Vitamin D Repletion Reduces the Progression of Premalignant Squamous Lesions in the NTCU Lung Squamous Cell Carcinoma Mouse Model

Sarah A. Mazzilli, Pamela A. Hershberger, Mary E. Reid, Paul N. Bogner, Kristopher Atwood, Donald L. Trump, and Candace S. Johnson

Abstract

The chemopreventive actions of vitamin D were examined in the N-nitroso-tris-chloroethylurea (NTCU) mouse model, a progressive model of lung squamous cell carcinoma (SCC). SWR/J mice were fed a deficient diet (D) containing no vitamin D3, a sufficient diet (S) containing 2,000 IU/kg vitamin D3, or the same diets in combination with the active metabolite of vitamin D, calcitriol (C; 80 μg/kg, weekly). The percentage (%) of the mucosal surface of large airways occupied by dysplastic lesions was determined in mice after treatment with a total dose of 15 or 25 μmol NTCU (N). After treatment with 15 μmol NTCU, the percentages of the surface of large airways containing high-grade dysplastic (HGD) lesions were vitamin D-deficient + NTCU (DN), 22.7% (P < 0.05 compared with vitamin D-sufficient + NTCU (SN)); DN + C, 12.3%; SN, 8.7%; and SN + C, 6.6%. The extent of HGD increased with NTCU dose in the DN group. Proliferation, assessed by Ki-67 labeling, increased upon NTCU treatment. The highest Ki-67 labeling index was seen in the DN group. As compared with SN mice, DN mice exhibited a three-fold increase (P < 0.005) in circulating white blood cells (WBC), a 20% (P < 0.05) increase in IL6 levels, and a four-fold (P < 0.005) increase in WBC in bronchial lavages. Thus, vitamin D repletion reduces the progression of premalignant lesions, proliferation, and inflammation, and may thereby suppress development of lung SCC. Further investigations of the chemopreventive effects of vitamin D in lung SCC are warranted. Cancer Prev Res, 8(10); 1–10. ©2015 AACR.

Introduction

Lung cancer kills more than 160,000 individuals in the United States annually. Reduction of lung cancer–related mortality may be achieved by identification and treatment of higher risk individuals with effective cancer-preventive agents. Candidate chemopreventive agents should have favorable activity and safety in preclinical models that mirror human disease (1). Clinical chemoprevention studies in lung cancer often examine the effects of agents on premalignant squamous lesions in patients at risk; such lesions can be regularly monitored by bronchoscopy (1).

In SWR/J mice, N-nitroso-tris-chloroethylurea (NTCU) induces premalignant lesions that progress to frank lung squamous cell carcinoma (SCC). This process resembles the stepwise progression observed during the development of lung SCC in humans.
Ligand-activated VDR binds vitamin D response elements (VDRE) within gene promoter regions (7). Supporting an antitumor role for vitamin D signaling in lung cancer are findings by us and others that VDR expression is associated with improved survival in non–small cell lung cancer (NSCLC), and 1,25D3 significantly inhibits growth of VDR-expressing NSCLC cell lines (15–17). VDR is expressed within premalignant lesions of the central airway (18). Therefore, it is plausible that VDR signaling may be activated within premalignant lesions by dietary vitamin D or calcitriol to prevent their progression.

Beyond its effects on the bronchial epithelium, vitamin D may also modify lung carcinogenesis by modulating the immune system (19, 20). Calcitriol is locally produced from circulating 25D3 in immune cells within the lung resulting in monocyte activation and the suppression of lymphocyte proliferation, cytokine synthesis, and nuclear factor kappa B (NF-kB) signaling through indirect and direct VDR/VRE gene regulation (21–24). The success of immune-targeted therapies has renewed interest in the role of the immune system in development of lung cancer. However, little attention has focused on the relationship between vitamin D and immune regulation in lung cancer. It has been shown that vitamin D enhances the antimicrobial response to the respiratory pathogen, Mycobacterium tuberculosis (22). In addition, vitamin D deficiency (0 IU/kg) promotes pulmonary inflammation in response to ovalbumin in mice (25). These studies indicate the need for further investigation into the relationship of vitamin D, inflammation, and lung cancer.

To determine the potential of vitamin D to prevent progression of SCC, we examined the effects of dietary vitamin D3 intake and systemic calcitriol administration on dysplasia, nuclear localization of VDR, proliferation, and inflammation in mice treated with NTCU. Our data show that the development of high-grade dysplasia (HGD) is significantly reduced in vitamin D–sufficient mice, compared with mice that are vitamin D deficient. To the best of our knowledge, this is the first study to demonstrate the impact of vitamin D on the development of premalignant lesions of the lung.

