Research Article

N-nitroso-tris-chloroethylurea Induces Premalignant Squamous Dysplasia in Mice

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Abstract

Squamous cell carcinoma (SCC) and premalignant endobronchial lesions have been difficult to study in murine models. In this study, we evaluate the topical N-nitroso-tris-chloroethylurea (NTCU) murine SCC model, determine the extent to which resulting premalignant airway dysplasia develops, discuss clinicopathologic grading criteria in lesion progression, and confirm that immunohistochemical (IHC) staining patterns are consistent with those observed in human endobronchial dysplasia and SCC. Male and female FVB mice were treated biweekly with topical NTCU (4, 8, or 40 mmol/L) or vehicle for 32 weeks. Following sacrifice, squamous cell lesions were enumerated and categorized into the following groups: flat atypia, low-grade dysplasia, high-grade dysplasia, and invasive SCC. The 40 mmol/L NTCU concentration produced the entire spectrum of premalignant dysplasias and squamous cell carcinomas, but was associated with poor survival. Concentrations of 4 and 8 mmol/L NTCU were better tolerated and produced only significant levels of flat atypia. Squamous origin of the range of observed lesions was confirmed with IHC staining for cytokeratin 5/6, p63, thyroid transcription factor-1 (TTF-1), and Napsin-A. This study shows that topical application of high-dose NTCU produces endobronchial premalignant lesions with classic squamous characteristics and should allow for improved preclinical evaluation of potential chemopreventive agents.

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Introduction

Lung cancer is the leading cause of cancer deaths in males and females in the United States with more than 200,000 incident cases and more than 150,000 deaths occurring in the United States in 2010 (1). Adenocarcinoma (AC) and squamous cell carcinoma (SCC) are the most common forms of non–small cell lung cancer (2). SCC chemoprevention trials in humans have focused on endobronchial dysplasia as an endpoint (3, 4) and until relatively recently, this field has lacked a reproducible animal model of airway dysplasia that could aid in the evaluation of chemopreventive agents prior to the initiation of clinical trials. Although multiple, well-characterized models of murine AC exist [initiator-promoter carcinogenesis (5), mutant KRAS, and the use of strict carcinogens (6–8)], SCC models are fewer in number and poorly characterized (9, 10).

Similar to other common cancers, a reproducible SCC model would advance preclinical studies and the understanding of premalignant biology. The N-nitroso-tris-chloroethylurea (NTCU) murine model presents an opportunity to expand current understanding of carcinogenesis in human smokers as it is has been reported to induce murine SCC and airway dysplasia (9). Recently, this model has been used to show both the inhibitory effects of pioglitazone (11), a mixture of Chinese herbs (12), and pomegranate fruit extract (13) in the progression of SCC and lung tumorigenesis. The present study aims to better characterize this model in FVB/N mice, to improve upon the reported dosing schedule, and to compare immunohistochemistry (IHC) staining patterns of SCC lesions in mouse lung with human endobronchial dysplasia. We confirm the squamous origin of these lesions by conducting IHC with markers standard for SCC and AC identification. In this study, we show that high-dose NTCU exposure results in an array of premalignant lesions, but lower doses only lead to earlier grade lesions (flat atypia).

Methods

NTCU administration

The FVB/N murine strain selection was based on previous reports of susceptibility to NTCU (9). Strains such as FVB and Balb/C classically exhibit an intermediate degree of...
sensitivity to lung carcinogens (14–16), and display many similarities to human AC (17–19). Male and female wild-type FVB/N mice were purchased from the Jackson Laboratory and NTCU treatment was initiated at approximately 10 weeks of age. Mice were housed in a negative airflow biohazard caging system and received normal rodent diet (Modified Rodent LabDiet 5001, TestDiet) ad libitum under a 12-hour diurnal light-dark cycle. Topical application of NTCU (Toronto Research Chemicals, Inc.) occurred biweekly for 32 weeks at concentrations of 4, 8, and 40 mmol/L, diluted with acetone (Sigma-Aldrich). Following dorsal coat shaving, a 25 μL volume was applied to exposed skin. Control animals received acetone alone.

