Estrogen promotes tumor progression in a genetically defined mouse model of lung adenocarcinoma

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Abstract
Numerous epidemiological observations point to sex differences in lung cancer etiology and progression. The present study was aimed at understanding the bases of these sex differences. To test the effect of estradiol on tumor progression, we used a mouse model based on conditional Kras expression and concurrent deletion of Tp53 following inhalation of an adenoviral vector expressing Cre recombinase (AdeCre). Ovariectomized females and males were treated with estradiol via a continuous-release capsule. Tumor multiplicity, tumor volume, and histological grade were determined at 10 weeks after AdeCre administration. Cell proliferation was monitored by Ki67 immunohistochemistry at 4 and 10 weeks after AdeCre administration. At 10 weeks, female mice had more than twice the number of tumors evident on the surface of the lungs than male mice; ovariectomy eliminated this sex difference. The estrogen treatment significantly increased tumor number and volume in ovariectomized females and in males. Histological character of the tumors ranged from adenoma to adenocarcinoma. Ovary-intact females exhibited higher grade tumors than ovariectomized females or males. Progression to higher histological grade was stimulated by estrogen in male mice but not in ovariectomized females. At 10 weeks after AdeCre administration, tumor cell Ki67-labeling varied widely, precluding assessment of an estrogen effect; however, at 4 weeks, Ki67 labeling of lung parenchymal cells was increased 3.5-fold by estrogen. In conclusion, estrogen acts as a promoter for lung adenocarcinoma in a genetically defined lung cancer model; estrogen-induced cell proliferation in the oncogene-initiated cells is likely to play a role in this tumor promoter activity.

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Introduction
Lung cancer is the leading cause of cancer mortality in both males and females. Each year, it is estimated that more than 180 000 new cases of non-small cell lung cancer are diagnosed in the US, with about 165 000 patients dying of the disease. While cigarette smoking remains the primary risk factor for lung cancer, several reports have suggested a role for estrogens in the development and/or progression of lung cancer, especially in women (Stabile & Siegfried 2003, Patel et al. 2004). Female smokers are more likely to develop adenocarcinoma of the lung than males (Muscat & Wynder 1995, Thun et al. 2006) and nonsmokers who develop lung cancer (predominantly adenocarcinoma) are thrice more likely to be female (Parkin et al. 2005).

The effect(s) of hormone replacement therapy is controversial, with some studies suggesting that estrogens decrease survival while other studies point to a protective effect (Taioli & Wynder 1994, Kreuzer et al. 2003, Schabath et al. 2004, Ganti et al. 2006, Schwartz et al. 2007). On the other hand, women with early menopause had a decreased risk, suggesting that endogenous hormones are detrimental (Taioli & Wynder 1994). Thus, both endogenous and exogenous estrogen may play a role in the development of lung cancer, especially adenocarcinoma. Both estrogen receptor (ER) α and β are expressed in normal and cancerous lung tissues and estrogens stimulate growth of lung cancer cell lines in culture (Kaiser et al. 1996, Stabile & Siegfried 2003, Hershberger et al. 2005, Dougherty et al. 2006).
In addition, estrogen blockade has an antiproliferative effect in lung cancer cells (Stabile et al. 2005).

Epidermal growth factor (EGF), its receptor (EGFR), and downstream signaling molecules, such as KRas and BRaf, have all been shown to be important in the etiology of lung cancer (Ahrendt et al. 2001, Yokota & Kohno 2004, Shigematsu & Gazdar 2006, Takeuchi et al. 2006). In addition, activating mutations in each of these signaling molecules have been shown to be more prevalent in lung adenocarcinomas found in women than in men (Graziano et al. 1999, Nelson et al. 1999, Shigematsu & Gazdar 2006). A high percentage of human lung adenocarcinomas exhibits activating mutations in KRas and concurrent inactivating mutations in TP53 (Ahrendt et al. 2001, Yokota & Kohno 2004, Shigematsu & Gazdar 2006, Takeuchi et al. 2006).

