Rosiglitazone prevents the progression of preinvasive lung cancer in a murine model

Christopher M.Lyon, Donna M.Klinge, Kieu C.Do, Marcie J.Grimes, Cindy L.Thomas, Leah A.Damiani, Thomas H.March, Christine A.Stidley and Steven A.Belinsky*

Lung Cancer Program, Lovelace Respiratory Research Institute, 2425 Ridgecrest Drive SE, Albuquerque, NM 87108, USA and 1Department of Internal Medicine, University of New Mexico, Albuquerque, NM 8710, USA
*To whom correspondence should be addressed. Tel: +505 348 9465; Fax: +505 348 4971; Email: sbelinsk@lrri.org

There is a critical need to identify efficacious chemopreventive agents for lung cancer that can be taken chronically with no side effects and whose mechanisms of action do not involve genotoxicity that could drive, rather than impede, cancer progression. We evaluated the ability of a chemopreventive cocktail that included selenium (antioxidant), rosiglitazone (peroxisome proliferator-activated receptor gamma agonist), sodium phenylbutyrate or valproic acid (histone deacetylase inhibitors) and hydralazine (cytosine-demethylating agent) to prevent the progression of lung cancer in A/J mice treated with NNK. Agents were administered alone or in various combinations. Effects of the chemopreventive agents were quantified based on the proportion of hyperplasias and adenomas within the mouse lung. Significant effects on tumor progression were seen in all treatment groups that included rosiglitazone as reflected by a 47–57% increase in number of hyperplasias and a 10–30% decrease in adenomas. Cell proliferation was also reduced in these treatment groups by ~40%. Interestingly, while treatment with rosiglitazone alone did not significantly affect lesion size, striking effects were seen in the combination therapy group that included sodium phenylbutyrate, with the volume of hyperplasias and adenomas decreasing by 40 and 77%, respectively. These studies demonstrate for the first time that chronic in vivo administration of rosiglitazone, used in the management of diabetes mellitus, can significantly block the progression of premalignant lung cancer in the A/J mouse model.

Introduction

Lung cancer is the leading cause of cancer-related deaths in the USA and is projected to reach epidemic levels worldwide during the 21st century (1). Chemotherapy has been largely ineffective in advanced disease and >85% of patients with lung cancer eventually succumb to the disease. Mortality could be reduced greatly by identifying people at high risk for cancer and developing effective interventions to impede or reverse respiratory carcinogenesis. Both genetic and epigenetic changes in oncogenes and tumor suppressor genes are clearly important for cancer initiation and progression. Ding et al. (2) screened 623 genes in lung adenocarcinoma and identified >1000 somatic mutations, mostly at prevalences <10%. Genome-wide scans of the epigenome have also identified hundreds of genes that are silenced through promoter hypermethylation (3,4). The development of lung cancer in the smoker proceeds through a process called field carcinogenesis that involves the generation of multiple independently initiated sites that harbor allelic loss, gene mutation and methylation throughout the respiratory epithelium of smokers (5). The large number of genes and pathways altered early in the genesis of this disease presents an obstacle for chemopreventive agents that affect at most a few pathways, making it unlikely that these compounds alone will impede the development of lung cancer in the high-risk smoker. The other major obstacle to this field is the identification of highly efficacious agents that can be taken chronically with no side effects and whose mechanisms of action do not involve genotoxicity that could drive, rather than impede, cancer progression. There have been hundreds of studies demonstrating activity of chemopreventive agents in animal carcinogenesis models. Our goal was to investigate combinations of agents that showed efficacy in multiple organs. The essential trace element selenium was associated with protection against cancers of the lung, prostate, esophagus and colon (6). Studies in the F344 rat have also corroborated the epidemiologic finding of a protective effect for selenium toward human colorectal cancer and a derivative of this element was found to block lung cancer in the mouse (7–9). Multiple mechanisms of action for the cancer prevention effects seen by selenium have been proposed and include induction of glutathione peroxidase, inhibition of lipoxigenase, induction of apoptosis, modulation of cell signaling molecules and inhibition of cytosine DNA methyltransferase (7,8,10–12). Another promising class of chemopreventive agents is the thiazolidinediones (including pioglitazone, rosiglitazone and troglitazone). These agents bind to peroxisome proliferator-activated receptor gamma (PPARγ), a member of the superfamily of nuclear hormone receptors that heterodimerize with retinoid X receptor to bind the PPAR response element leading to transcription of downstream genes that can induce cell differentiation and apoptosis (13,14). The thiazolidinediones have been used in the treatment of type 2 diabetes mellitus because activation of PPARγ regulates the expression of insulin-responsive genes involved in the regulation of glucose and fatty acid metabolism. Troglitazone was effective in preventing N-methyl-N-nitrosourea-induced gastric cancer in mice (15). PPARγ agonists also induce cell cycle arrest and apoptosis of lung cancer cell lines in vitro and inhibit growth of xenografts on nude mice (16,17).

