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Plasma and Esophageal Mucosal Levels of Vitamin C: Role in the Pathogenesis and Neoplastic Progression of Barrett's Esophagus

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Antioxidants may protect against the development of esophageal adenocarcinoma. Blood samples and endoscopic biopsies (squamous, Barrett's, and gastric mucosa) were obtained from 48 Barrett's esophagus (BE) patients, while 48 age- and sex-matched controls provided blood samples only. Plasma concentrations of vitamins A, C, and E were measured in all subjects, while vitamin C was measured in relation to the type of mucosa. Plasma total vitamin C level, but not vitamin A or E, was lower in BE patients compared to controls ($P = 0.014$). Tissue levels of total vitamin C were significantly lower in Barrett's compared with squamous mucosa ($P = 0.047$). A positive association was observed between plasma vitamin C and dietary intake of vitamin C, while there was an inverse association with alcohol consumption. The lower levels of vitamin C in plasma of BE patients and in Barrett's mucosa compared with squamous mucosa are consistent with oxidative stress being of importance in the pathogenesis and neoplastic progression of BE.

KEY WORDS: Barrett's esophagus; vitamin C; gastroesophageal reflux disease; oxidative stress.

Barrett's esophagus (BE), a condition where the normal squamous epithelium of the lower esophagus is replaced by a metaplastic columnar epithelium, complicates 3 to 5% of cases of gastroesophageal reflux disease (GERD) and is associated with an incidence of the order of 0.5 to 1% per annum of adenocarcinoma of the esophagus (1). The increase that has been observed in the incidence of esophageal adenocarcinoma in the last 30 years in the West (2) indicates that environmental factors are involved in the etiology of this tumour. In this context, the role of diet in BE and esophageal adenocarcinoma has been examined. Case–control studies have shown positive relationships with the intake of fat and red meat, while inverse associations have been identified with the consumption of fruit, vegetables, and fiber (3–6). Furthermore, it has been suggested that micronutrients such as the antioxidant vitamin C may have a protective role against the development of esophageal adenocarcinoma (7, 8).

Antioxidant vitamins, including vitamins A, C, and E, act as scavengers of reactive oxygen species, which are thought to contribute to the development of a variety of cancers, particularly those associated with chronic inflammation (9, 10). Reactive oxygen species have also been implicated in the pathogenesis of reflux esophagitis and BE. Correlations have been reported between markers of oxidative stress and the presence of esophagitis and its complications both in humans (11–13) and in animal models (14, 15).

Despite the evidence implicating oxidative mechanisms in reflux esophagitis and linking antioxidant nutrients with esophageal adenocarcinoma, little is known about the antioxidant vitamin levels in the plasma, but particularly esophageal mucosa, of patients with BE. The aim of this

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study was to measure dietary intake, plasma levels, and esophageal and gastric mucosal concentrations of vitamin C and plasma levels of vitamins A and E in a group of patients with histologically confirmed specialized intestinal metaplasia. Plasma concentrations in BE patients were compared with those of age- and sex-matched controls and the relationships among tissue, plasma, and dietary levels of vitamin C were explored in the patient group. Possible associations with clinical, pathological, and demographic characteristics of the patients were also examined.

MATERIALS AND METHODS

Subjects and Samples. The study protocol was approved by the local ethical committee and written informed consent was obtained from all subjects that participated. A total of 57 subjects were identified over a period of 12 months among patients undergoing endoscopic surveillance for BE at the Leeds General Infirmary. Children, frail elderly patients, mentally impaired patients, and patients on anticoagulants were excluded. Eligibility for inclusion was dependent on the presence of endoscopic proximal extension of columnar mucosa, which was confirmed as Barrett's specialized epithelium, i.e., with intestinal metaplasia, on histology of the esophageal biopsies obtained when patients were recruited to the current study. Among the 57 BE patients initially identified, a total of 48 met the above histological entry criterion, while for the other 9 patients, histology revealed only gastric-type metaplasia in the columnar segment.

Each case with histologically confirmed intestinal metaplasia ($n = 48$) was individually matched by age (± 5 years) and sex to one control selected from patients undergoing day case surgical procedures during the same period. Endoscopies or pH monitoring studies were not performed on the control group. Patients taking acid-suppressing therapy were excluded from being a control, as were subjects with symptoms suggestive of GERD, as determined from a detailed medical history. Patients with known chronic inflammatory conditions such as inflammatory bowel disease or rheumatoid arthritis and patients on vitamin supplements were also excluded. While we cannot exclude that a small number of controls had undiagnosed esophagitis or BE, the prevalence would be expected to be low in this population ($<5\%$) and would therefore be unlikely to compromise the comparison with BE patients. Regular medication and smoking and drinking habits were recorded in BE patients and control subjects.