The present study was designed to determine whether sex differences exist in lung tumorigenesis in a mouse model based on genetic alterations that are relevant to the human condition and to determine whether estrogen might be the basis for those differences. We developed a mouse model of lung tumorigenesis in which expression of oncogenic Kras and deletion of Tp53 are conditionally regulated. Tumorigenesis was compared in ovary-intact females and males, and ovariectomized females and males treated with estrogen. The results suggest that estrogen acts as tumor promoter in this genetically defined lung cancer model.

Materials and methods

Animals and treatments

All animal work was done under approval from the Institutional Animal Care and Use Committee of the Indiana University School of Medicine. Two strains of mice were procured from the NCI Mouse Repository: LSL-KrasG12D (B6;129 background) and Tp53-floxed (FVB;129 background). The LSL-KrasG12D mice, originated by Jackson et al. (2001), have a knock-in transgene for expression of the oncogenic form of Kras in which codon 12 is mutated from glycine to aspartic acid; expression of the KrasG12D transgene is under control of a floxed-stop signal (LSL: loxP-STOP-loxP) in the promoter region. Upon removal of the floxed-stop signal by Cre recombinase, oncogenic KrasG12D is expressed. These mice are maintained heterozygous for LSL-KrasG12D, as the homozygous state behaves as a Kras knockout, which is embryonic lethal. The Tp53-floxed mice were developed by Jonkers et al. (2001); the majority of the coding region of the Tp53 gene is flanked by loxP sites so that upon excision via Cre recombinase the gene is deleted. The Tp53-floxed gene is maintained in a homozygous state. The two strains of mice were bred to produce mice that were homozygous Tp53-floxed/heterozygous LSL-KrasG12D (Tp53+/lsl-KrasG12D); the offspring were maintained in the mixed genetic background.

The adenoviral vector that expresses Cre recombinase (AdeCre) was procured from the Gene Transfer Vector Core of the University of Iowa. The vector was supplied at a titer of $1 \times 10^{12}$ pt/ml. At 6–8 weeks of age, mice were lightly anesthetized with Avertin and 50 µl of the viral suspension at either a high dose ($5 \times 10^{10}$ pt) or low dose ($1 \times 10^{9}$ pt) were administered to each mouse intra-nasally. Groups of female mice were left ovary intact or they were ovariectomized 1 week prior to viral instillation; a third group was ovariectomized and administered a slow-release Silastic capsule of crystalline estradiol that emits $\sim 2$ µg hormone/day (Steinmetz et al. 1996). Male mice were left intact and either treated with an estrogen capsule 1 week prior to viral instillation or were left untreated. For tumorigenesis experiments, animals were killed by carbon dioxide inhalation at 10 weeks after virus administration. For analysis of the effect of estrogen on cell proliferation of oncogene-initiated lung parenchyma, male mice that had been treated with or without estradiol were killed at 4 weeks after AdeCre. The lungs were excised and fixed in a buffered picric acid/paraformaldehyde solution. After fixation, the lungs were held in 70% ethanol until processing.

Tissue processing and tumor measurements

Lungs were examined visually and tumor nodules on the surface of each lobe were counted by two technicians who were blinded to treatment information. The largest lobe was then processed for histological examination of hematoxylin- and eosin-stained sections.

To assess tumor volume, a mid-sagittal section of the lung was viewed on a dissecting microscope at $\sim 4 \times$ magnification and multiple images were captured to cover the entire section. The area of the lung occupied by tumor was determined by allowing the image analysis program (IPLab, Scanalytics Inc., Vienna, VA, USA) to differentiate between the darkly stained tumor areas and the pale non-tumorous tissue (see Fig. 1B). The area of tumor was divided by the entire area of the lung section and expressed as a percentage.