Due to dampened survival rates in the initial 40 mmol/L treatment group (summarized in the results section), a behavioral index scoring system was developed to track the health of animals over time in all treatment groups: (a) weight loss: 10%–15% (3 points), >15% (euthanize); (b) piloerection, disheveled appearance, blepharitis, fecal/urine changes (1 point); (c) decreased activity, ataxia (1 point); (d) inappetence, anorexia (1 point); and (e) labored respiration (1 point). Animals of the 4 and 8 mmol/L groups were weighed and scored at the time of each NTCU application throughout the course of the study. At any point during the study, animals reaching a score of 3 points were suspended from further treatments and monitored daily until a change in score was observed; animals exceeding 3 points were removed from the study and euthanized; animals showing improved symptoms were removed from the study and euthanized according to protocols.

After 32 weeks of NTCU application animals were euthanized and tissues were collected. At the time of sacrifice, the right lower lobe was removed for prostaglandin metabolite level measurement. The remaining 4 lobes were insufflated and tissues were collected. At the time of sacrifice, the right lower lobe was removed for prostaglandin metabolite level measurement. The remaining 4 lobes were insufflated, preserved in formalin, and used for lesion quantitation and IHC.

**Lesion grading and quantitation**

Lesion grading was carried out under bright field microscopy on hematoxylin and eosin (H&E)-stained tissue of 10 animals from each of the 4 treatment groups (4, 8, 40 mmol/L, and acetone control). Three separate H&E slides were examined per animal, taken from a single paraffin block, each spaced 50 μm apart. Lesion counts are reported per area of lung tissue as calculated using ImagePro Plus software (version 7.0, Media Cybernetics).

Endobronchial lesions were quantitated and classified by 2 independent, blinded observers (D.M. and L.O.) using the following clinicopathologic grading criteria (photomicrographs contained in Fig. 1): (a) flat atypia—bronchial epithelium composed of a single cell layer with normal bronchial epithelial cells interspersed between atypical cells that show enlarged and hyperchromatic nuclei, but generally maintaining the luminal cytoplasmic clearing and/or ciliation seen in normal cells; (b) low-grade dysplasia—stratified nonciliated squamous epithelial layer with maturation in upper layers as indicated by horizontal nuclear orientation and decreased nuclear-to-cytoplasmic (N:C) ratios. Atypia was confined to the lower one-third to one-half of the epithelium; (c) high-grade dysplasia—stratified, nonciliated squamous epithelial layer without maturation showing high N:C ratios throughout the epithelium, high degrees of nuclear pleomorphism, variably sized nuclei, hyperchromasia, a lack of orientation, occasional distinctive nucleoli, and atypia that extended into the upper half or involves the full thickness of the epithelium; and (d) invasive squamous cell carcinoma—nests of cells with cytologic similarity to high-grade dysplasia but showing invasion through the bronchial basement membrane. Tumor nests show evidence of squamous differentiation with distinct cell membranes or intercellular bridges, central keratinization in tumor nests, and/or the presence of dyskeratotic cells. Adenomas adenocarcinomas were observed in all experimental groups at a low frequency, but not included in the analyses.

**Immunohistochemistry**

To determine the cellular origin of NTCU-induced lesions, representative lesions were identified from H&E slides and stained for the classic SCC markers cytokeratin 5/6 (CK5/6, 1:200, Biocare Medical), and p63 (1:50, Biocare Medical), and the AC markers thyroid transcription factor 1 (TTF-1, 1:100, Biocare Medical) and Napsin-A (1:100, Biocare Medical). These markers are routinely employed to differentiate between SCC and AC in human tumor samples (20).