Materials and Methods

Reagents
Calcitriol (1,25D3; PCCA) was prepared in 100% ethanol at a concentration of 2.4 mmol/L (1 mg/mL), stored at −80°C, and further diluted in sterile saline weekly for treatment. NTCU (Toronto Research Co.) was aliquoted into 10 to 20 mg amounts, further diluted in sterile saline weekly for treatment. NTCU was purged with argon to reduce oxidation, and stored at −80°C for isolation of RNA.

NTCU mouse studies
All animal studies were carried out in accordance with Institutional Animal Care and Use Committee (IACUC)–approved protocols in a pathogen-free Association for Assessment and Accreditation of Laboratory Animal Care-certified facility. SWR/J mice (The Jackson Laboratory) were selected on the basis of susceptibility and tolerance to NTCU (2). The subcapsular region of the mice was shaved prior to the first NTCU treatment (2). Aliquots of NTCU (25 μL of 40 mmol/mL NTCU = 0.28 mg NTCU = 1 μmol) or vehicle (acetone) were applied to the shaved area once weekly for the intervention studies (40 mmol/L 1 ×/wk of 40 mmol/L) or twice weekly for the acute studies (at 3.5-day intervals; 80 mmol/L 2 ×/wk of 40 mmol/L), throughout the duration of each experiment. For the intervention study, 12-week-old female mice (n = 180) were randomized into two groups to receive diets that were identical, with the exception of vitamin D3 content (0 or 2,000 IU/kg vitamin D3; Harlan Tekland). Female mice were chosen as all published studies demonstrate the efficacy of NTCU in female mice (2, 26). One thousand IU/kg is the amount of vitamin D3 recommended for mouse diets (27). Diet modification was initiated at 12 weeks of age. Five weeks later, when vitamin D deficiency was established, mice on each diet were further randomized into three groups [15 mice/group/time point (15 and 25 weeks)] to receive control (acetone), NTCU, or NTCU with calcitriol. The calcitriol groups received 26.7 μg/kg Monday, Wednesday, and Friday (total weekly dose = 80 μg/kg) by intraperitoneal (i.p.) injection and +NTCU groups received equal volumes of i.p. saline on the same schedule.

Mice were weighed biweekly. Toxicity from the carcinogen was manifested as weight loss, superficial skin lesions, and lethargy. If weight loss was greater than 10%, treatment was interrupted and mice were allowed to recover. Missed treatments were added to the end of the study in order to maintain a total delivered dose of 15 μmol or 25 μmol NTCU. Mice were euthanized and lung lobes were either inflated with 10% neutral buffered formalin (VWR) and paraffin-embedded or flash-frozen in liquid nitrogen and stored at −80°C for isolation of RNA.

Blood collections for analysis of metabolites and white blood cell counts

Retro-orbital bleeds and cardiac punctures. Serial blood samples were collected by retro-orbital bleeding. Approximately 100 to 150 μL of whole blood per mouse per bleed was collected. Larger volumes of blood were collected by cardiac puncture upon study termination.

White blood cell isolation and enumeration. Whole blood was collected into EDTA and diluted in RBC lysis buffer. Following wash with PBS, cells were pelleted. Recovered white blood cells (WBC) were resuspended in 200 μL of 0.6 mmol/L EDTA phosphate-buffered saline (E-PBS). One hundred microliters of the WBC suspension was cytopspun and stained with Diff Quick (Dade Behring). The number of lymphocytes and neutrophils in approximately 500 cells per treatment group was determined. The remaining WBC suspension was further diluted in 400 μL of E-PBS and counted using a Beckman Coulter Vi-Cell cell counter to determine the cell number/total volume of blood collected.

Bronchoalveolar lavage
E-PBS (1 mL) was instilled into the trachea and then aspirated. The solution was reinstituted eight times. The volume of lavage solution that was recovered was noted, and the lavage dispensed into a second tube for cell enumeration. Once the cells were counted, the volume recovered from the instillation was used to calculate total cell number.