Tumors were graded according to the system devised by Jackson et al. (2005) as follows: Grade 1, uniform nuclei (adenoma); Grade 2, uniform enlarged nuclei with prominent nucleoli; Grade 3, enlarged, pleomorphic nuclei with prominent nucleoli and with nuclear molding present; Grade 4, very large
pleomorphic nuclei with atypia (abnormal mitoses, hyperchromatism) and with multinucleated giant cells present; Grade 5, the same as grade 4 plus desmoplastic stroma surrounding nests of tumors. To develop a grade for each animal, the grade of the individual tumors and the relative area of tumor represented by that grade were multiplied and added to the fraction of all tumor grades present, thereby generating an average tumor grade for each animal. This procedure was performed by two technicians who were blinded to treatment and the scores of the two were averaged.

Immunoblotting for ER protein

Lung tissues from animals that had been left untreated or were treated with AdeCre 10 weeks earlier were homogenized in a cell lysis buffer containing protease inhibitors (Cell Signaling, Danvers, MA, USA). Ovaries were also collected and homogenized; ovarian lysate served as a positive control for both ERα and ERβ. After centrifugation, the solubilized proteins were separated by 12% SDS-PAGE and blotted onto nitrocellulose membranes. The blots were blocked with 5% dry milk solution and incubated with primary antibody, anti-ERα (H-184, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ERβ (PA-310B, Affinity BioReagents, Golden, CO, USA), or anti-eIF4E (Cell Signaling). After washing, the blots were treated with horseradish peroxidase-conjugated secondary antibodies. Immunostaining was detected by incubating the blot in a chemiluminescent substrate solution (Pierce, Rockford, IL, USA) and visualized on X-Ray film (Kodak Biomax, Fisher Scientific, Pittsburgh, PA, USA).

Ki67 immunostaining

Lung tissue was harvested at 4 and 10 weeks from male mice that had been treated with AdeCre, with or without an estradiol implant. Formalin fixed, paraffin-embedded tissue was sectioned at 6 μm and deparaffinized. The sections were subjected to an antigen retrieval procedure by heating in 10 mM citrate buffer, pH 6.0 at 95 °C for 10 min, and then stained with a rabbit monoclonal antibody against Ki67 (SP6, LabVision, Cheshire, UK), using a horseradish peroxidase-conjugated secondary antibody (Envision Plus System HRP Labeled Polymer, Dako, Carpinteria, CA, USA), and diaminobenzidine chromogen (ICN Chemicals, Costa Mesa, CA, USA) to visualize positive staining. Ki67-labeling indices were generated by determining the percentage of stained cells.

Statistical analysis

Tumor counts, tumor volume, and grade were all analyzed by ANOVA followed by Newman–Keuls multiple comparison tests to compare individual treatments or by t-test with Welch’s correction if the variances differed between groups. Statistical significance was set at $P < 0.05$; values were also reported as ‘tending to differ’ when $0.05 < P < 0.10$ to indicate borderline significance.

Results

Inhalation of AdeCre induced multiple tumors that were apparent on the surface of the lungs 10 weeks later (Fig. 1A). Low power magnification of histological sections allowed determination of the relative area occupied by tumor as a measure of tumor volume (Fig. 1B). Histological appearance of the tumors varied from adenomas to highly differentiated adenocarcinomas to moderately undifferentiated adenocarcinomas (Fig. 2).

The number of tumor foci apparent on the surface of the lungs and the relative tumor volume depended on the dose of AdeCre administered (Fig. 3). When a high dose of AdeCre ($5 \times 10^{10}$ viral particles) was administered, tumor counts differed significantly between
intact females and males ($P<0.05$) and they tended to be different between ovariectomized females and intact females ($P=0.087$; Fig. 3A). Tumor volume was decreased by ovariectomy ($P<0.05$) and estradiol treatment increased both tumor counts ($P<0.05$) and tumor volume ($P<0.01$) in the ovariectomized mice induced with the high dose of AdeCre (Fig. 3A and B). Estradiol increased tumor counts in males induced by either the high ($P<0.01$) or low dose ($P<0.05$) of AdeCre (Fig. 3A and C). Although the increase in tumor volume in male mice treated with estradiol was not statistically different in animals induced by the high dose of virus (Fig. 3B), it was increased by estradiol in males ($P<0.05$) induced with a low dose of AdeCre (Fig. 3D).

