Short Communication

Lack of Effect of Daily *N*-Acetylcysteine Supplementation on Mutagen Sensitivity[1]

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Abstract

The European Organization for Research and Treatment of Cancer multicenter Euroscan trial was set up to prevent the occurrence of second primary tumors in the upper aerodigestive and respiratory tract in patients cured for early stage head and neck squamous cell carcinoma. One randomized group of patients receive daily *N*-acetylcysteine, an antioxidant that may be protective especially in the early steps of carcinogenesis. Mutagen sensitivity, measured as sensitivity to bleomycin in peripheral blood lymphocytes, has been found to be increased in head and neck squamous cell carcinoma and is hypothesized to reflect cancer susceptibility. The aim of this study was to investigate whether mutagen sensitivity is influenced by oral *N*-acetylcysteine supplementation and can therefore be used as intermediate end point in chemoprevention. Patients ($n = 19$) who had various periods of *N*-acetylcysteine supplementation (600 mg daily for 3–9 months) were analyzed. In addition, a patient group ($n = 14$) that did not receive *N*-acetylcysteine supplementation was analyzed for comparison. Our results show no evidence that administration of *N*-acetylcysteine did influence the mutagen sensitivity level. The only explanatory variable in the analysis of the difference between two samples of one person was the b/c value of the first measurement. Moreover, the variability in these repeated measurements (coefficient of variation of 14%) indicates that additional studies should be performed to minimize this variability and to optimize the testing of mutagen sensitivity to accurately identify individual patients at high risk for the development of multiple primary tumors.

Introduction

During the last decade, the survival of HNSCC[4] has only marginally increased, despite better treatment modalities (1). One of the reasons for this unchanged survival is the occurrence of MPT in the respiratory and upper digestive tract. The development of MPT, which occur at a constant rate of ~3% per year for the first five years, is a major clinical concern (2).

To explain the development of MPT, the concept of field cancerization has been suggested. The whole mucosa of the upper aerodigestive tract has been exposed to carcinogenic agents, and when one tumor is diagnosed, the whole area is at increased risk to develop more primary tumors (3, 4). To prevent or delay the process of carcinogenesis after the treatment of the first primary tumor, synthetic and natural compounds are administered to the patients in so-called chemoprevention trials. One example is the Euroscan trial, a cooperative multicenter European Organization for Research and Treatment of Cancer trial (5), in which curatively treated patients are randomized with early stage HNSCC. A 2 × 2 factorial design is used to test two agents; to achieve this, the patients were randomized in four groups: (*a*) 300,000 IU retinyl palmitate (vitamin A) daily during 1 year, and half of this dose during the second year; (*b*) 600 mg NAC daily for 2 years; (*c*) both retinyl palmitate and NAC; and (*d*) no treatment. End points of Euroscan are the number and time of occurrence of second primary tumors in the respiratory and upper digestive tract, local/regional recurrences and distant metastases, as well as long-term survival. The randomization of patients is finished, and more than 2500 patients have been entered in the trial. Within a few years, the results of Euroscan will be known.

The identification of biological markers that predict or monitor the development of a tumor is important because these markers will shorten the length of a trial and thereby increase the efficiency of chemoprevention trials. The benefit of the drug can be monitored before the end point of the trial (*e.g.*, the occurrence of a second primary tumor) is reached. Another factor that will improve the efficiency of chemoprevention trials is the selection of patients at the highest risk. This will render a high number of cancer incidences over a relatively smaller time interval (6). Besides these improvements of the trials, the fact that for these high-risk subjects side effects of the chemopreventive agents are more easily justified is also important.

An endogenous risk factor (7) for HNSCC patients who are at high risk to develop MPT is mutagen sensitivity (8, 9). This risk factor reflects chromosomal instability after the induction of damage with a clastogenic agent (bleomycin). A hypersensitive phenotype as measured with this assay, in com-

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[4] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; MPT, multiple primary tumors; NAC, *N*-acetylcysteine; b/c, breaks per cell, mean value in 100 metaphases.

