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Neutrophil Elastase/α_1-Proteinase Inhibitor Complex Levels Decrease in Plasma of Cystic Fibrosis Patients during Long-Term Oral β-Carotene Supplementation[1]

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ABSTRACT

Lung inflammation in cystic fibrosis (CF) is associated with an increased release from activated neutrophils of oxidants and proteinases. Free radical generation is not efficiently neutralized, and the major anti-proteinase, α_1-proteinase inhibitor (α_1-PI) is thought to be oxidatively inactivated. We hypothesized that enhanced antioxidant protection could represent an additional long-term strategy to attentuate the host inflammatory response. The effect on plasma neutrophil elastase/α_1-PI (NE/α_1-PI) complex levels (as a marker of lung inflammation) and plasma malondialdehyde concentrations (as a marker of lipid peroxidation) of additional oral β-carotene supplementation was studied in 33 CF patients who had already received long-term vitamin E supplementation. In the presence of a more than 10-fold increase in plasma β-carotene concentrations (mean ± SEM) (0.09 ± 0.01 to 1.07 ± 0.19 μmol/L; $p < 0.0001$), a small increase in plasma α-tocopherol concentrations (23.8 ± 1.31 to 28.4 ± 1.81 μmol/L; $p = 0.02$), and a more than 50% decrease in plasma malondialdehyde concentrations (1.00 ± 0.07 to 0.46 ± 0.03 μmol/L; $p < 0.0001$), plasma NE/α_1-PI complex levels decreased from 102.2 ± 16.0 to 83.0 ± 10.4 μg/L; ($p = 0.02$). Plasma retinol concentrations increased (1.05 ± 0.06 to 1.23 ± 0.07 μmol/L; $p = 0.0001$) due to conversion of β-carotene to retinol, which could have contributed to the decrease in NE/α_1-PI complex levels. Based on these results, we speculate that efficient antioxidant supplementation could attenuate lung inflammation in CF. (***Pediatr Res* 40: 130–134, 1996**)

Abbreviations

α_1-PI, α_1-proteinase inhibitor
CF, cystic fibrosis
ELF, epithelial lining fluid
MDA, malondialdehyde
NE, neutrophil elastase
TNF-α, tumor necrosis factor-α

Lung disease in CF is characterized by chronic bacterial infection and a profound influx of neutrophils into the lower respiratory tract. Autoinjury from the host inflammatory response is supposed to play an even more important role in disease progression than the total bacterial burden (1). Uninhibited elastase released from activated neutrophils is thought to significantly contribute to the destruction of lung connective tissue (2–4). However, NE exerts a number of additional actions (5–8), such as induction of IL-8 gene expression (which further augments neutrophil recruitment) and goblet cell hyperplasia, enhancement of secretions by submucosal glands, and impairment of ciliary beating and complement-dependent phagocytosis of pathogens, all of which could contribute to the severity of CF lung disease.

There is evidence of a proteinase-antiproteinase imbalance in favor of the former in CF airway secretions. Activated neutrophils release high amounts of NE and oxidants via NADPH or myeloperoxidase (9). Even though considerable quantities of the major antiproteinase α_1-PI are present in ELF and sputum of CF patients, only a small proportion is complexed to NE, and uninhibited NE activity is still detected (2–4), suggesting that α_1-PI is not fully functioning (3, 4, 10). Both proteolytic and oxidative inactivation of α_1-PI have been discussed as underlying mechanisms (3, 11–13). α_1-PI is easily oxidized at its methionyl residue, which reduces or completely abolishes its binding capacity (14, 15).

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NE/α_1-PI complex levels in peripheral blood are elevated in CF patients with pulmonary exacerbations and have been proposed as an early and sensitive marker of airway inflammation in CF (16, 17). Large amounts of active NE are found in sputum and bronchoalveolar lavage fluid even after i.v. antibiotic treatment, and plasma levels of NE/α_1-PI complexes are not corrected to normal (4, 18) and increase rapidly after cessation of antibiotic therapy (4). Thus, additional strategies to depress pulmonary inflammation long-term may be of significant importance.