Using a grading system based on histological characteristics (Fig. 2) as described by Jackson et al. (2005), we found that tumor grade was higher in ovari-intact females than males ($P<0.05$), that ovariectomy reduced tumor grade in females ($P<0.05$), and that estrogen increased tumor grade in males ($P<0.001$), but not in ovariectomized females (Fig. 4).

Figure 2 Histological grades of tumors. The lungs taken from animals at 10 weeks after AdeCre administration contained tumors that varied histologically from adenoma to adenocarcinoma. Tumors were graded according to criteria described by Jackson et al. (2005). (A) Grade 1 tumor with uniform nuclei (adenoma). (B) Grade 2 tumor exhibiting enlarged nuclei with prominent nucleoli. (C) Grade 3 tumor with large, pleomorphic nuclei and nuclear molding (arrowheads). (D) Grade 4 tumor with atypia (atypical mitoses, hyperchromatism) (white arrowheads) and a multinucleated cell (black arrowhead). Grade 5 (not shown) is the same as Grade 4 with the addition of tumor tissue surrounded by desmoplastic stroma. (Magnification for all panels shown by scale bar in A: 25 μm).

Both normal and tumorous lung tissues expressed ERβ, as demonstrated by a prominent band at ~59 kDa in the immunoblot analysis; the protein eIF4E, used as a loading control, shows that approximately equal amounts of lung lysate protein were applied to each lane (Fig. 5). The smaller bands present at ~40 kDa and lower are apparently due to proteolytic cleavage during tissue handling as they occur randomly in the various samples tested. When we examined the same protein preparation for ERα, there was no band apparent (not shown).

Estrogen induces cell proliferation in the endometrium and breast and this effect is believed to underlie the tumor promoter action of the hormone (Henderson & Feigelson 2000). We examined cell proliferation in the lungs of AdeCre-inoculated male mice, with or without estrogen treatment. The proportion of cells immunostaining for Ki67 was used as index of proliferation. When the tumor foci were examined at 4 and 10 weeks after AdeCre inoculation, there was no apparent pattern of Ki67 staining correlated to estrogen treatment; in fact there was a wide variation of Ki67 staining in tumors within the same lung (Fig. 6A) and at 10 weeks much of the lung was occupied by tumor. We therefore examined the lung parenchyma that was not involved in tumor foci at the earlier time point (4 weeks) after AdeCre inoculation. In this case, there was a dramatic increase in Ki67 labeling in response to estradiol (Fig. 6B–D).

Discussion

While estrogen is recognized to play an important role in the development and/or progression of certain cancers, most notably breast and endometrial cancers, the precise role of estrogens in lung cancer has not yet been elucidated. However, with the increasing rates of lung cancer in women and the potential increased susceptibility of women to the detrimental effects of tobacco, estrogen has been hypothesized to play a role in the pathogenesis of lung cancer (Stabile & Siegfried 2003, Patel et al. 2004, Ben-Zaken Cohen et al. 2007). Both ERα and ERβ have been reported to be present in cultured lung cancer cells and these cells show a biological response to estrogens and antiestrogens (Stabile et al. 2002, Dougherty et al. 2006). Furthermore, combined targeting of the ER and the EGFR demonstrated antiproliferative effects in non-small cell lung cancer cell lines grown in culture or as xenografts in immunodeficient mice, thus pointing to a possible mechanism by which estrogens can influence the pathogenesis of lung cancer (Stabile et al. 2005). Although studies based on human cell lines can point to a potential role of estrogen in tumor progression, animal models are required to investigate the array of in vivo controls that may be affected by hormones during tumorigenesis. In this report, we used a murine model of lung adenocarcinoma and have demonstrated...
the effects of estrogen manipulation on tumor growth. To our knowledge, this is the first demonstration of estrogen responsiveness in an animal model of lung adenocarcinoma. We have shown that the tumor burden and the degree of differentiation of tumors are affected by estrogen manipulation. These results lend further support to the notion that the pathogenesis of lung cancer, particularly lung adenocarcinoma, is influenced by estrogens.