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bination with exposure to carcinogenic agents such as cigarette smoke, drastically increases the risk for HNSCC (10).

Although mutagen sensitivity has been proposed to be constitutional (7), some authors have suggested that it can be used to monitor the efficacy of chemopreventive agents (11, 12). When this latter finding holds true, then mutagen sensitivity may be used as an intermediate end point for chemoprevention studies. This can be studied in the group of patients from the Euroscan trial who are supplemented with NAC, a precursor of glutathione. This compound interferes mainly at the level of detoxification in the early steps of carcinogenesis due to its antioxidant properties. Administration of NAC increases the concentration of glutathione in the plasma of the patients (13). If the patients would be mutagen hypersensitive because of an underlying deficiency in free radical detoxification, the treatment of NAC could protect against this sensitivity. On the other hand, if NAC would not have an effect on mutagen sensitivity, this would be in line with the hypothesis that mutagen sensitivity is constitutional. The aim of this study was to monitor whether the mutagen sensitivity is influenced by daily NAC supplementation.

Materials and Methods

Subjects. Blood samples were analyzed on mutagen sensitivity before and after NAC supplementation of 19 patients who were curatively treated for a primary tumor in the mucosa of the upper aerodigestive tract. These patients were treated with an oral dose of 600 mg of NAC daily and were only a subset of the total number of patients that have entered Euroscan from The University Hospital, Utrecht and The University Hospital, *Vrije Universiteit*, Amsterdam. From the period the mutagen sensitivity assay was validated in our laboratory until the completing of Euroscan, these 19 patients were the last randomized to receive NAC in the trial. For comparison, a group of 14 patients was analyzed who were treated for their tumor but who were not randomized in the Euroscan trial and did not receive NAC supplementation. The number of smoked pack years was calculated for each subject as the number of years during which was smoked, multiplied by the number of cigarette packs smoked daily (assuming that one pack contains 25 cigarettes). A similar calculation was used for the estimation of alcohol drinking history, in which one unit is defined as one standard glass of alcoholic beverage consumed daily, assuming that one glass of beer or liquor contains a similar amount of alcohol (± 15 ml).

Mutagen Sensitivity. The assay was performed as described previously (14). In short, one-half ml heparinized whole blood was cultured for 72 h in 4.5 ml of RPMI 1640 (Flow Laboratories, Irvine, UK) with 2 mM L-glutamine (Life Technologies, Inc., Paisley, UK) supplemented with 15% FCS (Flow Laboratories), 1.5% phytohemagglutinin (Wellcome Diagnostics, Dartford, UK), 100 IU/ml penicillin and streptomycin (ICN Biomedicals Ltd., Irvine, UK). For each subject, duplicate cultures were used. Bleomycin (Lundbeck, Copenhagen, Denmark) was added 5 h before cell harvest, ensuring that damage induced in the late S- and G_2 phase of the cell cycle could be evaluated at metaphase. Cells were arrested in metaphase by adding 100 μl, 50 μg/ml Colcemid (Sigma, St. Louis, MO) 1 h before harvesting. The cells were collected by centrifugation (300 × *g*, 5 min) and treated with a hypotonic solution (0.06 M KCl) for 20 min. For fixation and washing (×3) of the cells, Carnoy's fixative (methanol/glacial acetic acid, 3:1, v/v) was used. Fixed cells were dropped on wet slides. After air drying, slides were stained with 5% Giemsa solution (Merck, Darmstadt, Germany), coded, and scored under a light microscope at ×1250. Duplicate cultures of each patient were performed at each time point, and spread slides were prepared of each culture metaphase. From each coded slide, 50 metaphases were evaluated for the presence of chromatid breaks. So, at each time point, the b/c value is based on the scoring of 100 metaphases.

Statistical Analysis. Differences between repeated measurements of b/c values were calculated using the paired Student's *t* test. Differences between patient groups were estimated using the two-sample Student's *t* test. The relation between variables was analyzed with the Pearson correlation coefficient. ANOVA was performed to estimate the influence of variables on the difference between the first and the second measurement. The coefficient of variation was calculated for each person as the SD divided by the mean b/c value of the two measurements × 100%. Reported here are the means of the coefficients of variation for both patient populations.