Tissue sections were deparaffinized and rehydrated in graded alcohol and placed in 1X Rodent Decloaking buffer (Biocare Medical) in a pressure cooker for 5 minutes at 125°C. Sections were processed with the MM-Polymer Kit (Biocare Medical) and incubated in primary antibodies at 37°C for 1 hour. Controls of antibody staining consisted of the application of the secondary antibody without the primary antibody, in which case no staining was detected. To differentiate between squamous cell carcinoma and adenocarcinoma, the antibodies CK5/6, p63, TTF-1, and Napsin-A were applied to both a known human squamous cell carcinoma adenocarcinomas. Indication of squamous cell carcinoma was positive staining with CK 5/6 and p63; negative staining with TTF-1 and Napsin-A. The standard for determining lung adenocarcinoma is positive staining with TTF-1 and Napsin-A, and negative staining with CK5/6 and p63. All images were captured using an Olympus BX51 microscope with a DP72 digital camera via CellSens Entry image capture software (version 1.3, Olympus).

**Statistical analysis**

Lesion quantitation is reported graphically as the mean ± SEM. Comparisons between cohorts were made using a paired, 2-tailed Student t test. Survival curves were established using the Kaplan–Meier method and compared using the log-rank test. Results were considered significant at P ≤ 0.05. All statistical analyses and associated graphics were generated using Graphpad Prism (version 4.02, Graphpad Software, Inc.).
Results

NTCU (40 mmol/L) severely reduces survival

Although animals in the 40 mmol/L did exhibit progressive weight loss during NTCU treatment, weight loss was not observed in the 4 and 8 mmol/L treatment groups (Fig. 2). We observed survival rates of 45.0% (18/40) in 40 mmol/L NTCU-treated animals, 77.8% (21/27) in 8 mmol/L, 92.3% (24/26) in 4 mmol/L, and 84.6% (22/26) in acetone control animals (Fig. 3). The log-rank test of survival curves of all cohorts revealed a significant difference in survival of the 40 mmol/L cohort compared with all other treatment groups ($P < 0.05$). A large initial drop in survival of the 40 mmol/L cohort was observed during the 6th week of treatment (day 39), after which the survival curve remained significantly different from other treatment groups. The survival rates in the acetone, 4 and 8 mmol/L cohorts did not significantly differ from each other. On basis of the excessive toxicity with the 40 mmol/L dose, decisions were made to alter the planned twice weekly NTCU application, periodically suspending animals from treatment. The observed toxicity also prompted the investigation of NTCU doses reduced 5- and 10-fold (8 and 4 mmol/L) and implementation of the behavioral index outlined in the methods section for these groups. Initially, the animals of the 40 mmol/L cohort did gain weight when treatment was withheld (arrows in Supplementary Fig. S1), but after the NTCU was completely stopped (arrowhead) due to excess mortality weight loss continued. This group was divided into 2 waves (Supplementary Fig. S1, panels A and B), with differing periods of suspensions throughout the study. The number of animals in each score category was tabulated (Supplementary Table S1) and the scores used as endpoints in determining treatment suspension and termination in high-scoring animals.

NTCU induces flat atypia, endobronchial dysplasia, and invasive SCC

Histopathologic analysis of serial lung sections revealed a range of endobronchial squamous lesions, pending animals from treatment. The observed toxicity also prompted the investigation of NTCU doses reduced 5- and 10-fold (8 and 4 mmol/L) and implementation of the behavioral index outlined in the methods section for these groups. Initially, the animals of the 40 mmol/L cohort did gain weight when treatment was withheld (arrows in supplementary Fig. S1), but after the NTCU was completely stopped (arrowhead) due to excess mortality weight loss continued. This group was divided into 2 waves (Supplementary Fig. S1, panels A and B), with differing periods of suspensions throughout the study. The number of animals in each score category was tabulated (Supplementary Table S1) and the scores used as endpoints in determining treatment suspension and termination in high-scoring animals.