The murine model used in the present study is similar to the one initially developed in the laboratory of Tyler Jacks and associates (Jackson et al. 2001, 2005). In this model, a compound mutant mouse is generated whereby conditional expression of mutations in the Kras proto-oncogene and the Tp53 tumor suppressor gene lead to the development of lung adenocarcinoma in the context of an otherwise normal animal. Using this model, we were able to consistently induce lung adenocarcinoma. In our experience, however, tumorigenesis was more rapid than that reported by Jackson et al. (2005). In their report, Jackson et al. examined the tumor burden after 19–26 weeks of growth while several of our animals died of disease by 10 weeks. The difference in time course is likely due to differences in viral dose used between

Figure 3 Effect of estradiol on tumorigenesis. Female mice were left intact or ovariectomized (ovxd); males were left intact. Mice were treated with AdeCre at (A and B) a high dose (5 × 10⁷ viral particles) or (C and D) a low dose (1 × 10⁶ viral particles). At the time of AdeCre administration groups of ovxd females and males received a Silastic implant of estradiol. At 10 weeks after AdeCre, the lungs were removed and examined. Tumors visible on the surface of the lungs were counted (A and C) and the relative tumor volume was determined by measuring the area occupied by tumor relative to total lung tissue in a mid-sagittal H&E-stained section (B and D). Means ± s.e.m.; n = 10–13 animals/group in (A and B), n = 4 animals/group in (C and D); *P < 0.05 versus corresponding untreated control; **P < 0.05 versus ovary-intact females.
studies. The total dose of virus administered in our high dose study ($5 \times 10^{10}$ particles) was 10 times that used by Jackson et al. Using a lower doses of virus ($1 \times 10^9$ particles/animal), we found that tumor count was relatively low at 10 weeks and the tumor foci were very small. On the other hand, Jackson et al. did not indicate whether animal sex affected tumor growth, and it is possible that the difference in the rapidity of tumor growth between our experiments and theirs was affected by animal sex, for most of the animals that died of disease by 10 weeks in the high virus dose study were female.

Higher tumor multiplicity in females relative to males has been reported in carcinogen-induced animal models of lung cancer (Singh et al. 1998, Imai et al. 2002). In the present study, there were clear sex differences in tumorigenesis and experimental manipulation showed that estrogen is likely to play a role in these differences. Furthermore, tumor progression was sensitive to estrogen as indicated by the observations that estrogen increased tumor grade in males and tumor grade was higher in intact females than ovariecromized females. However, estrogen did not increase grade in ovariecromized females; this observation suggests that additional hormones and/or factors may be required for the full sex dichotomy in lung tumor progression. The ovaries of intact females cyclically produce estrogen and progesterone; these two steroid hormones have been shown to synergize in promotion of mammary tumors (Bigsby 2002). Although it has not been studied in lung, androgen and progesterone have been shown to exhibit overlapping gene regulatory action (Ghatge et al. 2005). Lung cells have been shown to express receptors for and be responsive to both progesterone (Press & Greene 1988, Hagen et al. 1990) and androgen (McDoniels-Silvers et al. 2002, Card et al. 2006, Montgrain et al. 2007) and therefore, it may be that circulating androgen in the male substitutes for progesterone, enhancing the effect of the administered estrogen. On the other hand, circadian patterns of other hormones, such as growth hormone, differ between males and females (Chowen et al. 2004) and these too may have effects on tumor progression.

