Modulation of Proliferating Cell Nuclear Antigen in the Bronchial Epithelium of Smokers

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Abstract
Clinical chemoprevention trials seek to intervene in the carcinogenic process to suppress, reverse, or delay the development of invasive cancer. Dysregulated cell growth is a hallmark of epithelial carcinogenesis, and proliferating cell nuclear antigen (PCNA) is a marker of dysregulated proliferation that is highly expressed in non-small cell lung cancers. Squamous metaplasia of the bronchial epithelium is found in chronic smokers and has been considered an early premalignant change. To evaluate the effect of 13-cis-retinoic acid (13-cRA) on PCNA modulation, we evaluated PCNA expression in a total of 706 bronchial biopsy specimens from histologically normal, hyperplastic, metaplastic, and dysplastic bronchial tissues obtained from 86 healthy smokers at baseline, of whom 69 subjects had completed 6 months of treatment on a randomized placebo-controlled chemoprevention trial of 13-cRA and had repeat bronchoscopic biopsies. PCNA expression was evaluated with respect to bronchial metaplasia and as an intermediate end point for response in the trial. In the bronchial biopsies obtained from six standardized pretreatment and posttreatment sites, high PCNA expression correlated significantly with more advanced histological grade ($P < 0.001$). Furthermore, smoking cessation during therapy correlated well with reduced PCNA expression ($P = 0.006$), although multivariate analysis indicated that this reduction in PCNA expression was associated with the reversal of squamous metaplasia. The level of PCNA expression appeared to correlate with the level of epidermal growth factor receptor expression both at baseline and at 6 months. In those patients who ceased smoking during the intervention, the 13-cRA also appeared to be more effective than placebo in reducing PCNA expression ($P = 0.034$ in all of the layers; $P = 0.026$ in basal layers). The efficacy of 13-cRA in the down-regulation of PCNA in quitters was independent of baseline PCNA expression levels. Our study demonstrated that increased PCNA expression was associated with histological progression from normal bronchial epithelium to squamous metaplasia and dysplasia. The modulation of PCNA by 13-cRA in patients who quit smoking suggests a potentially important role for regulating this proliferation marker in retinoid chemoprevention studies of former smokers.

Introduction
In the United States, 164,100 new cases of lung cancer will be diagnosed in 2000, making the lung the second most prevalent cancer site after the prostate in men and the breast in women (1). However, an estimated 156,900 people will die of lung cancer in the United States in 2000, more than the combined total number of people who will die of breast cancer, prostate cancer, and colorectal cancer. Although the 5-year survival rate in lung cancer has improved over the past three decades (2), substantial progress has yet to be made against a disease for which the 5-year survival rate is only 14% (2). Even in those patients who are surgically cured of early stage non-small cell lung cancer, a major cause of mortality is the development of second primary tumors, which occurs at an annual rate of 3–4%/year, thus providing an impetus for the clinical study of chemopreventive compounds (3).

Definitive chemoprevention trials currently rely on the end point of cancer incidence (4). These trials require thousands of subjects, incur high costs, and generally take from 5 to 10 years to complete. Three major clinical trials have evaluated the efficacy of different retinoids in the reduction of second primary tumors in patients cured of a primary aerodigestive cancer (head and neck cancer or lung cancer; Refs. (5–7). Two of these trials demonstrated a statistically significant reduction in the incidence of second primary aerodigestive tumors (5, 6), whereas the third trial failed to demonstrate a significant difference between the retinoid and control arms (7). Large trials have recently been completed seeking to define the long-term benefit of retinoids in lung cancer chemoprevention (8, 9). The future development of chemoprevention for cancers of the lung and other sites may well depend on intermediate end point biomarkers that may significantly reduce trial size, duration, and, subsequently, cost (10). Clinical-laboratory translational biomarker studies also may significantly augment our understanding of carcinogenesis and the mechanism of action of chemopreventive agents. The careful selection and testing of appropriate biomarkers is currently underway.

