N-Acetylcysteine suppression of the proliferative index in the colon of patients with previous adenomatous colonic polyps

Richard D. Estensen\textsuperscript{a}, Michael Levy\textsuperscript{b}, Steven J. Klopp\textsuperscript{a}, Arthur R. Galbraith\textsuperscript{a}, Jack S. Mandel\textsuperscript{c}, Joyce A. Blomquist\textsuperscript{c}, Lee W. Wattenberg\textsuperscript{a,}\textsuperscript{*}

\textsuperscript{a}Department of Laboratory Medicine and Pathology, University of Minnesota, Lions Research Building, Rm. 183, 2001 6th Street, S.E., Minneapolis, MN 55455, USA

\textsuperscript{b}Park Nicollet Clinic, St. Louis Park, MN 55416, USA

\textsuperscript{c}Division of Environmental and Occupational Health, School of Public Health, University of Minnesota, Minneapolis, MN 55455, USA

Received 3 June 1999; received in revised form 21 July 1999; accepted 21 July 1999

Abstract

This investigation is part of an effort to develop chemoprevention for carcinogenesis of the large bowel. The agent investigated is N-acetylcysteine (NAC). We used as a predictive biomarker, the proliferative index (PI), in a short-term human study. Patients with previous adenomatous colonic polyps are a cohort with increased risk for colon cancer and an increased PI of colonic crypts. They were randomly assigned to an experimental group given 800 mg/day of NAC for 12 weeks or a placebo group. Using proliferative cell nuclear antigen immunostaining, the PI of colonic crypts was measured prior to and after the treatments. The PI of the NAC group was decreased significantly ($P < 0.02$) while the placebo group showed no difference ($P > 0.45$). Since this decrease in PI may be an indicator of decreased risk of colon cancer, more extensive studies of the potential of NAC as a chemopreventive agent for colon cancer appear warranted. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Chemoprevention; Carcinogenesis; Large bowel; Biomarker; Proliferative index; N-Acetylcysteine

1. Introduction

N-Acetylcysteine (NAC), long used both as a pharmaceutical mucolytic and in the treatment of acetaminophen overdose, has recently been shown to be an effective cancer chemopreventive agent in animal models of carcinogenesis when administered by diet [1–5]. NAC may prevent tumors by acting as a blocking agent, i.e. preventing initiation or promotion steps in carcinogenesis as a result of direct interaction with the carcinogen or its metabolic products [6,7]. NAC is also taken into the cell, is deacylated and incorporated into the glutathione synthetic cycle. By becoming intracellular glutathione, or by interacting directly with environmental carcinogens outside the cell or other cellular effects [7], NAC produces a decrease in the incidence of tumors in animal models of carcinogenesis of the lung [1], colon [2,3], and urinary bladder [4]. In studies of mouse skin, NAC added after initiation interferes with the promotion phase of multistage carcinogenesis [5]. The data from animal experiments have encouraged the study of NAC in human populations to determine its potential for decreasing lung tumors in smokers [8].

The objective of the present study was to assay the effects of NAC on the proliferative index (PI) of colo-
nic epithelial cells, a biomarker closely correlated with colon carcinogenesis. Using the effect on this biomarker in a short-term test, we hoped to find if the agent would be a candidate for longer term studies. The PI was determined by identifying dividing cells by immunostaining with proliferating cell nuclear antigen (PCNA). We chose patients with both an increased PI as well as an increased risk for colon cancer i.e. patients who have had an adenomatous polyp removed.

In the protocol employed, the patients were randomized into two virtually identical groups with regard to age and sex. All subjects had an initial rectal biopsy. Subsequently, one group was given a daily oral administration of NAC (experimental) or a placebo (control). They remained on these regimes for 3 months at which time the oral administrations were discontinued and a second rectal biopsy was performed. Both before entry into the study, and at the time of their last biopsy, patients filled out a Willett dietary history questionnaire and were evaluated for consumption of animal fat, vegetable fat, total fat, crude fiber, calcium, vitamin D, and folate.

The results of this study show that in patients who were practically identical as to age, sex, and diet, NAC significantly ($P < 0.02$) reduced the proliferative index of colonic epithelial cells.

2. Materials and methods

2.1. Capsule preparations

NAC, USP, in gelatin capsules, was obtained from Ogden Bioservices Corp. (Rockville, MD). Each capsule contained 200 mg NAC, 90.2 mg lactose NF, 80 mg cornstarch NF, and 1.8 mg ascorbic acid USP. Identical placebo capsules contained 290.2 mg lactose NF, 80 mg cornstarch NF, and 1.8 mg ascorbic acid USP. Capsules sufficient for 3 months of daily administration at a dose of 800 mg of NAC/day and a like number of placebo capsules were placed in coded vials prior to the study.