Patients with BE were asked to stop acid-suppressing drugs 2 weeks before their endoscopy according to a standard protocol. All endoscopies were performed in one consultant's (I.G.M.) weekly endoscopic sessions. Following a thorough examination of the esophagus, stomach, and duodenum, the length and appearance of the columnar segment were recorded. Four-quadrant biopsies were obtained at 2-cm intervals from throughout the columnar lined esophagus using standard biopsy forceps and the specimens were processed for histology. Additional biopsies, one from the middle part of the columnar epithelium and one from either adjacent squamous epithelium or proximal gastric mucosa, were collected for the measurement of tissue ascorbic acid and total vitamin C. The specimens were immediately frozen in liquid nitrogen and stored until further analysis.

Prior to endoscopy (patients with BE) or surgical procedure (controls), a blood sample was collected in a Li-heparin tube. All samples were obtained in the afternoon with the patient fasting for 6–8 hr. following a light breakfast. Plasma was separated immediately and divided into two 0.5-ml aliquots. For ascorbic acid measurement 1 ml of 2% metaphosphoric acid (MPA) was added to the first plasma sample, and for total vitamin C measurement 1 ml of 2% MPA and 9 mg of dithiothreitol (DTT) was added to the other sample. MPA precipitates protein and stabilizes the ascorbic acid, while DTT reduces dehydroascorbic acid to ascorbic acid and enables measurement of total vitamin C (ascorbic acid and dehydroascorbic acid). All samples were stored at -80°C until further analysis.

Vitamin Analysis. Tissue samples were thawed, blotted dry, weighed, and homogenized in 0.5 to 1 ml of 2% MPA. Each sample was divided into two parts and DTT was added to one part to a final concentration of 6 mg/ml. Tissue and plasma levels of ascorbic acid and total vitamin C were measured as previously described (16, 17) by reversed-phase HPLC with electrochemical detection. The HPLC system consisted of a Waters LKB pump, a Gilson 231/401 autosampler, and a Waters 4μ Nova-Pak C_{18} cartridge (8×100 mm) connected to an EG & G electrochemical detector. Plasma levels of vitamins A and E were measured by the method of Thurnham *et al.* (18) with a minor modification, hexane being used for the extraction instead of heptane. All chemicals were obtained from Sigma Chemical Co. Plasma vitamin levels are expressed as micromoles per liter and mucosal levels are expressed as micromoles per kilogram of wet weight tissue.

Dietary Assessment. Patients with BE were invited for interviews that were conducted by one of the authors (A.F.) using a standard questionnaire. The height and weight of each patient were measured and the body:mass index (BMI) was calculated. Detailed information was obtained on sociodemographic factors, occupation, medical and drug history, and use of alcohol and tobacco. Dietary assessment was made using a previously evaluated food frequency questionnaire, which consists of a list of 219 food items and additional questions on typical portion size and cooking methods (19). Patients were asked to recall their frequency of consumption (i.e., times per day, week, or month) of specific food items or groups of similar food items over the previous 12 months. Any dietary changes since the diagnosis of their BE were recorded. Nutrient intakes were calculated based on portion weight and nutrient composition according to previously published values and the subject's frequency of consumption of each food item.

Statistical Analysis. The Kolmogorov–Smirnov goodness-of-fit test was used to test the data for normality of distribution. As some of the data were skewed, nonparametric tests were used for all univariate and bivariate analyses. Comparisons of plasma antioxidants between cases and controls were performed with the Wilcoxon signed ranks test for paired data. Although Barrett's mucosal samples were collected from all patients in the study group, only squamous esophageal or gastric mucosal samples were available from each patient for comparison. Therefore, tissue antioxidant data from all patients were pooled together in their respective groups and analysis of variance was made with the Kruskal–Wallis test for multiple independent samples. Comparisons between pairs of groups were made using the Mann–Whitney U test. Correlation coefficients were calculated by the nonparametric Spearman rank test.

As the concentration of vitamin C in Barrett's mucosa is likely to be influenced by a number of interrelated factors, stepwise multiple linear regression analysis was performed to verify the associations among tissue levels, plasma levels, and dietary intake of vitamin C while controlling for the effects of other variables. Two models were constructed. Plasma concentration of vitamin C was used as the dependent variable in the first model and analyzed against the independent variables of age, sex, dietary intake of vitamin C, length of Barrett's segment, BMI, cigarette smoking, and alcohol use. Mucosal vitamin C was entered into the second model as the dependent variable and plasma vitamin C was added to the independent variables listed above. Continuous variables containing heavily skewed data were incorporated into the linear models by log-transforming them or by creating dichotomous variables. Statistical significance of <0.1 was used for the inclusion of a variable in the model and a value of <0.15 was used for removal of a variable that was already entered.