Vitamin D metabolites and calcium
25D3 and 1,25D3 were measured in sera pooled for each group at multiple time points. 25D3 levels were measured using liquid chromatography–mass spectrometry at the University of Pittsburgh or Heartland Assays (Ames). 1,25D3 levels were also measured by Heartland Assays using a radio-immunnoassay. Serum calcium was measured with a colorimetric assay (Point
Immunohistochemistry
For histologic assessment of lesions, formalin-fixed lung tissue was paraffin-embedded and cut into 5-μm thick sections. Three sections at least 100-μm apart were stained with hema-toxylin and eosin (H&E) and scored to allow for adequate sampling of the lung. H&E staining was conducted using a standard protocol (28). Standard immunohistochemistry techniques were used to stain for Ki-67, cytokeratin 5 & 6 (CK 5/6), thyroid transcription factor (TTF-1), p63, and F4/80 (26). The IHC technique used for VDR is similar to the IHC method cited above except that slides were processed with an ABC kit (Vector Labs) to enhance the visualization of VDR (18). Primary antibodies were used at the following concentrations: VDR, 1:250 MA1-710 (Affinity Bio); p63, 1:1,500 SC-8431 (Santa Cruz Biotechnology); TTF-1, 1:400 M3675 (Dako); and F4/80, 1:100 AB6640 (Abcam). See Supplementary Fig. S1 for immunohistochemistry controls.

Scoring histopathology and staining
Lung histology was assessed in an unblinded fashion by two observers. There was ≥90% concordance between observers for all lesions. Histologic classifications of NTCU induced premalignant lesions, discussed in detail by Hudish and colleagues (26), were used as a reference. Lung histology was assessed in the large airways (trachea and left and right main stem bronchi) in 3 H&E sections per mouse. Ten to 15 mice per treatment group were scored. The mucosal surfaces of large airways occupied by normal epithelium, atypia, low-grade dysplasia (LGD; inclusive of squamous metaplasia and mild dysplasia), and HGD (moderate and severe dysplasia) were determined. Subsequently the percentage of surface area of the large airway occupied by each lesion was enumerated in increments of 25% per section. For example, the area in one cross-section of a large airway may contain 50% LGD, 25% atypia, and 25% normal tissue, thus taking into account the variation in histologic subtypes within a single airway. Then, the mean percentage of airway occupied by each lesion was calculated per mouse. Finally, data were averaged for all mice within a treatment group to determine the mean percentage of airway occupied by each lesion type per treatment group. This is the first study to quantify the lesions in the larger airways apart from the small airways. Small airways were scored per bronchial opening based on the predominant lesion present because little variation was seen within most small airways.

VDR staining was assessed using a published method (18). VDR staining was scored by counting the number of positively stained cells in a section of large airway. Five hundred cells were counted, and the percentage of positive cells was characterized as follows: absent, <10% positive, 10% to 50% positive, or >50% positive (1 section per mouse and 5 mice per treatment group were analyzed). Ki-67 staining was scored by a standard technique (29). The number of positively stained nuclei in 500 cells in sections of large airway was determined (1 section per mouse and 5 mice per treatment group).

RNA isolation and qRT-PCR
Total RNA was isolated and purified from lung tissue using the TRIzol method and standard techniques (30). Total RNA (500 ng/sample) was reverse transcribed into cDNA using random hexamers and the Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative PCR was performed using Applied Biosystems TaqMan Gene Expression Array reagents and the ABI Prism 7300 Real Time PCR System by standard techniques. Primer probe sets IL6 (Mm00446190_m1) and GAPDH (Mm99999915_g1) were used. GAPDH served as the internal control. The relative changes in gene expression were calculated using the comparative Ct method (31).

Statistical analysis
The frequency and the percentage of lesions were compared between groups using the Kruskal–Wallis ANOVA test. When appropriate, the Dunn test was used to conduct post-hoc pairwise comparisons. VDR quantitation was compared using the Fisher exact test, while the Ki-67 quantitation was compared using the Wilcoxon rank-sum exact test. All other comparisons were made using the Student t test. A significance level of 0.05 was considered for all analyses.

Results
Effects of dietary vitamin D3 and calcitriol (1,25D3) on serum vitamin D metabolites and calcium in the NTCU mouse model
Pilot NTCU dosing studies revealed that cutaneous applications of 25 μL of 40 mmol/L NTCU (N) once weekly were well tolerated, permitting long-term studies. To examine the effects of vitamin D on progression of SCC, mice were randomized into two groups to receive diets containing either 0 (deficient: D) or 2,000 IU/kg (Sufficient: S) vitamin D3. Mice on each diet were further subdivided into three groups [15 mice/group/time point (15 and 25 weeks)] and treated with acetone control (D or S). NTCU only (DN or SN) or NTCU + calcitriol (DN+C or SN+C; Fig. 1A). All groups that received a vitamin D–deficient diet had mean serum 25D3 levels <4 ng/mL (P < 0.0001). Among groups on a vitamin D–supplemented diet but not receiving calcitriol, the mean 25D3 serum level was 19 ng/mL. In the SN + C group, the mean serum 25D3 level was 10 ng/mL (P < 0.005; Table 1). Serum 1,25D3 levels, measured between 8 and 30 weeks after the start of diet, were decreased in mice receiving the deficient diet (DN) compared with the supplemented diet (SN), as expected (32). To assess the effect of calcitriol on 1,25D3 levels, blood was obtained 1 hour after calcitriol treatment. Regardless of the diet, 1,25D3 levels of nearly 300 pg/mL were achieved (Table 1). Serum calcium remained within normal ranges in all groups (Table 1; ref. 32). Parathyroid hormone (PTH) was suppressed in the calcitriol-treated groups (data not shown). Mice in all groups treated with NTCU gained or maintained weight within 10% of initial body weight throughout the experiments (Table 1). However, as noted in Tables 2 and 3, the number of surviving mice out of the initial 15 mice per treatment arm in the 25 μmol NTCU group on the vitamin D–deficient diet (DN) was reduced by 47% compared with the NTCU group on the sufficient diet (SN).
The frequency and percentage of airway occupied by HGD is reduced in vitamin D3–sufficient mice