Targeting the epigenome may also prove to be an effective chemopreventive strategy. Our own studies have shown that treatment of A/J mice following exposure to 4-methylnitrosamino-1-(3-pyridyl)-1-butane (NNK) with the demethylating agent, 5-aza-2’-deoxyctydine (DAC), combined with the histone deacetylase (HDAC) inhibitor sodium phenylbutyrate reduced lung tumor development by >50%, whereas modest to no effect was seen when these agents were used alone (18). The mutagenic effects of DAC preclude its use in primary prevention in humans; however, the antihypertensive drug hydralazine has been reported to induce cytosine demethylation and gene reexpression in vitro and in vivo (19,20). Moreover, the growth inhibitory effect of the HDAC inhibitor valproic acid was potentiated by hydralazine in breast and colon tumor cell lines (21). The HDAC inhibitors valproic acid, used as a chronic therapy for epileptic disorders, and phenylbutyrate, used in the chronic management of patients with urea cycle disorders, have been proposed as anticancer therapies and are currently being evaluated in Phase I and Phase II clinical trials (22,23). HDAC inhibitors induce histone deacetylation and chromatin decondensation to increase expression of genes epigenetically silenced through chromatin modification in the absence of DNA cytosine methylation within gene promoters (24,25). HDAC inhibitors can also cooperate with DNA-demethylating agents such as DAC to induce reexpression of genes with densely methylated CpG islands (26).

The agents discussed above meet our criteria as candidates for a chemoprevention cocktail as either they are natural compounds or they have been used to treat non-neoplastic diseases and the doses used in humans have biological activity for their proposed mechanism of action for cancer prevention. The A/J mouse, because of its high susceptibility to carcinogen-induced lung cancer, is a common model used for assessing the efficacy of chemopreventive agents (27). Therefore, the objective of this study was to test combinations of selenium, rosiglitazone, hydralazine, valproic acid and phenylbutyrate for chemoprevention of lung tumor progression in A/J mice treated with the tobacco carcinogen, NNK, common model used to assess the efficacy of different interventions.

Abbreviations: HDAC, histone deacetylase; i. p., intraperitoneal; PPARγ, peroxisome proliferator-activated receptor gamma.
Materials and methods

Animal treatment and histopathology

Female A/J mice (n = 135) (4–6 weeks old) obtained from Jackson Laboratory (Jackson, MS) were treated three times [every other day, 50 mg/kg, intraperitoneal (i. p.)] with NNK (Chemogen Science Laboratories, Lenexa, KS) dissolved in saline or with saline alone (0.1 ml). Animals were held for 42 weeks following carcinogen exposure to allow for the development of preinvasive lesions: alveolar hyperplasias and adenomas (28). Mice were then separated into nine groups of 15 mice for treatment with either a combination or the agents. The agents used were hydralazine [2.5 mg/kg, i. p. (Sigma, St Louis, MO)], selenium [seleno-L-methionine, 16 p.p.m. in drinking water continuously (Sigma)], sodium phenylbutyrate [300 mg/kg, i. p. (Scandinavian Formulas, Kirk, PA)], valproic acid [120 mg/kg, i. p. (Sigma)] and rosiglitazone [10 mg/kg, i. p. (GlaxoSmithKline, King of Prussia, PA)]. Not all combinations could be evaluated due to the limited availability of rosiglitazone. Mice were treated three times each week (Tuesday, Wednesday and Thursday) with rosiglitazone, hydralazine, phenylbutyrate and valproic acid, a protocol used in our previous study (18).