NTCU induces flat atypia, endobronchial dysplasia, and invasive SCC

Histopathologic analysis of serial lung sections revealed a range of endobronchial squamous lesions,
similar to those present in airways of smokers (21). These were classified as flat atypia, low-grade dysplasia, high-grade dysplasia, and invasive carcinoma (Fig. 1). A lesion similar to murine flat atypia has not been reported in human studies of smoking-related endobronchial damage. The 40 mmol/L NTCU dose induced the entire spectrum of premalignant dysplastic lesions, including invasive SCC in experimental animals (Fig. 4). However, it was associated with excessive mortality (Fig. 3). An analysis of differences in lesion number between the males and females of this group revealed a significantly higher number of low-grade and high-grade dysplasias in the females (Supplementary Fig. S2, P < 0.05).

Animals of the 4 and 8 mmol/L groups did not develop high-grade dysplasia or invasive SCC. After 32 weeks of treatment they did, however, have large amounts of flat atypia (in fact, more than the animals of the 40 mmol/L group). Significant low-grade dysplasia was found only in animals that received 8 and 40 mmol/L NTCU. Significant high-grade dysplasia and invasive carcinoma were only detected in the 40 mmol/L group (Fig. 4).

The presence of flat atypia did not seem to have a clear relationship with the development of SCC in the 40 mmol/L group (Fig. 5). The presence of high-grade dysplasia, however, was associated with a significant increase in SCC, (P < 0.05). Although the presence of low-grade dysplastic lesions was associated with increased high-grade dysplasia, the relationship only trended toward significance (P = 0.10).

As a result of necessary treatment suspensions, 10 animals in the 40 mmol/L group were available for lesion enumeration (6 received 33 doses and 4 received 46 doses). Although only significant regarding flat atypia, lesion multiplicity was higher in all lesion categories of the 40 mmol/L animals that received more doses of NTCU over the course of the study (Fig. 6). No squamous lesions of any type were detected in acetone control animals.

Adenomas/adenocarcinomas were observed in all of the treatment groups at the following rates: 40 mmol/L, 22.2% (4/18); 8 mmol/L, 19.0% (4/21); 4 mmol/L, 8.3% (2/24); and acetone, 9.1% (2/22). The majority of animals with an AC had a single tumor (11/12). Pulmonary tumors were confirmed as AC via examination of H&E-stained tumor sections and positive IHC for TTF-1 and Napsin-A (shown in Supplementary Fig. S3).

**NTCU-induced lesions express squamous cell IHC markers**

To better characterize the origin of squamous lesions, IHC was carried out for a panel of markers commonly used to classify human lung cancer, including cytokeratin 5/6 (CK5/6), p63, TTF-1, and Napsin-A. CK5/6 are intermediate-size basic keratins that are expressed in squamous and nonkeratinizing epithelia (22), and are routinely used to identify dysplasias and carcinomas of stratified epithelia

![Figure 2. Animal weights for all treatment groups. The higher average weight of the animals of the 40 mmol/L group at the beginning of the study is a result of a 3-week age difference; animals of the 40 mmol/L groups began NTCU application at a mean age of 11 weeks and those of the other groups began application at a mean age of 8 weeks.](image1)

![Figure 3. Kaplan–Meier survival curve in NTCU-treated animals. The log-rank test of survival curves of all cohorts revealed significant survival differences when comparing the 40 mmol/L with all other treatment groups (P < 0.05). The survival rates in the acetone, 4 and 8 mmol/L cohorts did not significantly differ from each other. Euthanized animals and those found dead were recorded as events rather than censored subjects. *, NTCU treatment suspended.](image2)

![Figure 4. Multiplicity by lesion type. Significant flat atypia was detected in all treatment groups. Significant low-grade dysplasia was detected in middle- and high-dose groups, and high-grade dysplasia and SCC was only detected in the high-dose group. * * * , P < 0.05.](image3)
Lesion multiplicity was higher in all categories of which improves diagnostic accuracy of human lung cancer specimens (31, 32). All of the observed murine SCC lesions consistently stained positive for CK5/6 and p63, and negative for TTF-1 and Napsin-A, analogous to human endobronchial lesions (Fig. 7).