Invasive squamous cell carcinomas of the lung often arise in association with areas of squamous metaplasia, dysplasia, or carcinoma in situ (11, 12). In association with chronic smoking...
and lung cancer, various histological changes ranging from loss of cilia, cellular atypia, reserve cell hyperplasia, squamous metaplasia, and dysplasia to carcinoma in situ have been reported in the bronchial epithelium (13). One widely held view has been that potentially reversible squamous metaplasia can progress in a multistep fashion to squamous metaplasia with atypia, then to carcinoma in situ, and finally to invasive carcinoma (14). However, several investigators who have used squamous metaplasia or sputum atypia as end points in clinical trials have noted the spontaneous regression of both of these markers (15–20), particularly in individuals who cease smoking (20). Therefore, distinguishing an easily reversible form of metaplasia or atypia from metaplasia or atypia that proceeds step-wise along the neoplastic pathway may be important with regards to their ability as biomarkers.

Several investigators have attempted to use morphological changes found in sputum cytology or on bronchial biopsy specimens as intermediate end points for lung cancer chemoprevention trials (15–20). In 1987, we began a randomized, placebo-controlled chemoprevention trial evaluating the efficacy of 13-cRA for reducing squamous metaplasia in chronic smokers’ bronchial epithelium, as assessed by serial histological evaluation of bronchial biopsy specimens obtained from six standardized sites (20). We also followed changes in bronchial metaplasia associated with smoking status, because previous studies had not examined the effect of smoking cessation on bronchial metaplasia in a prospective fashion. The results of this clinical trial revealed that smoking cessation was associated with reversal of squamous metaplasia but failed to demonstrate that 13-cRA significantly induced the reversal of squamous metaplasia or dysplasia.

Because dysregulated cell growth is a hallmark of epithelial carcinogenesis, we evaluated PCNA expression in bronchial epithelium within the context of this prospective trial to evaluate its potential as an intermediate end point in lung cancer chemoprevention trials. PCNA/cyclin, a 36,000 intranuclear polypeptide (21), is an auxiliary protein of DNA polymerase-δ (22), which is critically associated with cell proliferation (23). Its level of expression fluctuates during the cell cycle. It appears in trace amounts in the G0 phase, increases to its maximum in S phase, and slowly declines thereafter (24, 25).

Previous studies (26) from our group have demonstrated that PCNA expression increases significantly with progression of histology in the head and neck from hyperplastic to dysplastic to cancer. In addition, increased PCNA expression in head and neck tumors at baseline appears to portend a poorer prognosis than low baseline PCNA expression (27).

Therefore, we correlated PCNA expression with histological progression and examined the impact of retinoid therapy. We also attempted to correlate PCNA expression, a marker of cellular proliferation, with EGFr expression to correlate dysregulation of the EGFr signaling pathway with enhancement of proliferation. Our hypothesis was that increased dysregulation of the EGFr signaling pathway, as expressed by increasing EGFr staining, could be associated with greater proliferation, as manifested by an increase in the percentage of PCNA expression. We further predicted that PCNA might be more sensitive than histology as an assay of retinoid- or smoking-cessation-induced reduction in proliferation. Finally, given the preponderance of data from our previous trial suggesting that 13-cRA was ineffective in reversing squamous metaplasia in active smokers, we examined whether PCNA might better define a trend toward enhanced reversal of metaplasia in those individuals who successfully ceased smoking and received 13-cRA.