The development of lung SCC is a multistep process. The mechanism whereby NTCU induces SCC in the lungs of mice has not been elucidated. NTCU did not induce the development of lesions or tumors other than those reported. However, <5% of all mice spontaneously developed lung adenoma/adenocarcinomas, as has been well described in the SWR/J mouse (33). One mouse in the DN group developed a lymphoma. To assess chemopreventive actions of vitamin D, the lesions in the large airways were quantified. Similar numbers of large airways were scored in all groups. Changes in the frequency and percentage of airway occupied by HGD were used as endpoints to compare groups.

All NTCU-treated groups had significantly more lesions after treatment with 15 μmol of NTCU than corresponding acetone controls, in which there was no evidence of bronchial epithelial changes. The development of lesions in NTCU-treated mice was marked by the loss of cilia and columnar morphology and increased chromatin material, characteristic of squamous metaplasia, and dysplastic lesions (Fig. 1B). Dietary vitamin D3 supplementation resulted in approximately 50% reduction in the frequency of HGD after 15 μmol of NTCU: SN (43%) and SN+C (50%), compared with the DN group (100%; P < 0.05; Table 2). Treatment with calcitriol had no significant impact on HGD occurrence in vitamin D–sufficient group. As illustrated in Fig. 1B, the predominate lesions in the SN, SN+C, and DN+C groups were LGD. The DN group developed more HGD, which is distinguished from LGD by the increased variation in nuclei shape in more than half to all of the thickness of the lesion. Calcitriol was also protective, as 58% of mice in the DN+C group developed HGD after 15 μmol of NTCU, compared with 100% in the DN group (P < 0.05; Table 2). However, HGD frequency in the SN+C group remained at the same level as was seen after 15 μmol of NTCU (50%; Table 2).

In addition to reducing the frequency with which HGD occurred, the percentage of airway occupied by HGD lesions after treatment with 15 μmol of NTCU was significantly less in the SN (8.7%), SN+C (6.6%), and reduced in the DN+C (12.3%) groups compared with the DN (22.7%; P < 0.05) group (Table 3). It was
also noted that the percentage of LGD in mice that received 15 μmol NTCU was on average higher in the SN group than the DN group. The relative increase in LGD and decrease in HGD in SN mice compared with DN mice indicates that vitamin D repletion prevents the progression of LGD to HGD. As compared with the 15 μmol NTCU treatment, the percentage of HGD lesions after treatment with 25 μmol of NTCU increased in both the deficient groups, DN (35.5%) and DN+C (21.3%). The percentage of HGD in the sufficient groups remained less than the deficient groups. Moreover, little dose-dependent increase was observed in the percentage HGD lesions in the SN (8.4%; P < 0.05) and SN+C (11.8%) groups (Table 3). The prominent lesion in the small airways of NTCU-treated mice was atypia, regardless of vitamin D status or NTCU dose (Supplementary Table S1).

To confirm that the lesions that developed were squamous in nature, immunohistochemistry markers that are used to identify human lung SCC (CK 5/6 and p63) and adenocarcinoma (TTF-1) were examined. The lesions that developed in the large airways of NTCU mice had increased expression of CK 5/6 and p63, whereas the expression of TTF-1 decreased (Fig. 2A–C). The expression of CK 5/6 and p63 were greater in the DN group compared with the SN group after treatment with 25 μmol of NTCU, consistent with our observation that deficient mice have more advanced squamous lesions (Fig. 2B and C).