The threshold of statistical significance was set at 0.05 and all tests were two-tailed. Statistical analysis was carried out using SPSS 8.0 for Windows (SPSS Inc., USA).

RESULTS

Thirty-five male and 13 female patients with BE were recruited (median age, 61 years; range, 35 to 75 years). The median length of the columnar segment was 5 cm (range, 1 to 12 cm). Three of the BE patients had histological evidence of low- or high-grade dysplasia in addition to specialized intestinal metaplasia. There were no cases of esophageal adenocarcinoma in this patient series.

Plasma Antioxidants. Within the group of BE patients, lower levels of plasma ascorbic acid were found in males compared to females (median, 42.75 versus 51.87 μmol/L, respectively) but the difference was not statistically significant (Mann–Whitney, $P = 0.14$). There were also no significant differences in plasma ascorbic acid or total vitamin C between smokers and nonsmokers (Mann–Whitney, $P > 0.05$) or any correlation with age, length of Barrett's segment, or BMI (Spearman, $P > 0.05$). However, two of the three subjects with intestinal metaplasia and dysplasia did have particularly low levels of ascorbic acid (46.74, 19.95, and 11.40 μmol/L) and total vitamin C (51.87, 23.37, and 14.25 μmol/L).

BE patients were individually matched on age and sex to a control group selected from patients undergoing minor or intermediate surgical procedures (day cases). The two groups were similar with respect to smoking habits, alcohol intake, and use of nonsteroidal antiinflammatory drugs (Table 1). Plasma levels of both ascorbic acid and total vitamin C were significantly lower in BE patients compared with controls (Wilcoxon, $P = 0.013$ and $P = 0.014$, respectively; Table 2). In contrast, there were no significant differences between cases and controls in the plasma levels of vitamins A and E (Table 2).

TABLE 1. BASELINE CHARACTERISTICS OF PATIENTS WITH BARRETT'S ESOPHAGUS (CASES) AND MATCHED CONTROLS

	Cases	*Controls*
Number	48	48
Sex (male/female)	35/13	35/13
Mean age (years)	61	61
Current smokers*	7	11
Alcohol intake >15 units†/week*	12	9
Use of NSAIDs*	10	10

Note. NSAIDs, nonsteroidal antiinflammatory drugs.
*Number of patients.
†"Unit" of alcohol refers to 10 ml of pure alcohol, typically 1.5 units for half a pint of beer or a glass of wine.

Tissue Ascorbic Acid and Total Vitamin C. The concentrations of ascorbic acid and total vitamin C in squamous, Barrett's, and gastric epithelium are summarized in Table 3. Analysis of variance revealed significant differences among the three mucosal types for both ascorbic acid and total vitamin C (Kruskal–Wallis, $P = 0.044$ and $P = 0.021$, respectively). Post hoc comparisons between pairs of groups showed decreases of approximately 20% in both ascorbic acid and total vitamin C in Barrett's compared to squamous mucosa (Mann–Whitney, $P = 0.071$ and $P = 0.047$, respectively). The differences between Barrett's and gastric mucosa were not significant.

No significant differences were seen between men and women or between smokers and nonsmokers in Barrett's mucosal levels of ascorbic acid and total vitamin C (Mann–Whitney, $P > 0.05$) and there were no significant correlations with age, length of Barrett's segment, BMI, or alcohol intake (Spearman, $P > 0.05$).

Correlations Between Various Estimates of Vitamin C. We examined correlations between dietary vitamin C intake and plasma and Barrett's mucosal vitamin C levels. The estimates of daily dietary vitamin C intakes in the BE patients were similar in men (mean, 123.4 ± 63.4 mg) and women (136.2 ± 63.3 mg). There was a strong correlation between Barrett's mucosal levels of total vitamin C and ascorbic acid ($r = 0.87$, $P < 0.001$). A positive correlation was also found between total vitamin C level in plasma and Barrett's mucosa ($P = 0.017$). There was a weak positive correlation between plasma vitamin C and estimates of dietary intake of vitamin C (Table 4).