Patients and Methods
Selection of Tissue Samples. A total of 152 chronic smokers (87 men and 65 women) with a smoking history of at least 15 pack-years were enrolled in the study. Bronchoscopic biopsies were performed at six predetermined bronchial sites from 149 participants including the carina and right upper, right middle, and lower lobes, as well as the left upper and lower lobe bronchi. Tissue samples were formalin fixed, paraffin embedded, mounted, and stained with H&E, and the slides were reviewed by one pathologist (J. Y. R.). Metaplastic lesions were identified according to criteria defined previously (20). The histological criteria of normal bronchial epithelium, basal cell hyperplasia, and squamous metaplasia, with and without dysplasia, are as follows. In normal epithelium, the basal cell layer consists of one cell layer located just above the basement membrane; the parabasal cell layer consists of one to two cell layers located above the basal cell layer and below the superficial layer of ciliated superficial cells. Basal cell (reserve cell) hyperplasia is defined as more than three cell layers of basal and parabasal cells. Squamous metaplasia is indicated by polygonal cells with abundant cytoplasm covered by flattened squamous cells on the surface; intracellular bridges may be seen. In squamous metaplasia, the surface ciliated columnar cells are no longer present. Dysplasia is defined as cells showing variation in size and shape, hyperchromatic nuclei, and loss of polarity.

Of the 149 study participants, 86 patients who have dysplasia or a metaplasia index of ≥15% were randomized to receive treatment with 13-cRA (1 mg/kg/day) or placebo. Sixty-nine patients completed the study and had repeat bronchoscopic biopsies at 6 months (20). Included in this analysis are the bronchial biopsy samples obtained at baseline from the 86 subjects who were randomized and the 69 subjects who completed 6 months of treatment.

Immunohistochemistry. Paraffin sections of 4-μm thickness taken from 10% formalin fixed tissues were stained according to the avidin-biotin complex technique using the ABC Elite kit (Vector Laboratories, Burlingame, CA). The primary antibody used in the study was PCNA (clone 19 A2) from Biogenex (San Ramon, CA). Briefly, the sections were first deparaffinized and rehydrated through a xylene and alcohol series and then immersed in 3% H2 O2 in methanol for 15 min to block the endogenous peroxidase activity. As the pretreatment, the slides were microwaved in 1% zinc sulfate in distilled water for 3 min to eliminate variation in size and shape, hyperchromatic nuclei, and loss of polarity.

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Sections were viewed under high power (×200), and PCNA expression was semiquantitatively assessed on the basis of staining intensity (grades 0–4). The PCNA index was cal-

5 The abbreviations used are: 13-cRA, 13-cis-retinoic acid; PCNA, proliferating cell nuclear antigen; EGFr, epidermal growth factor receptor.
PCNA expression was assessed separately in the basal layer, parabasal layer, and superficial layer of cells of the bronchial epithelium. Only cells with definitive nuclear staining were counted positive, and cytoplasmic staining was interpreted as negative. Staining was repeated if there was any detectable fluctuation in the PCNA index of the control. Methods for EGFr staining were reported by us previously (28).

**Statistical Analysis.** Site-specific PCNA expression, metaplasia status, histology, and EGFr expression were measured for each patient. Both the subject and the biopsy site were used as the analysis unit. The Pearson χ² test was applied to assess the association between PCNA expression and biopsy site. The Kruskal-Wallis test was applied to compare the PCNA expression in histologically normal, hyperplastic, metaplastic, or dysplastic groups. For the analysis of changes in PCNA expression over time, paired t tests were used. To compare changes in PCNA expression over time between groups, the 2-sample t test and the linear mixed effect model were applied. A two-way ANOVA test was applied to compare the PCNA change by treatment and smoking cessation status. Regression analysis was used to incorporate covariates into modeling the change in PCNA expression. The association between EGFr and PCNA was analyzed by the Spearman’s rank correlation (29).

**Results**

**PCNA Expression in the Bronchial Epithelium by Biopsy Site and Histology.** The demographic information of the participants is listed in Table 1. Of 152 chronic smokers, 86 (57%) were eligible by virtue of their metaplasia index being ≥15% or having dysplasia. Of these 86, 69 patients completed 6 months on trial and underwent bronchoscopy at 6 months. A total of 706 biopsy sites were evaluable for PCNA, 381 pre-therapy sites and 325 post-therapy sites. The χ² test showed that there was no correlation between biopsy site and PCNA expression. Table 2 shows the percentage of PCNA staining at baseline grouped into five categories stratified by histology. Of the 27 specimens with histologically normal bronchial epithelium without a parabasal cell layer (with one basal cell layer and one ciliated columnar cell layer, denoted as Normal I in Table 2), only two specimens exhibited more than 5% PCNA-positive cells, and eight (36.7%) had greater than 1% PCNA-positive cells. Of specimens possessing up to two layers of parabasal cells (still considered normal and denoted as Normal II in Table 2) and specimens showing basal cell hyperplasia (more than three layers of basal and parabasal cells), more than 1% PCNA-positive cells were detected in 35.4% (40 of 113) and 85.4% (82 of 96), respectively. The corresponding figures for >5% PCNA positivity were 2.7% (3 of 113) and 57.3% (55 of 96). There was no significant increase in PCNA activity in the areas of goblet cell hyperplasia examined, as opposed to marked increases seen in basal cell hyperplasia.