VDR expression increases in a lesion and ligand-dependent manner

Vitamin D metabolites bind to VDR to elicit anticancer activity. We therefore examined the effect of carcinogen treatment and dietary intervention on VDR levels. Comparing the S to SN or D to DN groups, we find that VDR is upregulated by carcinogen exposure. This agrees with human data, where nuclear VDR increases with disease progression (18). However, our data indicate that disease progression is not the sole determinant of VDR expression. If we compare SN to DN after 15 μmol of NTCU, we see that VDR is higher in the SN group (P < 0.05; Fig. 2D and E). This occurs despite the fact that lesions are more advanced in the DN group. No differences in VDR mRNA expression were detected in lung tissues regardless of disease or vitamin D status (data not shown). The most likely explanation for the elevation in VDR expression in SN mice compared with DN mice is that ligand upregulates/stabilizes VDR protein (34, 35). These data suggest that vitamin D repletion is needed to maintain VDR expression and presumably function in premalignant lesions.

Proliferation is reduced in NTCU-induced bronchial lesions in vitamin D3–sufficient mice

Numerous studies have demonstrated that calcitriol and its analogues inhibit cell proliferation through VDR-dependent transcriptional processes (36). To examine the effects of vitamin D status on proliferation within lesions, we used immunohistochemical staining of Ki-67, which is classically used to measure proliferation in the development of bronchial dysplasia. There was an increase in Ki-67 staining in all of the NTCU-treated groups after 15 μmol of NTCU treatment compared with S (P < 0.01). There was more Ki-67 staining in the DN group compared with SN (P < 0.05). Calcitriol-treated groups (SN and DN) had approximately a 5% reduction in Ki-67 in comparison with their

Table 1. Body weight and serum 25-hydroxyvitamin D3, 1,25-dihydroxyvitamin D3, and calcium

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dietary vitamin D3 (IU/kg)</th>
<th>Body weight (g)</th>
<th>Serum 25D3 (ng/mL)</th>
<th>Serum 1,25D3 (pg/mL)</th>
<th>Serum calcium (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone (S)</td>
<td>2.000</td>
<td>22.8 ± 0.9</td>
<td>20 ± 1.1</td>
<td>88 ± 18.3</td>
<td>9.90 ± 0.05</td>
</tr>
<tr>
<td>NTCU (SN)</td>
<td>2.000</td>
<td>18.1 ± 0.4b</td>
<td>19 ± 0.5</td>
<td>88 ± 17.8</td>
<td>9.90 ± 0.27</td>
</tr>
<tr>
<td>NTCU + calcitrol (SN+C)</td>
<td>2.000</td>
<td>18.5 ± 0.4</td>
<td>10 ± 1.1</td>
<td>290 ± 12.7ac</td>
<td>9.97 ± 0.32</td>
</tr>
<tr>
<td>Acetone (D)</td>
<td>0</td>
<td>22.7 ± 0.9</td>
<td>&lt;4 ± 0.2ab</td>
<td>43 ± 1.6</td>
<td>10.30 ± 0.20</td>
</tr>
<tr>
<td>NTCU (DN)</td>
<td>0</td>
<td>19.4 ± 0.6</td>
<td>&lt;4 ± 0.2a</td>
<td>40 ± 0.6</td>
<td>10.23 ± 0.27</td>
</tr>
<tr>
<td>NTCU + calcitrol (DN+C)</td>
<td>0</td>
<td>19.0 ± 0.4b</td>
<td>&lt;4 ± 0.2a</td>
<td>288 ± 26.5bc</td>
<td>9.40 ± 0.80</td>
</tr>
</tbody>
</table>

NOTE: Body weight is the mean ± SEM of the final weight of mice within a given group after 25 weeks of NTCU or control. Serum 25D3 and calcium values are the mean ± SEM collected throughout the course of the experiment (4–30 weeks). Serum 1,25D3 values are the mean ± SEM collected at 8, 24, and 30 weeks.

*P < 0.05 compared with Acetone (S).
**P < 0.005 compared with Acetone (S).
***P < 0.0001 compared with Acetone (S).

Table 2. Frequency of normal histology, atypia, LGD, and HGD in the large airways after NTCU treatment [N mice with at least 1 lesion/N total mice in group (% of total)]