(p53, the murine homologue of the tumor suppressor p53, is essential in airway epithelial development (24) and can be used as an aid in SCC diagnosis (25). TTF-1 is a transcription factor expressed in type II pneumocytes, Clara cells (26, 27), and ciliated epithelial cells in the lung (28). TTF-1 is often used in conjunction with p63 in differentiating SCC from AC or small-cell carcinoma of the lung (29). Napsin-A is an aspartic proteinase involved in the maturation of surfactant protein B (30). TTF-1 and Napsin-A both positively stain AC and negatively stain SCC, the routine use of which improves diagnostic accuracy of human lung cancer specimens (31, 32). All of the observed murine SCC lesions consistently stained positive for CK5/6 and p63, and negative for TTF-1 and Napsin-A, analogous to human endobronchial lesions (Fig. 7).

Although multiple different pathologies were observed in the 40 mmol/L group, only flat atypia was detected in animals of the 4 mmol/L group at a significantly higher rate than control animals. In the 8 mmol/L group, significant flat atypia and low-grade lesions were detected, but the latter at a much lower frequency compared with the 40 mmol/L group. Of note, flat atypia was found at significantly higher rates in the 4 and 8 mmol/L groups, raising the question whether these early lesions may have progressed had the duration of the experiments been extended. Although histologically distinct from normal airway, flat atypia is not a strongly associated precursor to cancer. It may represent a reactive cytologic response to NTCU application. When comparing animals with and without SCC, the presence of SCC correlated with higher numbers of high-grade dysplastic lesions (but not low-grade or flat atypia). The relationship between the frequency of high-grade dysplastic lesions and the presence of SCC lends support to the theory that these are truly premalignant lesions that are associated with the development of SCC.

The toxicity we observed at the highest dose, coupled with the length of treatments, translated into fewer animals completing the protocol. We speculate that a more intermediate dose, for example, 20 mmol/L NTCU applied twice a week, or a higher dose (40 mmol/L) applied weekly may prove to be a better model. These experiments are currently in progress.

Treatment suspensions among animals of the 40 mmol/L cohort presented an opportunity to observe the effects of...
differential dosing over the course of our 32-week study and
to determine if there was a threshold NTCU dose required to
induce SCC. Our experimental animals fell into 2 distinct
dosing groups, those that received 33 doses \( n = 6 \), and
those that received 46 doses \( n = 4 \). In this comparison
(Fig. 6), a threshold dose (a minimum NTCU concentration
necessary for SCC induction) was not discernible, and it
remains to be seen if optimal NTCU dosing (dose strength
and number of applications) can reveal such a threshold.
Although our study did show that endobronchial lesions
mostly flat atypia) are inducible through a topical chemical
application that does not adversely affect animal survival,
advanced SCC was only observed with higher NTCU concen-
trations. Improvements also remain to be made in the
development of more standardized dosing schedules and
whether animals receiving lower doses should be main-
tained longer. The use of small-animal imaging has been
reported (11, 33). Advanced animal imaging may help to
determine the optimal timing for tumor development, but
the resolution to detect precancerous lesions (pre-SCC) is
thus far insufficient.

Experimental animals also developed lung adenomas at a
low but constant rate, and this is consistent with previous
reports of spontaneous alveolar and bronchiolar carcino-
mas in the lungs of aged male (45.0%) and female (52.8%)
FVB/N mice (34). There are no known reports of sponta-
neously occurring endobronchial dysplasia in mice and
multiple AC models employed in our laboratory have failed
to induce the dysplastic lesions seen in NTCU-treated mice.
Overall, the NTCU SCC model improves preclinical
modeling of endobronchial dysplasia, a main target of
many current and future human lung cancer chemopreven-
tion trials. Here, we report on well-tolerated NTCU dosing
schedules with adequate premalignant lesion frequency.
The NTCU model will improve our ability to test novel
agents before initiating human trials and improve our
understanding of the molecular events accompanying the
development and progression of premalignant lesions.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.
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References

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