Table 5 summarizes the results of multiple linear regression analysis. Plasma vitamin C was again positively associated with dietary intake of vitamin C but negatively associated with alcohol intake. Plasma vitamin C was the only independent predictor of mucosal vitamin C while controlling for the effects of possible confounding variables. Similar results were obtained when ascorbic acid was incorporated into the models as the dependent variable (data not shown).

TABLE 2. PLASMA ANTIOXIDANT LEVELS IN PATIENTS WITH BARRETT'S ESOPHAGUS (CASES) AND MATCHED COTROLS ($n = 48$)

	Cases	*Controls*	*Paired difference*	P *value**
Ascorbic acid	43.89 (26.22 to 58.43)	58.43 (34.49 to 76.38)	17.96 (−8.27 to 32.49)	0.013
Vitamin C	47.31 (31.92 to 64.13)	59.57 (36.77 to 83.05)	15.96 (−5.24 to 31.92)	0.014
Vitamin A	2.40 (2.20 to 2.60)	2.23 (1.92 to 2.57)	−0.15 (−0.50 to 0.40)	>0.05
Vitamin E	33.55 (28.80 to 39.90)	34.90 (31.02 to 40.77)	2.20 (−8.30 to 8.65)	>0.05

Note. Values represent medians, with interquartile ranges in parentheses (μmol/L).
*Wilcoxon signed-rank test.

DISCUSSION

In recent years, considerable attention has focused on oxidative mechanisms in carcinogenesis (20, 21) and the protective role of dietary antioxidants (9, 22). Previously little or no information has been available on the plasma and tissue concentrations of antioxidant vitamins in BE patients. Here we report lower plasma levels of ascorbic acid and total vitamin C in BE patients with specialized intestinal metaplasia compared with matched controls. In addition, lower levels of vitamin C were found in Barrett's mucosa compared to squamous mucosa in the BE patients. Both these observations suggest that there may be impaired antioxidant status in BE patients.

The observation of lower plasma vitamin C levels in BE patients compared to the control group has a number of possible explanations. It is important to note that it is unlikely to be explained by variations in smoking habits, alcohol consumption, or use of nonsteroidal antiinflammatory drugs (factors that may influence oxidant activity) because the two groups were similar in these respects. In addition, BE patients refrained from taking acid-suppressing drugs 2 weeks prior to the study and control subjects were only included if not taking this type of drug. This is important because it is possible that proton pump inhibitors could influence vitamin C levels (23). One possible reason for the lower plasma vitamin C level in the BE patients is lower dietary intake of vitamin C. There was a weak correlation between dietary intakes of vitamin C and plasma levels, consistent with previous reports (24). Data supporting the hypothesis of lower intakes in BE patients come from preliminary results of a study of diet, BE, and esophageal adenocarcinoma (25). However, the fact that other plasma vitamin levels did not differ between BE patients and controls in our study tends to contradict the idea of a general deficiency in dietary vitamin intake. In addition, the estimated mean intake of vitamin C (127.2 ± 62.9 mg) in the BE patients does not appear to be low for the U.K. population (26). Alternative explanations for the observed differences in plasma vitamin C levels include the possibility that those individuals in the population with a reduced ability for absorption of vitamin C are predisposed to develop BE or that the presence of BE affects plasma vitamin C levels, perhaps through altered absorption or turnover. However, we cannot draw conclusions regarding these possibilities in the current study. In addition, we included a relatively small number of subjects (48 cases, 48 controls) and therefore larger studies with more extensive consideration of factors which may modulate vitamin C intake, absorption, or metabolism are merited.

TABLE 3. TISSUE LEVELS OF ASCORBIC ACID AND TOTAL VITAMIN C IN RELATION TO BIOPSY SITE

	Ascorbic acid	*Total vitamin C*
Squamous	600.78 (445.17–722.76) $n = 23$	664.05 (512.83–852.43) $n = 24$
Barrett's	495.90 (393.30–577.98) $n = 47$	553.18 (460.67–658.06)* $n = 48$
Gastric	386.74 (293.66–546.63) $n = 22$	473.67(388.45–583.79) $n = 22$

Note. Values represent medians, with interquartile ranges in parentheses (μmol/kg).
*Significantly different from squamous mucosa (see text).