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Normal</th>
<th>Atypia</th>
<th>LGD</th>
<th>HGD</th>
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</thead>
<tbody>
<tr>
<td>15 weeks of NTCU (15 μmol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>11/71 (100)</td>
<td>12/14 (86)</td>
<td>14/14 (100)</td>
<td>6/14 (43)</td>
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<tr>
<td>SN</td>
<td>13/14 (93)</td>
<td>13/14 (93)</td>
<td>7/14 (50)</td>
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<tr>
<td>SN+C</td>
<td>15/15 (100)</td>
<td>10/10 (100)</td>
<td>9/10 (90)</td>
<td>10/10 (100)</td>
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<tr>
<td>D</td>
<td>4/10 (40)</td>
<td>10/10 (100)</td>
<td>12/12 (100)</td>
<td>7/12 (58)</td>
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<tr>
<td>25 weeks of NTCU (25 μmol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>14/14 (100)</td>
<td>1/14 (7)</td>
<td>9/13 (69)</td>
<td>4/8 (50)</td>
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<tr>
<td>SN</td>
<td>5/13 (38)</td>
<td>13/13 (100)</td>
<td>8/8 (100)</td>
<td>4/8 (50)</td>
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<tr>
<td>SN+C</td>
<td>2/8 (25)</td>
<td>8/8 (100)</td>
<td>4/8 (50)</td>
<td>4/8 (50)</td>
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<tr>
<td>D</td>
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<td>2/10 (20)</td>
<td>5/7 (71)</td>
<td>7/7 (100)</td>
</tr>
<tr>
<td>DN</td>
<td>2/7 (29)</td>
<td>5/7 (71)</td>
<td>10/10 (100)</td>
<td>10/10 (100)</td>
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<tr>
<td>DN+C</td>
<td>3/10 (30)</td>
<td>10/10 (100)</td>
<td>10/10 (100)</td>
<td>10/10 (100)</td>
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</table>
Table 3. Percentage of the surface area of the large airways with normal histology, atypia, LGD, and HGD after NTCU treatment

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>N</th>
<th>Normal</th>
<th>Atypia</th>
<th>LGD</th>
<th>HGD</th>
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<tr>
<td>15 weeks of NTCU (15 μmol)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>11</td>
<td>100 ± 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SN</td>
<td>14</td>
<td>26 ± 17.9</td>
<td>63.5 ± 17.5</td>
<td>8.72 ± 9.5</td>
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</tr>
<tr>
<td>SN+C</td>
<td>14</td>
<td>38.5 ± 27.1</td>
<td>53.3 ± 23.3</td>
<td>6.6 ± 7.7</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>15</td>
<td>100 ± 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DN</td>
<td>10</td>
<td>3.8 ± 6.7</td>
<td>25.3 ± 10.5</td>
<td>48.3 ± 9.2</td>
<td>22.7 ± 8.8b</td>
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<tr>
<td>DN+C</td>
<td>12</td>
<td>0.8 ± 1.8</td>
<td>23.2 ± 21.3</td>
<td>63.1 ± 20.4</td>
<td>12.3 ± 13.9</td>
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<tr>
<td>25 weeks of NTCU (25 μmol)</td>
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<td></td>
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<tr>
<td>S</td>
<td>14</td>
<td>99.7 ± 1.1</td>
<td>0.3 ± 1.1</td>
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<tr>
<td>SN</td>
<td>13</td>
<td>4.9 ± 8.0</td>
<td>38.2 ± 16.2</td>
<td>48.5 ± 14.1</td>
<td>8.4 ± 7.7</td>
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<td>SN+C</td>
<td>8</td>
<td>6.4 ± 15</td>
<td>41.4 ± 28.7</td>
<td>40.4 ± 29.1</td>
<td>11.8 ± 15.6</td>
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<td>97.9 ± 5.3</td>
<td>2.1 ± 15.3</td>
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<td>DN</td>
<td>7</td>
<td>3.5 ± 6.6</td>
<td>12.5 ± 35.6</td>
<td>48.5 ± 27.4</td>
<td>35.5 ± 21b</td>
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<td>DN+C</td>
<td>10</td>
<td>5.42 ± 9.2</td>
<td>72.9 ± 14.9</td>
<td>21.3 ± 10.3</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Percentage of lesions is represented as the mean of total airways scored per group ± SEM; 3 sections (each ~100 μm apart) were scored per mouse.

*Significant P < 0.05 compared with S.

**Significant P < 0.05 compared with SN.

respective NTCU treated groups (Fig. 2F and G). Following 25 μmol of NTCU treatment, the percentage of positively stained cells increased approximately 2-fold in all treatment groups. However, staining in the DN group was 30% higher than the SN group (P < 0.001). The addition of calcitriol continued to reduce proliferation in the DN+C (12% decrease; P < 0.01) and the SN+C groups (6% decrease; Fig. 2G). These data suggest that vitamin D deficiency reduces proliferation, and that calcitriol partially overcomes the enhanced proliferation observed in vitamin D–deficient mice.

NTCU-induced inflammation is reduced in vitamin D3–sufficient mice

Inflammation is associated with the development of SCC (37), and vitamin D has the potential to modify inflammation (23). Therefore, we examined the local and systemic effects of NTCU administration on inflammation in vitamin D–sufficient and -deficient mice. This was done by measuring IL6 plasma levels systemically and local pulmonary IL6 mRNA expression. In addition, we examined the early inflammatory events induced by NTCU treatment in vitamin D–deficient and -sufficient mice by studying mice after 2 weeks of treatment with NTCU.