Mice were killed by exsanguination; lungs were inflated and fixed with 4% buffered paraformaldehyde for 18–24 h and then transferred to 70% ethanol for routine histological processing and staining of paraffin sections with hematoxylin and eosin. A single standardized section was prepared from all lungs that included the five lung lobes. Pulmonary lesions were classified as hyperplasia or neoplasia (adenoma or carcinoma) as described (18,28). Hyperplastic lesions were microscopically and involved a minimum of 5–10 alveoli in a focus lined by hyperplastic type II epithelial cells. Adenomas were characterized by a monomorphic growth pattern and were generally composed of well-differentiated cells. Carcinomas were composed of cells with varying degrees of differentiation and were characterized by complete loss of normal architecture.

Morphometry and immunohistochemistry

Digitized color images (640 \times 480 pixels) containing alveolar hyperplasias and adenomas were acquired using a 3×-charge-coupled device video camera (DEI-470; Optronics Corp., San Diego, CA), a frame grabber (LG-3; Scion Corporation, Frederick, MD) and a Macintosh G4 computer with ImageJ software (National Institutes of Health). The camera was connected to the phototube of an Olympus BH2 microscope (Olympus America, Center Valley, PA) with a ×4 objective lens and light source set at 4.5 V. Captured color images were processed with ImageJ using the threshold command to yield binary (black and white) images. Processing yielded images of solid black masses with outlines that approximated the perimeters of the lesions as seen by light microscopy; alveolar septa and other structures were made part of the white background. The area occupied by the lesion in a processed image was measured with the Analyze menu (analyze particles), where the scale in ImageJ was set at 260 pixels/mm with the aid of a stage micrometer. By comparing what was visible in the field, only measurements that corresponded to a specific lesion were transferred to a Microsoft Excel file for summing areas of individual masses. Assuming that tumors were spherical and that the imaging captured lesions were at their maximal diameter, an estimation of individual tumor volume was made from the area measurements.

Tissue sections (5 μm) were placed on Probe-On Plus slides and deparaffinized in xylene and rehydrated in a graded series of ethanol/H2O baths. Sections were incubated for 30 min at room temperature in 1% hydrogen peroxide in methanol, rinsed in H2O and incubated in boiling antigen retrieval citra solution (BioGenex, San Ramon, CA). Sections were incubated for 1 h at room temperature in goat serum (7.5%) followed by overnight incubation at 4°C with the primary antibody to Ki67, Ready to Use Mix (Lab Vision, Fremont, CA; RM-9106-R7), or cleaved Caspase-3 (1:20 dilution; Cell Signaling, Burlington, MA). A biotinylated secondary antibody was added for 1 h and avidin–biotin complex formed using the Vectastain ABC kit (Vector Labs, Burlingame, CA). Sections were developed using diaminobenzidine (DAB Peroxidase Substrate Kit; Vector Labs) and counterstained with hematoxylin. Ki67 and Caspase-3 staining was quantified in adenomas from the different treatment groups. A set of five randomly selected Ki67- or Caspase-3-stained sections stained with hematoxylin and eosin that included all lung lobes (28). Sections were developed using diaminobenzidine (DAB Peroxidase Substrate Kit; Vector Labs) and counterstained with hematoxylin. Ki67 and Caspase-3 staining was quantified in adenomas from the different treatment groups. A set of five randomly selected Ki67- or Caspase-3-stained sections were processed with ImageJ using the threshold command to yield binary (black and white) images. Processing yielded images of solid black masses with outlines that approximated the perimeters of the lesions as seen by light microscopy; alveolar septa and other structures were made part of the white background. The area occupied by the lesion in a processed image was measured with the Analyze menu (analyze particles), where the scale in ImageJ was set at 260 pixels/mm with the aid of a stage micrometer. By comparing what was visible in the field, only measurements that corresponded to a specific lesion were transferred to a Microsoft Excel file for summing areas of individual masses. Assuming that tumors were spherical and that the imaging captured lesions were at their maximal diameter, an estimation of individual tumor volume was made from the area measurements.