One suggested mechanism by which (lack of) vitamin C is involved in the pathogenesis of BE is by modifying the response of esophageal cells to the noxious effects of refluxed acid and bile. We did not examine whether the lower plasma vitamin C levels in BE patients were reflected by lower esophageal mucosal levels compared to control subjects in this study. However, it is significant that the ascorbic acid and total vitamin C levels in the Barrett's mucosa were reduced compared to squamous epithelium. The association of BE with reflux esophagitis is well established (27) and a mixed inflammatory cell infiltrate, composed primarily of neutrophils and lymphocytes, can be a feature of the metaplastic mucosa (28). Inflammatory cells produce a battery of reactive oxygen species that can inflict oxidative DNA damage (29). Indeed, increased

TABLE 4. CORRELATIONS AMONG VARIOUS ESTIMATES OF VITAMIN C

	Barrett's mucosal ascorbic acid	*Barrett's mucosal total vitamin C*	*Plasma total vitamin C*	*Dietary vitamin C*
Barrett's mucosal ascorbic acid	$r = 1$ — $n = 47$	$r = 0.87$ $P < 0.001$ $n = 47$	$r = 0.25$ $P = 0.086$ $n = 47$	$r = -0.04$ $P = 0.769$ $n = 46$
Barrett's mucosal total vitamin C		$r = 1$ — $n = 48$	$r = 0.34$ $P = 0.017$ $n = 48$	$r = 0.09$ $P = 0.528$ $n = 47$
Plasma total vitamin C			$r = 1$ — $n = 48$	$r = 0.25$ $P = 0.097$ $n = 47$
Dietary vitamin C				$r = 1$ — $n = 47$

Note. r, correlation coefficient; P, probability value; n, sample size.

production of oxygen-derived free redicals and oxidative damage have been reported in studies of reflux esophagitis and BE (11, 13). As vitamin C is a potent quencher of free radicals in vivo (30), an increased rate of consumption in redox reactions, combined perhaps with insufficient replenishment due to low plasma levels, would explain the reduced concentration of vitamin C seen in Barrett's mucosa. Decreased levels of mucosal vitamin C in the presence of ongoing reflux and inflammation would render Barrett's mucosa more susceptible to oxidative DNA damage, mutations in key cell cycle and growth regulatory genes, and ultimately malignant transformation (29, 30). It should be noted that vitamin C levels in Barretts mucosa are intermediate between those of esophageal squamous mucosa and gastric mucosa (Table 3). This may indicate that columnar-type epithelium per se has lower levels than squamous mucosa and that this altered differentiation thus results in esophageal cells with lower antioxidant capacity.

Interestingly, male patients with BE had a tendency to lower plasma levels of ascorbic acid and total vitamin C compared with females ($P = 0.07$). This observation is of potential relevance to the strong male predominance seen in esophageal adenocarcinoma. It is noteworthy that men, but not women, with low plasma levels of ascorbic had a significantly higher risk of dying from cancer of all sites (31). The observed negative association of vitamin C with consumption of more than 15 units of alcohol per week was independent of dietary intake of vitamin C and may be due to an effect of alcohol on the absorption and metabolism of vitamin C. In contrast with previous studies (32), no significant difference in plasma vitamin C was seen between smokers and nonsmokers. This most likely reflects the limited statistical power of the study, given that only 7 of 48 patients in this sample were smokers.

TABLE 5. MULTIPLE LINEAR REGRESSION ANALYSIS OF PLASMA VITAMIN C AND BARRETT'S MUCOSAL VITAMIN C

Variable	*Regression coefficient (β)*	*Standard error*	P *value*
Plasma vitamin C*			
Dietary vitamin C	2.32	1.19	0.05
Alcohol intake	−3.01	1.36	0.03
Mucosal vitamin C†			
Plasma vitamin C	2.58	1.16	0.03

*Dependent variable—model I. The analysis was adjusted for age (in 10-year categories), male sex (yes/no), vitamin C intake (mg/day), body mass index (kg/m^2), cigarette smoking (yes/no), alcohol intake >15 units per week (yes/no), and length of Barrett's segment (cm).
†Dependent variable—model II. Plasma vitamin C (μmol/L) was added to the independent variables listed above.

In conclusion, we have demonstrated lower plasma levels of ascorbic acid and total vitamin C in BE patients compared with controls. In addition, in BE patients the metaplastic epithelium contains lower levels of vitamin C compared to squamous esophageal mucosa. These results are consistent with a role for oxidative mechanisms in the pathogenesis of BE and progression to adenocarcinoma, perhaps by modulating levels of DNA damage in esophageal mucosa. This is further supported by recent observations of higher DNA damage levels in BE tissue compared to matched squamous epithelium from the same patient (33). The data provide a plausible explanation for epidemiological evidence showing a strong association between low consumption of fruit and vegetables and increased risk of esophageal adenocarcinoma. Furthermore, a mechanistic understanding of this association contributes to a scientific rationale for prevention studies in BE patients.

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