Systemic inflammation. NTCU increases plasma IL6 levels in DN mice compared with SN at each time point investigated (P < 0.05; Fig. 3A). These data suggest that NTCU elicits an enhanced inflammatory state in the DN mice. Consistent with this idea, 2 weeks following NTCU treatment, the WBC count in the DN-80 mmol/L group was markedly increased compared with all other groups (P < 0.05). There was a dose-dependent increase in the number of WBCs in the SN group (Fig. 3B). Differential staining indicated that the increase in WBCs consisted predominately of neutrophils in the SN-80 mmol/L group (P < 0.01) and the DN-40 mmol/L and DN-80 mmol/L groups (P < 0.05). Little change was seen in the SN-40 mmol/L group compared with S alone (Fig. 3B and C). The large increase in the number of WBCs in the DN-80 mmol/L group consisted of neutrophils, monocytes, and lymphocytes.

Local inflammation. The expression of IL6 in lung tissue was increased after 15 μmol of NTCU compared with control (S) in the SN (P < 0.01), SN+C (P < 0.05), and DN (P < 0.05) groups. In the DN mice, there was an average 6-fold increase in expression. The magnitude of NTCU induction of IL6 was reduced in the 25 μmol NTCU groups. The DN and DN+C groups had higher expression than SN or SN+C groups, further suggesting persistent inflammation in vitamin D–deficient mice (P < 0.05; Fig. 3D). In addition, bronchoalveolar lavages (BAL) were collected following 2 weeks of treatment with 40 or 80 mmol/L NTCU. The number of macrophages in the BALs of mice treated with 80 mmol/L NTCU was increased compared with control (P < 0.05). The acute, proinflammatory effect of NTCU was enhanced in the deficient mice (P < 0.01; Fig. 3E). To evaluate local inflammation in the vitamin D–deficient NTCU-treated mice at later time points, lung tissue from S, DN, D, and DN after 25 weeks of treatment was stained with F4/80, a mouse macrophages marker. F4/80 was most readily detected in the DN group (Fig. 3F). Cumulatively, these data suggest an association between increased inflammation and vitamin D deficiency, which correlates with enhanced disease progression.

Discussion

Epidemiologic studies suggest that vitamin D may play a role in cancer prevention; numerous studies describe the inverse associations between 25D3 levels or UVB exposure and cancer risk. Mohr and colleagues (38) determined that lower levels of UVB exposure are independently associated with higher lung cancer incidence. Preclinical studies suggest that the active metabolite of vitamin D, 1,25D3, contributes to the prevention of adenocarcinoma of the lung (39). Lung cancer is a heterogeneous group of diseases, and the activity of vitamin D metabolites may differ among histotypes. Our studies focused on the role vitamin D in preventing the progression of premalignant squamous lesions. We discovered that the incidence and progression of premalignant lesions is decreased in vitamin D–sufficient mice as compared with vitamin D–deficient mice. Our data further suggest that vitamin D deficiency increases inflammation (both locally and systemically) and proliferation of premalignant cells, thereby enhancing progression of squamous lesions.

Using an approach analogous to that used in recent human trials of chemopreventive agents (iloprost and myoinositol) in bronchial carcinoma, we examined the differences in endobronchial histology by measuring the frequency and percentage of HGD that develop in the large airways of mice treated with NTCU. Our studies indicate that mice sufficient in vitamin D3 (20 ng/mL...
serum 25D3) had fewer HGD lesions than mice on a vitamin D3–deficient diet (<4 ng/mL serum 25D3). We also found that systemic administration of calcitriol reduces the development of premalignant squamous lesions in vitamin D3–deficient mice. These findings suggest that vitamin D supplementation among subjects with deficiency may be an effective approach to block or reduce premalignant epithelial change in individuals at risk for lung cancer. With regard to this point, we propose that individuals diagnosed with chronic obstructive pulmonary disease (COPD) represent an appropriate population to target with a preventive vitamin D–based intervention (40). COPD patients are at significantly elevated risk for developing lung cancer (41), and vitamin D deficiency is a common problem in COPD patients: 31% have 25D3 levels <20 ng/mL, and 7% have severe deficiency (25D3 levels ≤ 10 ng/mL; ref. 42).