2.2. Study design

The outline of the study design can be seen in Fig. 1. Subjects for this study were volunteers who had a histologically confirmed adenomatous polyp removed. They were patients of the Park Nicollet Clinic. The selection of the subjects was based on a review of pathology reports and hospital charts within a year of their entry into the study. Excluded were patients with invasive cancer of the large bowel, those with familial polyposis, Gardner’s syndrome, any other malignancy, intestinal malabsorption syndromes, chronic pancreatitis, pancreatic insufficiency, active liver diseases, inflammatory bowel disease, and those on vegetarian or macrobiotic diets. All patients were English-speaking who could comprehend and comply with the study protocol. The age and sex of all subjects was recorded and each volunteer was given a Willett diet history questionnaire which they filled out prior to both the first and second biopsies. Subjects were randomized into two groups, Experimental (receiving NAC) and Control (receiving placebo). There were 34 subjects in the experimental group and 30 in the control group at the start of the study. The slight overloading of the experimental group was done to increase the likelihood of an adequate number finishing the trial in the event that withdrawals occurred due to adverse effects. Subjects were blinded as to whether they were receiving NAC or placebo. Likewise, those scoring the PI were blinded as to which group the specimen came from.
from. Patients were asked to return unused pills to the study coordinator at the time of the second biopsy. Compliance was estimated by counting unused pills. Compliance in the control group was 92.8% and in the experimental group 93.1%.

Patients were advised to report all adverse reactions to the study coordinator. Only one patient reported an adverse reaction that resulted in withdrawal from the study. This patient reported headaches associated with taking the pills. Medication was withdrawn and restarted. The headaches disappeared with withdrawal and recurred when the pill regimen was restarted. The code was broken for this patient who was taking NAC. This patient withdrew from the study. A second patient, who was in the control group, withdrew for non-medical reasons, leaving 33 patients in the experimental group and 29 patients in the control group.

2.3. Rectal biopsies

Rectal biopsies from three sites were obtained through a flexible sigmoidoscope from each subject prior to being placed on NAC or placebo and again within 12 h of the termination of their treatment 3 months later. Biopsies were performed without colon preparation.

2.4. Histology and immunostaining

Biopsies were immediately placed, mucosa side up, on filter papers and fixed in 70% methanol. The specimens, which usually measured 3–6 mm long, 1–2 mm wide, and no more than 1 mm thick, were dehydrated with absolute methanol and embedded in Paraplast-Xtra. Orientation of the biopsies was such that the axis of the crypts were parallel to the block face. Blocks from pre- and post-treatment biopsies were processed together. Sections were cut at 6 μm and immunostained for proliferating cell nuclear antigen as follows: slides were deparaffinized, rehydrated, and incubated at room temperature in a blocking reagent for 30 min. The blocking reagent was prepared by adding 15 μl horse serum (Vector Laboratories, Burlingame, CA) per ml of phosphate-buffered saline (PBS), pH 7.4 containing 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO). After draining off the blocking reagent, slides were incubated for 2 h at room temperature with an anti-PCNA reagent (Signet Laboratories, Dedham, MA, clone PC-10, IgG2a). The working reagent consisted of 20 μl of the primary antibody, 15 μl horse serum, and 965 μl PBS. Slides were gently rinsed with PBS and then incubated for 30 min with a biotinylated second-step antibody (Vector Laboratories, anti-mouse IgG, rat absorbed). The working reagent was made by adding 5 μl biotinylated antibody and 15 μl horse serum to 980 μl PBS. After incubation, this was rinsed off with PBS and the slides were incubated with the third-step ABC label (Vector Laboratories, Elite) for 30 min at room temperature. The slides were then rinsed with PBS and colorized for 6 min in 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB) solution (Aldrich Chemical Co., Milwaukee, WI). The working solution consisted of 28 mg DAB, 24 μl of 30% H2O2, and 70 ml PBS. After colorization, the slides were rinsed thoroughly with water, brightly counterstained with hematoxylin, dehydrated, and coverslipped with Permount (Fisher Chemical Co., Pittsburgh, PA).

2.5. Determination of PI

Fifty crypt columns, a crypt column defined as beginning at the cell in the center of the base of a colonic crypt to the cell at the top of the crypt, were counted for each patient. A crypt column usually contained about 50 cells. The number and position of each labeled cell was noted and recorded. Data for each patient was analyzed for total cells, labeled cells, and labeled cells for each of five equal crypt compartments beginning at the base (compartment 1) to the luminal surface (compartment 5). The number of cells per crypt column did not change in any group during this study.

2.6. Statistical analyses

Total percentage labeled cells within each group in pre-treatment biopsies and post-treatment biopsies were compared. The means were analyzed using Student’s t-test for paired samples. Additionally, we analyzed mean percentages of labeled cells in each of the five crypt compartments of all crypts within each group pre- and post-treatment. Finally, mean differences between groups were analyzed using Student’s two-sample t-test with unequal, or equal variances if appropriate. With respect to diet components, the two groups were compared by Wilcoxon’s rank test. P-
values were for the two-sided tests. A $P$-value of $< 0.05$ was considered indicative of a significant difference. In the absence of statistical significance the groups were loosely termed similar, though in some instances the power may not have been adequate.