Histological analysis

Histological sections stained with hematoxylin and eosin that included all lung lobes (28) were analyzed using light microscopy. Lesions were classified as hyperplasia, adenoma or carcinoma. Our initial studies characterizing the NNK-induced A/J lung tumor model showed, through serial killing, that 14 weeks after carcinogen treatment, 100% of the lesions seen were hyperplasias. The progression to neoplasia involved a decline in the frequency of hyperplasias and an increase in adenomas, with 50% of adenomas seen arising within the hyperplastic lesion (27). Moreover, the absolute number of lesions (hyperplasias, adenomas and carcinomas) remained constant from 30 to 56 weeks post-carcinogen treatment. Thus, with this model, effects of a chemopreventive agent are quantified based on the proportion of hyperplasias and adenomas within the mouse lung. An increase in number of hyperplasias associated with a treatment is indicative of preventing progression to adenoma. Significant effects (P < 0.05) on tumor progression were seen in all groups in which rosiglitazone was included (Table I). This was reflected by a 47–57% decrease in lesion number from sham treatment, and by reductions in lesion count from groups treated with the combination of agents.

Statistics

The primary analysis of tumor development compared the mean number of hyperplasias, adenomas and carcinomas observed across the nine treatments. One-way analysis of variance was conducted. Since there were many treatments, some subgroups, such as those including rosiglitazone, were analyzed in separate models. These analyses were conducted both on the counts and on the ranks because of the asymmetric distributions of counts. Pairwise comparisons between sham and each treatment group were based on the least squares means from the analysis of variance. A mixed model with fixed treatment effects and random effects for animals was used to examine area and volume. A logarithmic transformation of area and volume was used since the distributions were non-normal. Thus, geometric means were used to summarize these variables. The percent of cells staining with Ki67 within adenomas also was compared across treatment groups using analysis of variance and pairwise comparisons. All analyses were conducted in SAS.

Results

Rigoslitazone inhibits progression of preinvasive lung cancer in the A/J mouse

A chemoprevention study was designed in which A/J mice were treated with the tobacco carcinogen NNK and monitored for 42 weeks to allow the development of preinvasive lesions: alveolar hyperplasias and adenomas (28). This study design mimics the situation for the chronic smoker who has developed field cancerization: preinvasive lesions containing loss of heterozygosity at many chromosome loci and genetic and epigenetic changes but is still clinically cancer free (5,29). After 42 weeks, mice were separated into nine groups of 15 mice and treated for 6 weeks with an individual agent or a combination of agents. Dose–response studies assessed the toxicity of the drug combination in sham adult mice treated for 2 weeks. Doses selected produced no systemic toxicity based on pathologic evaluation of lungs, kidneys, liver and gastrointestinal tract (data not shown). Not all combinations could be evaluated due to the limited amount of rosiglitazone available for treatment. Body weight was not affected during the treatment period at the doses selected. After killing, the number of lesions was determined for each animal by evaluating sections stained with hematoxylin and eosin that included all lung lobes (28).

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Table I. Inhibition of lung tumor progression in A/J mice by rosiglitazone

<table>
<thead>
<tr>
<th>Group</th>
<th>Hyperplasia</th>
<th>Adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>7.4 ± 1.1</td>
<td>7.9 ± 1.0</td>
</tr>
<tr>
<td>Se</td>
<td>10.8 ± 0.8</td>
<td>7.5 ± 0.7</td>
</tr>
<tr>
<td>Hyd</td>
<td>9.3 ± 1.1</td>
<td>7.6 ± 0.9</td>
</tr>
<tr>
<td>Se + Hyd</td>
<td>7.9 ± 0.7</td>
<td>6.1 ± 1.0</td>
</tr>
<tr>
<td>Se + Hyd + PB</td>
<td>8.5 ± 0.9</td>
<td>6.6 ± 1.0</td>
</tr>
<tr>
<td>Se + Hyd + VP</td>
<td>9.8 ± 1.2</td>
<td>5.9 ± 0.8</td>
</tr>
<tr>
<td>Ros</td>
<td>11.3 ± 1.4*</td>
<td>5.5 ± 0.6*</td>
</tr>
<tr>
<td>Ros + Se + Hyd</td>
<td>10.9 ± 1.3*</td>
<td>4.9 ± 0.8*</td>
</tr>
<tr>
<td>Ros + Se + Hyd + VP</td>
<td>11.6 ± 1.3*</td>
<td>7.1 ± 1.3</td>
</tr>
</tbody>
</table>

Hyd, hydralazine; PB, sodium phenylbutyrate; Ros, rosiglitazone; Se, selenium; VP, valproic acid.