The precise mechanisms by which estrogens influence the pathogenesis of lung cancer are unknown. However, several reports have provided insights into these mechanisms. It is known that, upon estrogen binding in target cells, ER undergoes a conformational change that allows for the association of estrogen–ER complexes with specific estrogen response elements in DNA; in addition, in their activated state, ER and its coactivator proteins are phosphorylated; these events lead to transcriptional regulation of target genes (McKenna & O’Malley 2002, Wu et al. 2005, Likhite et al. 2006). It was shown that the ERz is present in non-small cell lung cancer (NSCLC) specimens in the phosphorylated state and that inhibition of ERz or ERβ expression inhibits proliferation of lung cancer cell lines (Marquez-Garban et al. 2007). Furthermore, estradiol and EGF were able to elicit phosphorylation of the receptor coactivator, SRC-3, and combined treatment with an antiestrogen and an EGFR inhibitor blocked cell proliferation (Marquez-Garban et al. 2007).
Harshberger et al. (2005) proposed a model in which transcriptional responses to estrogen in lung cancer cells are generated by ERβ and GRIP1/TIF2, a p160 coactivator, while Dougherty et al. suggested that the DRIP205 and other coactivators may be responsible for sex-specific estrogen responsiveness of lung cancer cells. ERβ is the major form of ER in the mouse lung, but it is not clear that estrogen effects on the lung are mediated directly by the receptor in the lung parenchyma. ERβ transcripts are readily detectable in mouse lung homogenates (Couse et al. 1997, Kuiper et al. 1997) and protein is detectable by both immunoblot and immunohistochemical analyses (Patrone et al. 2003). Although small amounts of mRNA for ERα have been found in mouse lung homogenates (Couse et al. 1997, Kuiper et al. 1997), immunological analyses indicate that the protein is undetectable (Patrone et al. 2003). Estrogen stimulates expression of an artificial estrogen-responsive reporter gene in the mouse lung and deletion of ERβ reduces expression of endogenous genes (Patrone et al. 2003). However, it may be that ERα is also important for normal responsiveness of the lung. Massaro et al. (2007) showed that estradiol increased expression of cyclin D1-3 in the lungs of wild-type mice but not in mice deficient in either ERα or ERβ; together with the observations on ER expression, these results suggest that either minute, undetectable amounts of ERα are sufficient for lung cell responses or that the effects of hormone are mediated by an indirect effect of another ER expressing tissue.

Whatever the exact molecular pathways are that mediate estrogen’s effects, the key cellular response is likely to be hormone-induced proliferation. We found that estrogen-stimulated proliferation of the preneoplastic parenchymal cells in the lung. In a preliminary study, a single dose of estrogen had no effect on Ki67-labeling index in the lung at 16–24 h (data not shown); similarly, Massaro et al. (2007) found no increase in cell proliferation indices in mouse lung after a single dose of hormone but they did find that estrogen-enhanced expression of genes associated with cell proliferation. We found a dramatic effect of estradiol on the proliferative index of cells that had been initiated by expression of oncogenic Kras and concurrent deletion of Tp53. It may be that the kinase pathways induced by oncogenic Kras interact with ER in the lung, as has been shown in endometrial and mammary cells (Kato et al. 1995, Kato 2001, Lopez et al. 2001, Wu et al. 2005) and that this interaction produces a heightened response to estrogen stimulation.

In summary, sex differences occur in a mouse model of lung tumorigenesis based on activation of the Kras oncogene and depletion of Tp53; both tumor burden and histological grade were higher in females. Endocrine ablation and hormone replacement experiments indicate that, for the most part, estrogen underlies the mechanisms of these sex differences. Furthermore, estrogen-enhanced proliferation of preneoplastic cells suggests that the hormone acts as a promoter during early stages of tumorigenesis. Thus, our observations provide additional evidence for the role of estrogen in lung cancer tumorigenesis. In addition, the tumor model described will be useful in delineating the role of hormones in modifying molecular, cellular, and immunological parameters involved in progression of lung cancer.
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