Results

The two patient populations did not differ in mean b/c levels and age (Table 1). The interval time between the two measurements was significantly longer in the nontreated group compared to the NAC-treated group ($P < 0.01$), even when the interval for data censured at 12 months were fixed at 12. No correlations were found between the differences between repeated measurement and age, interval time, cumulative smoking, and alcohol use.

There was no difference between repeated measurements in both patient groups as measured with a paired Student's *t* test. The mean difference between the two measurements was 0.004 b/c (95% confidence interval; −0.145 to 0.153) for the NAC-supplemented patients and −0.06 b/c for the control patients (95% confidence interval; −0.260 to 0.130). The present data did not provide evidence for a mutagen sensitivity modulating effect by NAC administration.

A view of the variation in b/c values of the individual patients is visualized in Fig. 1. The coefficients of variation for the NAC-supplemented group and for the control patient group were 14.4 and 14%, respectively. This variation in b/c level between the two measurements could not be explained by the NAC intake as tested with the Student's two-sample *t* test. There was a significant correlation between first and second measurement ($r = 0.5$, $P < 0.01$). The relation of the two measurements with their difference can be explained by mathematical necessity (regression to the mean). This implies that, due to the clear interaction between the difference between two measurements and the value of the first measurement, this latter variable has to be included as covariate in the further ANOVA. This resulted in the finding that no other variable influences the difference between repeated measurements.

Discussion

In this study, the only significant interaction with the difference between repeated measurements was the actual b/c value of a person. This can be explained partly by "regression to the mean." However, whether other influences also play a role in this phenomenon requires further investigation in larger patient groups.

The implication of the present finding is that the intake of 600 mg of NAC daily does not influence the mutagen sensitivity, which is in contrast from what was expected from *in vitro* studies in which protective effects were reported on the interaction between bleomycin and NAC (11). However, the con-

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Table 1 Patient characteristics

NAC patient	Age	Smoking[a]	Alcohol[b]	Tumor site	Treatment	Interval[c]	Difference[d]
1	73	3	3	Oral cav	Surgery	9	−0.54
2	44	2	3	Oral cav	Surgery	6	−0.52
3	51	3	2	Oral cav	Surgery + RT[e]	3	−0.45
4	71	1	1	Larynx	RT	4	−0.07
5	68	3	2	Larynx	Surgery	3	0.39
6	69			Larynx	RT	2	0.46
7	65	3		Larynx	RT	5	0.07
8	72	3	2	Larynx	RT	3	0.09
9	57	3	1	Larynx	RT + sur + chemo	2.5	0.36
10	59	3	3	Oral cav	Surgery	4	−0.06
11	71	2	1	Larynx	RT	2	0.15
12	61	3	1	Larynx	Surgery	4	−0.11
13	39	1	1	Oral cav	Surgery + RT	4	−0.02
14	61	2		Larynx	RT	2.5	0.26
15	49	3	3	Oral cav	RT	7	0.19
16	65	3	2	Larynx	RT	9	0.13
17	57	2	2	Larynx	RT	3	0.02
18	69	2	3	Pharynx	Surgery	4	0.22
19	78	1	3	Oral cav	Surgery + RT	3	−0.49
Patients without NAC intervention							
1	38	2	2	Pharynx	Surgery + RT	≥12	0.2
2	74	3	3	Oral cav	Surgery	≥12	0.33
3	79	2	2	Larynx	Surgery	≥12	−0.7
4	59	3	3	Larynx	Surgery + RT	≥12	−0.29
5	66			Pharynx	Surgery + RT	≥12	−0.49
6	51	2	3	Pharynx	Surgery + RT	≥12	0.28
7	64			Pharynx	Surgery + RT	≥12	0.27
8	69	1	1	Oral cav	Surgery	≥12	−0.21
9	57	3	2	Pharynx	Surgery	≥12	−0.08
10	62	1	1	Oral cav	Surgery + RT	≥12	0.41
11	49	2	2	Oral cav	Surgery	0.5	−0.04
12	67	3	3	Pharynx	Surgery	0.5	−0.21
13	77	1	2	Oesophagus	Sur + RT + chemo	1	−0.35
14	45	2	2	Larynx	Surgery + RT	0.5	0.09