The timing at which such supplementation to reverse low vitamin D levels is effective in the murine and clinical situations remains to be investigated. Our studies also fail to address the important question of vitamin D dose-response. How much vitamin D repletion is optimal? Studies in the AOM/DSS murine colon cancer model suggest that a dose–response relationship exists; colonic dysplasia decreases as serum 25D3 levels increase from approximately 12 to 60 ng/mL (43). The most striking effect was seen when mice with serum 25D3 levels of 12 ng/mL (100 IU/kg diet) were compared with those having a level of 30 ng/mL (400 IU/kg diet). In a separate study in the Apc−/− mouse model, no difference in the incidence of small intestinal tumors was observed between mice with serum 25D3 levels of 26 ng/mL and those with serum levels of 290 ng/mL (44). When considered together, these colon cancer prevention studies indicate a benefit of overcoming vitamin D deficiency, but that enhancing vitamin D levels in individuals with "adequate" levels (26–30 ng/mL) may be of only modest additional benefit. This is consistent with our observation that calcitriol decreases the frequency and percentage of HGD in vitamin D–replete mice but has little effect on the same parameters in vitamin D–deficient mice.

In addition to evaluating the development of premalignant lesions, we examined vitamin D–regulated pathways. Many of the actions of vitamin D signaling pathways are mediated by the binding of a heterodimer composed of ligand-bound VDR with the retinoid X receptor (RXR) to DNA to modulate gene expression (7). Menezes and colleagues (18) demonstrated that nuclear VDR staining increases in premalignant squamous lesions in lung. Other studies show that VDR staining is increased in premalignant and well-differentiated lesions in the breast and colon.
(18, 45, 46). In contrast, VDR protein expression is reduced in invasive and poorly differentiated breast and colon tumors. Low serum 25D3 serum content is associated with poor prognosis in NSCLC (15, 45, 46). Together these studies suggest that a loss of serum 25D3 serum content is associated with poor prognosis in invasive and poorly differentiated breast and colon tumors. Low

The relationship between vitamin D status and the inflammatory response in these mice exposed to a lung carcinogen is also noteworthy. We observe persistent enhancement of inflammation both systemically and locally in NTCDU-treated, vitamin D–deficient mice. There is also an initial acute inflammatory response to NTCDU treatment in vitamin D–deficient mice. Others have demonstrated the expression of CYP27B1 and local production of 1,25D3 in lung epithelial and immune cells (49). It is possible that in sufficient mice, where 25D3 levels are adequate, an influx of CYP27B1-expressing WBCs to the lung may increase local production of 1,25D3. This may result in increased modulation of VDR target genes that control inflammation. 1,25D3 has been shown to increase the expression of inhibitor of NFkappaB subunit beta (IKKbeta) to inhibit NFkappaB activation (50). No such local production of 1,25D3 (and suppression of inflammation) would occur in vitamin D–deficient mice. Enhanced inflammation in the lungs is expected to promote the development of lung SCC, as shown in the mouse model of inactive kinase IKKalpha (37). The presence of heightened inflammation in vitamin D–deficient mice is suggested by the elevation in IL6 protein in the circulation and IL6 gene expression in the lung. This study was not powered to examine the inflammatory response to carcinogen as it relates to

| Figure 3. Immune modulation in response to vitamin D in the NTCDU model. A, plasma IL6 levels are elevated in the DN group throughout the vitamin D intervention study (S: SN or S: DN, P < 0.05 and SN: DN P < 0.05). B, the total of WBCs collected in whole blood after acute NTCDU treatment are significantly greater in the DN group (2 weeks of NTCDU: 40 mmol/L (2 μmol) and 80 mmol/L (4 μmol)). C, the percentage of neutrophils in WBCs increased after acute NTCDU treatment. D, IL6 expression was increased in the lungs of DN mice following NTCDU treatment. E, total WBC counted in BALs are significantly greater in the DN group after 2 weeks of acute NTCDU treatment, and F, F4/80 staining of mouse macrophages increased in the small airways after 25 μmol of NTCDU in the DN group. [Magnification, ×400; *, P < 0.05 and **, P < 0.005 compared with S.]
Vitamin D Repletion Reduces Premalignant Lesions in NTCU-Mice

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Acknowledgments
The authors thank the laboratory of Drs. Barbara Foster and Mike Moser for technical support.

Grant Support
This work was supported by NIH/NCCAM F31AT0006487 (to S.A. Mazzilli), NIH/NCI T32 CA009072 and NIH/NCI CA067267 (to C.S. Johnson), CCSR P50CA106056 (to D.L. Trump), and SPORE P50 CA09440 (to P.A. Hershberger, Project Leaders).

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Vitamin D Repletion Reduces the Progression of Premalignant Squamous Lesions in the NTCU Lung Squamous Cell Carcinoma Mouse Model

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Cancer Prev Res Published OnlineFirst August 14, 2015.

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Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-14-0403

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