Among the biopsy specimens that exhibited squamous metaplasia and dysplasia, 85.2% (115 of 135) of the metaplastic lesions and 90% (9 of 10) of the dysplastic lesions showed more than 1% PCNA-positive cells, respectively. Approximately half of the metaplastic (72 of 135; 53.3%) and dysplastic lesions (5 of 10; 50%) showed >10% of the cells with PCNA positivity. Overall, there was a good correlation between the mean proliferative activity and histological progression from normal (up to two layers of parabasal cells) to hyperplasia and ultimately metaplasia and dysplasia (see Fig. 1 and Fig. 2).

The distribution of PCNA expression at baseline and at 6 months was stratified by histology and plotted in Fig. 2. The trend from these plots indicated that PCNA expression increases in direct relation to histological progression. For the statistical analysis, metaplasia and dysplasia were combined because of the small number in the dysplastic group. The Kruskal-Wallis test revealed that the distribution of PCNA expression is significantly different among the histologically normal, hyperplastic, and metaplastic or dysplastic groups both at baseline and at 6 months (P < 0.001). Within the individual histological groups, no significant differences were observed in median PCNA expression in the different layers at baseline and at 6 months.

**Changes in PCNA Expression over 6 Months.** There were 250 pairs of samples evaluated at baseline and at 6 months for PCNA expression. Baseline PCNA versus 6-month PCNA was plotted in Fig. 3 for all of the layers combined, stratified by different histological change categories. From Fig. 3, we can see that among the sites in which histological change tended toward normal, PCNA expression generally decreased (P < 0.001; paired t test), and among the sites wherein histological change tended toward hyperplasia or metaplasia, PCNA expression increased (P < 0.001; paired t test). Thus, our hypothesis that increases in PCNA expression would be seen in those patients who demonstrated clear histological progression appears valid.

**Effects of Smoking Cessation on PCNA Expression.** PCNA changes between those who quit smoking during the trial (quitters, n = 16) and those who continued to smoke (nonquitters, n = 53) were compared using a 2-sample t test. A significant difference in PCNA expression was found in the basal layer (P = 0.004) and all of the layers (P = 0.006), but no significant difference was found in the parabasal layer (P = 0.87). Overall, subjects who ceased smoking were found to have more decrease in PCNA expression than those who continued to smoke. However, this overall decrease in PCNA expression was the reflection of metaplasia reversal after smoking cessation (see below).

**Effects of the 13-cRA Treatment on PCNA Expression.** When the subject was used as the analysis unit, the linear mixed effect model was applied with treatment, time, and their interaction as fixed effects and patient and site as random effects. The reduction in PCNA expression was highly significant (P = 0.0001) over time. However, the treatment effect and the treatment by time interaction were not significant, when the biopsy site was used as the analysis unit and the correlations between treatment and PCNA expression were examined within each histological category to control for changes in PCNA expression associated with metaplastic change. Compared with biopsy samples from placebo-treated subjects, biopsy samples from...
subjects treated with 13-cRA did not reveal significant changes in PCNA expression after 13-cRA treatment. However, when one adjusts for the baseline histology, there was a statistically significant difference in the reduction of PCNA expression between the 16 quitters and the 53 nonquitters by the intervention. By applying the ANOVA test, 13-cRA was more effective in reducing PCNA expression compared with the placebo arm. The reduction is statistically significantly larger in the 13-cRA group, both in the basal layer (\( P = 0.026 \)) and in all of the layers combined (\( P = 0.034 \); Fig. 4), especially in those samples showing metaplastic changes at baseline. These data fit well with our hypothesis that smoking is more likely to increase the labeling index in basal cells, whereas the parabasal layers may instead be more reflective of longer term changes that would impact on histological phenotype.