Values are number of lesions (mean ± SEM) from 13 to 15 mice per group. *P < 0.05 when compared with sham.
increase in number of hyperplasias and a 10–30% decrease in number of adenomas compared with sham. Carcinomas were too few to accurately assess treatment effects (data not shown). As expected, the total number of lesions (hyperplasia + adenoma) was not affected by any treatment since the effect of treatment was on the progression of hyperplasias to adenomas and not ablation of lesions. Combining selenium, hydralazine and phenylbutyrate or valproic acid with rosiglitazone did not further inhibit progression, supporting rosiglitazone as the active agent in affecting tumor progression.

Effect of preventive interventions on cell replication and size of hyperplasias and adenomas

All the agents evaluated in this chemoprevention study inhibit growth rates of cell lines derived from lung and other tumors (16,17,21,30,31). Therefore, cell replication and mean volume of hyperplasias and adenomas was quantified in the different treatment groups. The main analysis was restricted to the treatments associated with change in number of lesions, i.e. the three treatment groups that included rosiglitazone, with comparison to sham. Overall, significant differences \( P < 0.005 \) in volume for adenomas were seen for these three groups. While rosiglitazone treatment alone did not significantly affect the size of hyperplasias or adenomas, striking effects were seen in the combination therapy group that received rosiglitazone, selenium, hydralazine and phenylbutyrate, but not when valproic acid was used instead of phenylbutyrate. The volume of the hyperplasias and adenomas were decreased by 40 and 77%, respectively \( P < 0.06 \) and \( P < 0.001 \), respectively; Figure 1A). Similar effects were seen when examining lesion area. Selenium and hydralazine administered alone, together, or in combination with phenylbutyrate or valproic acid did not affect the size of the hyperplasias or adenomas (data not shown).

Rates of cell proliferation in mice receiving rosiglitazone were also quantified in adenomas based on staining of cells within adenomas with Ki67. An average of 13% of cells stained positive for Ki67 in adenomas from sham mice (Figure 1B). The percentage of positive staining cells was significantly reduced by 40 and 48% in mice treated with rosiglitazone alone or in combination with phenylbutyrate, respectively, whereas a more modest albeit significant reduction was seen when rosiglitazone was combined with valproic acid, consistent with the reduced volume of the adenomas seen across there three treatment groups (Figure 1A and B). The percentage of Caspase-3 positive cells was similar between groups (data not shown).

Discussion

These studies demonstrate for the first time that rosiglitazone administered chronically in vivo can significantly block the progression of premalignant lung cancer in the A/J mouse model by most probably effecting rates of cell proliferation. Our findings are consistent with a recent retrospective study that assessed the risk for cancers of the lung, prostate and colon among >87 000 veterans, of which ~11 000 were taking thiazolidinediones for diabetes mellitus (32). A 33% reduction in lung cancer risk among thiazolidinediones users was observed after adjusting for confounder interactions that included age, race/ethnicity, insulin use and drug–drug interactions. Previous in vitro studies with the lung adenocarcinoma-derived cell line A549 demonstrated a dose response for inhibition of cell growth by inducing cell cycle arrest with troglitazone and the induction of apoptosis by a relatively high dose (20 \( \mu \)M) of this inhibitor (16,17). We saw similar effects on growth inhibition and apoptosis in Calu-6 cells using the same high dose of rosiglitazone (Lyon and Belinsky, unpublished data).