3. Results

3.1. Compliance

Compliance in the control group was determined to be 312 pills/patient (92.8% pills taken) and in the experimental group, 313 pills/patient (93.1% pills taken), a virtually identical compliance rate.

3.2. Comparison of age, sex and diet of the experimental and control groups

Analysis of group composition showed that the experimental and control groups were similar with respect to age and sex. Additionally, groups did not differ significantly with regard to any category of diet either at the beginning or the end of the trial (results not shown).

3.3. Comparisons of total labeled cells

The data comparing the PI are shown in Table 1 which may be read from top to bottom or from left to right. To make comparisons of the mean percent labeled cells in the initial and the final biopsies within a group, read from top to bottom. The first vertical column of data shows that the mean percent of all cells labeled for the NAC group was 7.06 pre-treatment and 6.20 post-treatment. This decrease in the PI in the NAC treatment group is significant ($P < 0.02$). The second vertical column of data shows that the control group receiving the placebo had a labeling percentage of 6.34 pre-treatment and 6.70 post-treatment. A $P$-value of $> 0.45$ indicates that this increase is not significant.

Comparison between the pre-treatment NAC group (experimental) and the pre-treatment placebo (control) group can be seen on the top line of Table I moving from left to right. Note that the difference in the two pre-treatment groups is not significant ($P > 0.12$). A similar finding appears when comparing the post-treatment groups ($P > 0.27$). However, by comparing the pre- and post-treatment differences, another measure of a significant difference between the subjects receiving NAC and those given the placebo is noted ($P < 0.04$). Thus, while the mean proliferative indices at the beginning and at the end of the treatment were similar, the experimental group showed a significant difference with respect to the change in the PI.

3.4. Comparison of crypt compartments

Although all of the crypt compartments from NAC treated patients showed a post-treatment decrease in the mean percent of labeled cells (Table 2), only crypt compartment 4 demonstrated a significant decrease in the proliferative index, and this decrease was only borderline ($P < 0.05$). Possibly more patients would be necessary to show a significant change in the crypt

<table>
<thead>
<tr>
<th>Time of biopsy</th>
<th>Experimental group $^a$ (% labeled cells)</th>
<th>Control group $^b$ (% labeled cells)</th>
<th>Mean difference</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment</td>
<td>7.06 (1.78)$^c$</td>
<td>6.34 (1.86)</td>
<td>0.72</td>
<td>$&gt; 0.12$</td>
</tr>
<tr>
<td>Median</td>
<td>6.89</td>
<td>5.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-treatment</td>
<td>6.20 (1.56)</td>
<td>6.70 (1.97)</td>
<td>$- 0.50$</td>
<td>$&gt; 0.27$</td>
</tr>
<tr>
<td>Median</td>
<td>5.93</td>
<td>6.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean difference (pre – post)</td>
<td>$- 0.87$ (2.06)</td>
<td>$0.36$ (2.52)</td>
<td>1.23</td>
<td>$&lt; 0.04$</td>
</tr>
</tbody>
</table>

$^a n = 33$.
$^b n = 29$.
$^c$ Values are mean (standard deviation).

Table 1
Comparison of pre-treatment and post-treatment total proliferative index in experimental and control groups
compartments. Patients who received the placebo demonstrated no significant difference pre- and post-treatment.

4. Discussion

NAC produces a lowering of the proliferative index in the colonic crypt epithelium of human volunteers who previously had adenomatous polyps. This population is at increased risk for both developing a second polyp or adenocarcinoma of the colon [9]. The decrease of the proliferative index in trials of similar size has been utilized by others as an indication for further investigation of the chemopreventive potential of an agent in larger numbers of patients with either a second polyp or the appearance of adenocarcinoma as the endpoint [10]. NAC is now being investigated in a clinical trial in progress using prevention of the occurrence of colorectal polyps in subjects who had previous polyps removed endoscopically [11]. The relationship of the data obtained in that study to the use of the PI as a predictive biomarker in the present investigation will be of interest. NAC is also being studied in patients in Euroscan trials [8] to determine whether or not it can decrease the numbers of lung tumors. This cohort could be examined to see if patients receiving NAC have lower numbers of colon tumors, either polyps or carcinomas. If so, it would be an additional positive factor for undertaking additional studies of the capacity of NAC to prevent cancer of the large bowel.

In our study there was a single mild adverse effect (headache) associated with taking NAC and no serious toxic reactions. This is consonant with the broad experience with NAC as a pharmaceutical agent [8]. The lack of side effects [8] and possible efficacy, as indicated in this study, make NAC a good choice for further investigation as a chemopreventive agent in human colon cancer.

Acknowledgements

Our thanks to those who were volunteers for these studies, without whose unselfish cooperation the data obtained could not exist. Special thanks to Liz Kingsbury for her preparation and editing of the manuscript. This work was supported by Grant EDT-41B from the American Cancer Society and Grant 93-46 from the Methodist Hospital Foundation.

References


