with only 8% (1 of 12) of bexarotene-treated mice; an 83% decrease in tumor incidence. The number of SCLC per mouse was 0.59 ± 0.13 and 0.08 ± 0.08 in control and bexarotene-treated mice, respectively, representing an 86% decrease in mSCLC multiplicity (Fig. 2B). Average tumor load was 65.6 ± 21.9 and 5.5 ± 5.4 mm^3 in control and bexarotene-treated mice, respectively (Fig. 2B), which is a decrease of 92%. Budesonide had a moderate inhibitory effect with a decrease in tumor incidence by 41% (48% in control mice versus 28% in budesonide-treated mice; Fig. 2B). The average number of mSCLC tumors per mouse was 0.29 ± 0.18 in budesonide-treated mice, representing a decrease in tumor multiplicity by 51% (P = 0.141; Fig. 2B). The average tumor load was 30.4 ± 25.3 mm^3, representing a decrease in tumor load of 54% (P = 0.221; Fig. 2B).

The striking decrease in mSCLC growth is likely to be reflected in decreased proliferation and/or increased apoptosis. To investigate these two possible mechanisms, immunohistochemical assays with anti-Ki67 antibody for proliferative index and TUNEL assay for apoptotic index were done (Fig. 3A). Staining for Ki67 was present in 40.0%, 41.7%, and 1.8% of SCLC cells in control, budesonide, and bexarotene-treated tumors, respectively (Fig. 3A, *b*). The Ki67 labeling index was decreased by 96% after bexarotene treatment (Fig. 3A, *b*). TUNEL-positive cells were present in 2.2%, 4.0%, and 12.9% of mSCLC in control, budesonide-treated, and bexarotene-treated mSCLC samples, respectively (Fig. 3A, *d*). TUNEL labeling increased by <2-fold with budesonide and by almost 4-fold with bexarotene. These results indicate that treatment with bexarotene decreased the proliferative index and increased the apoptotic rate of the mSCLC cells. Finally, the presence of glucocorticoid receptor (GR) was determined in mouse SCLC using immunohistochemical staining with anti-GR antibody. GR staining was present in bronchial epithelial cells (Fig. 3B, *a-d*) and muscle cells (Fig. 3B, *d*) but was not detected in mSCLC cells (Fig. 3B, *a* and *c*).

Discussion

In this study, we used a recently developed model of SCLC (5) to test for the potential chemopreventive activity of two agents. We found that bexarotene is highly effective in inhibiting the development of mSCLC. Bexarotene is a RXR-selective agonist that minimally binds retinoic acid receptor receptors (18), and it is the first synthetic RXR-selective agonist to enter clinical trials for cancer therapy indications (19). The RXR receptors form heterodimers with a wide variety of nuclear receptors, including the peroxisome proliferator–activated receptors (PPARα, PPARγ, and PPARδ), the farnesoid X receptor (FXR), the constitutive androstane receptor (CAR) receptor, the RAR receptors (α, β, γ), the vitamin D receptors, and the liver X receptors (LXRα and LXRβ; refs. 20–22). The resulting heterodimers serve as transcription activators of a wide variety of genes. These receptors play major roles in glucose (PPARγ), triglyceride (PPARα), cholesterol (PPARδ, LXR), bile acid (FXR), and xenobiotic (CAR receptor) metabolism. Bexarotene has been shown to have antiproliferative activity in preclinical *in vitro* and *in vivo* models of many cancers. In the *N*-nitroso-*N*-methylurea–induced estrogen receptor (ER)–positive rat mammary tumor model, bexarotene caused a 90% reduction in tumor burden and tumor incidence compared with control rats (23). Similarly, bexarotene and other RXR agonists have proven highly effective in preventing ER negative mammary tumors in transgenic mice (24, 25). We and others have shown that the RXR agonist bexarotene is an effective chemopreventive agent in an adenocarcinoma model of lung cancer (13, 26). Other investigators have similarly shown that other RXR agonists are similarly effective in preventing lung non-SCLC in human (27). Rosati et al. (28) have shown that a RXR-selective synthetic retinoid, LG100153, was a potent agent in SCLC cell lines (28). The present finding that bexarotene profoundly reduced mSCLC tumor incidence and multiplicity, as well as decrease tumor size, is consistent with previous studies and suggests that bexarotene is a potent chemopreventive agent against mSCLC.

Budesonide treatment did not have a statistically significant effect on the chemoprevention of mSCLC in this model as measured by tumor incidence, tumor number, or tumor load (Fig. 2). Budesonide has been shown to be one of the most potent chemopreventive agents in mouse adenoma/adenocarcinoma models. It prevented lung adenoma formation in benzo(*a*)pyrene–induced A/J mice (wild-type) when delivered via diet (29) or by aerosol inhalation (30). It was also effective against lung adenoma/adenocarcinoma development in p53 and/or Ink4A/Arf mutant mice (12). One possible reason for the lack of efficacy of budesonide in this model is the absence of detectable GR in mouse SCLC (Fig. 3B). A correlation between decreased GR expression and resistance to the antiproliferative effects of GR in human SCLC has previously been observed (31, 32). A recent study found that human SCLC cells (DMS 79, DMS 53, and COR L24) are profoundly resistant to glucocorticoids primarily due to deficient GR expression (33).

In summary, we found that mouse SCLC development could be effectively prevented by the RXR agonist bexarotene. The glucocorticoid budesonide, which was highly effective in a mouse lung adenocarcinoma model, was ineffective in mouse SCLC likely due to a lack of GR expression in SCLC cells. The preventive effect of bexarotene is accompanied by decreased proliferation and increased apoptosis. To the best of our knowledge, this is the first chemopreventive investigation in a mouse model of SCLC. B5 (AJ × $Trp53^{F2\text{-}10/F2\text{-}10}$; $Rb1^{F19/F19}$) mice will be a valuable model for preclinical chemopreventive and potentially chemotherapeutic studies to identify agents active against human SCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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