Our initial goal for these studies was to develop a chemoprevention cocktail that would be superior to individual therapy. While the agents chosen did not appear to increase the efficacy of rosiglitazone for blocking tumor progression based on further reduction in absolute number of adenomas, there may have been added benefit to the inclusion of the HDAC inhibitor, sodium phenylbutyrate. The greater reduction of size of hyperplasias and adenomas in lungs from mice receiving rosiglitazone and phenylbutyrate compared with rosiglitazone may stem from the common effect of these agents on differentiation and cell proliferation, albeit through different pathways. The dose of valproic acid used in our prevention study was four times lower than the dose effective in modulating growth in a breast cancer xenograft model (25). This may account for the lack of effect of this HDAC inhibitor on lesion size. Previous studies by Chang and Szabo (31) showed enhanced growth inhibition (not additive or synergistic) of the lung adenocarcinoma cell lines, A549 and H358, with the combination of ciglitizone and phenylbutyrate corroborating our in vivo findings.

A study comparing the ability of 1,4-phenylenebis(methylene)selencyanate (p-XSC) and selenium (10 p.p.m.) to block tumor development (initiation phase) rather than progression in the A/J mouse (our study design) revealed a much stronger effect by p-XSC (33). The differential effect was attributed to a 10-fold greater retention of p-XSC than selenium in the mouse lung. Our study used a higher dose of selenium (16 p.p.m.) but also failed to demonstrate any activity of this trace element toward preventing cancer progression in the A/J mouse lung. In contrast, treatment of rats with similar doses of selenium during the initiation or promotion phase following treatment with \( N \)-methyl-N-nitrosourea blocked the formation of aberrant crypt foci, suggesting differences in pharmacokinetics between species (34). Hydralazine used alone or in combination with selenium and an HDAC inhibitor also did not have any activity toward preventing cancer progression in the A/J mouse lung. Hydralazine at a dose of 5 mg/kg (2-fold higher than that of our studies) was ineffective in blocking growth of a colon cancer cell line in nude mice but potentiated the effectiveness of valproic acid (21). When conducting pilot studies to identify maximum tolerated dose of hydralazine, we observed severe lethargy in 40-week-old, but not young (6-week-old), mice receiving 5 mg/kg. Thus, the need to restrict our dosing to 2.5 mg/kg could have impacted the efficacy of hydralazine in combination with an HDAC inhibitor. In addition, the effectiveness of this drug...
as a demethylating agent and its proposed mechanism for antineoplastic activity may be overstated (20,21). A recent study by Chuang et al. (35) failed to demonstrate any effect of hydralazine treatment of bladder, colon or prostate cancer cell lines on DNA methylation or expression of p16 and RARβ, genes shown to be responsive to this agent in these other studies. We conducted similar studies with the Calu-6 and H125 cell lines with hydralazine and also did not see any reexpression of the p16 or PAX5 β genes after treatment for 2 weeks (Belinsky, unpublished data).

Our study supports the potential for combining a PPARγ agonist with an HDAC inhibitor for lung cancer prevention. The PPARs have also been proposed as targets in the lung for treatment of chronic obstructive pulmonary disease, a risk factor of cancer, because of their anti-inflammatory and immunomodulatory properties (36,37). However, a recent meta-analysis of 40 clinical trials suggested that chronic use of rosiglitazone increases the risk for cardiovascular adverse events in persons with diabetes mellitus (38). Limiting use of this drug to persons without a history or risk factors for congestive heart failure may obviate this issue. The other obstacle to use of these drugs in humans is defining the effective dose. The dose of rosiglitazone in these animal studies was ~15 times the area under the curve for the maximum recommended human daily dose. Moreover, while phenylbutyrate has been used safely in clinical trials, its short half-life (~1 h) necessitates the oral consumption of grams of drug (23). The validation of this combination will necessitate dose–response studies in mice with these agents that are administered over a more chronic time period that would mirror sustained use of these agents by smokers. A potential solution to drug dose and half-life could be the development of an aerosol to directly deliver rosiglitazone and phenylbutyrate to the lungs. Aerosol delivery has the potential to localize drugs specifically to lung tissue, with significantly higher local concentrations and superior pharmacokinetic profiles (39). The continued improvement in nebulizers for drug deposition and development of nanoparticle systems for sustained release of drugs within the lung parenchyma (40) could represent a new avenue to achieve effective chemoprevention in smokers.

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References

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