Baseline PCNA Expression as a Predictor of Response to 13-cRA. Focusing on the treatment group, we evaluated whether baseline PCNA levels were predictive of metaplasia reversal after 13-cRA treatment (Table 3). The percentage of metaplastic sites that reverted to normal was not significantly different in cases from low (\(<1\%\)) to high (\(\geq 10\%\)) baseline PCNA expression levels, indicating that baseline PCNA expression levels are not predictive of metaplasia reversal in response to 13-cRA treatment.

Relationship between PCNA and EGFr. PCNA expression and EGFr expression at baseline and at 6 months were analyzed by computing the Spearman’s rank correlation coefficient \( \rho \). The overall data revealed that PCNA and EGFr are positively correlated at baseline (\( \rho = 0.29; P < 0.001 \)).
and at 6 months ($p = 0.28; P < 0.001$). Stratified by histology, PCNA and EGFr correlated significantly in hyperplastic and metaplastic lesions (except in metaplastic sites at 6 months) but not in histologically normal tissue (data not shown). This correlation also holds true for the basal and parabasal levels of expression (data not shown). As noted previously by our group (28), EGFr expression, like PCNA, appeared to decrease with smoking cessation but mainly because of the reversal of squamous metaplasia after smoking cessation.

Regression Analysis of PCNA Expression Change Adjusted by Covariates. Regression analysis was performed using reduction in PCNA expression over 6 months as an outcome variable (Table 4). The covariates measured during the 6-month period included treatment arm (13-cRA or placebo), smoking cessation, EGFr change, and histological change. In the simple linear regression analysis, smoking cessation, EGFr change, and histological change were statistically significant predictors of reduced PCNA expression ($P = 0.006$, $P < 0.001$, and $P < 0.001$, respectively). Treatment arm
versus placebo) alone did not correlate with decreased PCNA expression \((P = 0.92)\). In the multiple regression analysis, histology change and EGFr continued to correlate with changes in PCNA expression. However, smoking cessation failed to correlate with reduced PCNA expression after adjustment for EGFr change and histological (metaplasia) change \((P = 0.12)\). The treatment by smoking cessation was not statistically significant \((P = 0.13)\) in multiple regression analysis, because of the small number of participants who ceased smoking while on the study.

### Discussion

This placebo-controlled, translational study in chronic smokers evaluated PCNA expression as a potential biomarker of squamous metaplasia and retinoid response. This large-scale study included the analysis of 706 bronchial biopsy samples obtained from individuals who participated in a prospective chemoprevention trial. The data show that, in smokers, the cellular proliferative activity increases with progressive histological changes in the bronchial epithelia. In a previous study of esophageal premalignancy by Yang et al. (30) and in a study of subjects at high risk for colon cancer by Lipkin et al. (31, 32), the patterns of expression of the proliferation marker tritiated thymidine were similar to the patterns of PCNA expression that we observed in the bronchial tissue.

Our data revealed that PCNA expression was increased in metaplastic lesions and that subsequent reversal of bronchial metaplasia correlated significantly with a reduction in PCNA expression. Our study showed that 13-cRA treatment in active smokers failed to reduce PCNA expression compared with that in the placebo group. However, our data suggested that 13-cRA was associated with a greater decrease in PCNA expression than was placebo among the small group of patients who did quit smoking. Furthermore, this study effectively clarified the relationships between PCNA expression, smoking status, squamous metaplasia, and EGFr expression.