[a] Cumulative tobacco smoking was assessed as pack years. 1 = non smoker; 2 = <30 pack years; 3 = ≥30 pack years.
[b] Cumulative drinking was assessed as unit years. 1 = nondrinker; 2 = <100 unit years; 3 = ≥100 unit years.
[c] Interval = months between first and second measurements.
[d] Difference between b/c value of the first and second measurement.
[e] RT, radio therapy.

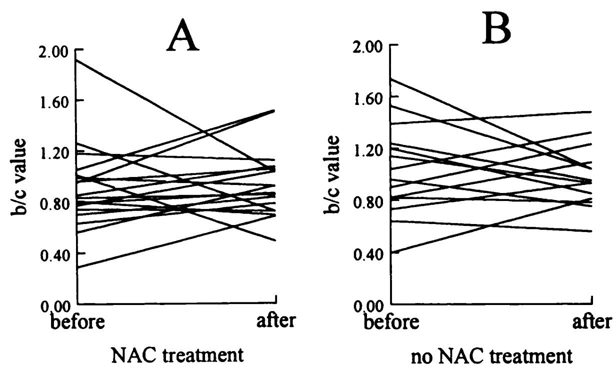

Fig. 1. The individual b/c levels are plotted of the first and the second measurement. *A* represents patients who obtained NAC supplementation, and *B* represents control patients without NAC supplementation.

centrations used in those experiments (10 mM NAC) may not be reached in the *in vivo* situation for which peak plasma levels of ±12 μM NAC were reported (15). Another reason for the lack of influence of NAC intake may be that during the 3 days of culturing the lymphocytes in the mutagen sensitivity assay, the influence of NAC is diluted. The plasma is diluted 10 times in the culture medium, and thiols can be metabolized at the time the bleomycin is added. Fortunately, the potentiative effect that NAC can directly have on bleomycin-induced DNA damage (16) was also not found in the present study. Besides antioxidant properties, NAC can also show pro-oxidant properties that potentiate the DNA-damaging effects of bleomycin. Moreover, it has become clear in the last few years that supplementation of antioxidants does not always exert the desired protective effect, and even in patient trials, the opposite effects have been described (17).

The results of this study, in addition, show us to be careful regarding the identification of high-risk individuals based on one mutagen sensitivity measurement. In practice, the repeated measurements of patients for risk estimation is not easily applicable. However, for those patients who are in a b/c area between 0.7 and 1.0, a second measurement may be worthwhile to prevent false stratification. More specific analysis of repeated sampling is necessary to substantiate this postulation, and has to be one of our main goals for future investigations on the concept of individualized high-risk identification using this mutagen sensitivity assay.

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The nonmodifiable character of mutagen sensitivity is in line with our earlier postulation that mutagen sensitivity is a constitutional factor. We could analyze only this small sample size (associated with the unique subset of patients), resulting in a power not exceeding 0.6. However, this study indicated that NAC does not exert a substantial protective effect on the mutagen sensitivity. This does, of course, not imply that mutagen sensitivity and/or NAC supplementation are not important factors for the occurrence of second primary tumors. This answer can become available only when the results of the Euroscan trial are known, which will at least be several years from now.

We hypothesize that mutagen sensitivity is a biomarker that cannot be modulated by external factors such as smoking, alcohol, or nutrients. The genetic trait of mutagen sensitivity has become more clear by this study because supplementation of NAC was not associated with large changes in mutagen sensitivity. Therefore, we conclude that mutagen sensitivity cannot be used to monitor the efficacy of NAC supplementation in protecting against carcinogenesis.

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