Studies (33) of animal airways have demonstrated that airway surface epithelium is replaced very slowly. Normally, less than 0.5% of cells are actively dividing at any given time (34, 35). In the normal pathogen-free rat, the basal cells form about 70% of the dividing cell population (35). Although earlier
studies demonstrated that both basal cells and mucous cells were capable of cell division, many investigators concluded that the basal cell is the stem cell for airway epithelium. Like the germinal (reserve) cells of the epidermis, the basal cells were thought to divide to form new basal cells and “intermediate” cells that were also capable of cell division and differentiation into mucous cells and ciliated cells (36, 37).

In humans, only a few cells are actively proliferating in normal bronchial epithelium (38), and our study confirmed that there is limited proliferation in the bronchial epithelium of individuals who quit smoking. PCNA-positive cells increased progressively and statistically significantly in parallel with histological progression from normal to hyperplasia to metaplasia to dysplasia. Also, proliferative activity ranged widely within individual histological categories. This finding suggests that PCNA expression may be more sensitive than histological progression in prediction, related, e.g., to the retinoid or smoking cessation. Whether PCNA can add valuable data to histological assessments has not been confirmed. However, our data suggest that PCNA status may be a useful marker, especially in individuals who quit smoking. In our analysis, PCNA was significantly correlated with EGFr. This relationship was consistently present in our multivariate analysis, suggesting a relationship during histological progression between PCNA (a marker of proliferation) and EGFr expression.

EGFr expression has been shown to be increased in both metaplastic lesions (28) and squamous cell lung cancers (39). The involvement of the EGFr signaling pathway in lung carcinogenesis has been described by numerous authors (40–44). In addition to playing a role in the development and maintenance of the squamous phenotype, EGFr may also control the proliferative activity of these cells (44). The complex relationship between PCNA and EGFr, two potentially important biomarkers of epithelial carcinogenesis, has yet to be fully elucidated.

One notable incidental finding of our study is the reduction in PCNA expression detected in patients who quit smoking and were treated with the retinoid. Adjusting for baseline histology, the retinoid appeared to be demonstrably more effective in reducing PCNA expression in individuals who ceased smoking than placebo, in all of the layers (P = 0.054) and in the basal layer where proliferation appeared greatest (P = 0.026). Although the number of quitters was small in this study, the trend was quite striking. Therefore, PCNA expression appears to provide additional information to histological change in the evaluation of retinoid response in the setting of smoking cessation. This is particularly important because both the intergroup lung study (8) and the Euroscan study (9) showed no benefit to retinoid chemoprevention in active smokers. Focus in this field has now shifted to retinoid chemoprevention of former smokers. It should be noted that the relative risk of lung cancer incidence and overall mortality for former smokers was less than one in the β-Carotene and Retinol Efficacy Trial study (45, 46).

Our findings suggest that PCNA may be a useful biological marker in clinical trials of retinoids in individuals who cease smoking. Changes in PCNA expression may well bear investigating in former smokers (defined as those individuals who have quit for at least 1 year who participate in retinoid-based chemoprevention trials). It is these individuals that may well be the most appropriate target for chemoprevention trials. The clinical findings from this trial revealed that there is a significant association between bronchial metaplasia and PCNA expression. We also saw that both of these biomarkers, although prevalent in active smokers, can be reversed to a high degree with smoking cessation. Therefore, the reversibility of these markers in this trial demonstrates that only a subpopulation of premalignant histological changes persists after smoking cessation. This stable subpopulation of altered cells may thus represent premalignant foci within bronchial epithelium. Future chemoprevention trials should focus on the detection and treatment of these stable lesions in former smokers.

In summary, the data presented in this study suggest that although retinoid treatment did not alter overall PCNA expression in bronchial epithelium, it appeared to reduce its expression in individuals who ceased smoking. Along with a panel of cellular and molecular markers, including EGFr expression, loss of heterozygosity at specific chromosomal loci (47–49), and point mutations of specific tumor suppressor genes, PCNA may well play a role in helping clinical investigators better evaluate the degree of underlying genetic damage in the lung tissue of former smokers (50–54).

Acknowledgments

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References