Due to an increased release of oxidants from activated neutrophils on one hand and combined antioxidant deficiencies, primarily due to not fully corrected fat malabsorption, on the other, CF patients exhibit an oxidant-antioxidant imbalance in favor of the former (19). As a result, excess lipid peroxidation occurs (20, 21), as documented by increased plasma concentrations of MDA (21), an end product of oxidation of unsaturated fatty acids with three or more double bonds (22). Normalization of the impaired β-carotene status of vitamin E-sufficient CF patients decreased lipid peroxidation to levels of healthy subjects (21).

We hypothesized that long-term supplementation with β-carotene in vitamin E-sufficient CF patients will not only decrease lipid peroxidation, but also reduce the oxidant burden and protect α_1-PI against oxidative inactivation. In this observational study, we investigated the effect of 16 mo of β-carotene supplementation on lipid peroxide and NE/α_1-PI complex levels in peripheral blood of vitamin E-sufficient CF patients.

METHODS

Subjects. Thirty-three patients (16 male, 17 female), ages 2.3–30.5 y (median, 9.1 y), under long-term care at the CF outpatient clinic of the Department of Pediatrics, University of Zurich, with plasma β-carotene concentrations below mean − 2 SD of controls of the same age range and enrolled in a 16-mo oral β-carotene supplementation study (23) were investigated. All but three patients had achieved plasma α-tocopherol concentrations >15.9 μmol/L (mean − 2 SD of Swiss population) (24) due to long-term oral α-tocopherol supplementation for at least 1 y before this study. Three patients received oral retinyl palmitate supplements (Arovit, Hoffmann La Roche Ltd.) and nine patients a multivitamin preparation (Protovit, Hoffmann La Roche Ltd.) on a long-term basis. These treatments were not changed during the study period. Thirteen patients were chronically infected with *Pseudomonas aeruginosa*. Only one patient required i.v. antibiotic treatment at one of the two evaluations; it was started after blood was drawn for the study. Continuous antibiotic therapy by aerosol or orally in 10 patients was not changed during the study period, and six additional patients received antibiotics orally either at baseline or at 16 mo. Shwachman scores (25) were 88 ± 11 at study entry and 83 ± 11 at 16 mo, indicating a fairly stable disease status. Patients were in good nutritional status compared with Swiss reference values (26) (z scores; median; minimum, maximum) (weight, −0.43; −2.8, 1.24; height, −0.28; −2.73, 1.86; upper arm circumference, −0.85; −2.31, 2.01).

Clinically healthy nonsmoking staff members (11 male, 23 female), ages 19.9–42.8 y (median, 30.5 y), of the same hospital volunteered for this study, as it was ethically not acceptable to draw the necessary amounts of blood from healthy children.

During the study period, patients continued to take oral vitamin E supplements, either RRR-α-tocopherol (Multaben, Roche Pharma Schweiz Ltd., Reinach, Switzerland) or all-rac-α-tocopheryl acetate (Ephynal, Hoffmann La Roche Ltd., Basel, Switzerland) without changes in dose (330 ± 120 IU) and preparation. After the baseline evaluation, patients received 0.5 mg/kg of body wt of β-carotene orally in a single daily dose at breakfast (BellaCarotin, 3M Medica, Ltd., Borken, Germany). Details of dose and preparation have been described previously (21).

The study was approved by the Ethics Committee of the Department of Pediatrics, University of Zurich, and informed consent was obtained from the patients or their parents as well as from healthy controls.

Plasma antioxidants. After an overnight fast, blood was drawn in sterile plastic tubes containing 1.6 mg/mL K-EDTA (Sarstedt Monovette, Nümbrecht, Germany), protected from light by aluminum foil and centrifuged immediately at 2000 × g for 8 min. Plasma was stored at −20°C for a maximum of 4 d before determination of plasma α-tocopherol, β-carotene, and lycopene by HPLC (27). Plasma α-tocopherol to cholesterol ratios were calculated to correct for differences in cholesterol levels between patients and healthy controls.

Plasma lipid peroxides. Another aliquot of EDTA plasma was kept at −70°C until determination of the MDA-thiobarbituric acid adduct by HPLC (28). Coefficients of variation of repeated MDA-thiobarbituric acid assays in our laboratory were 4.4% (within run) and 6.9% (from run to run), respectively (21). For four patients blood was not available for MDA determination either at baseline or at 16 mo.

Plasma NE/α_1-PI complexes. NE/α_1-PI complex levels were measured in plasma as described elsewhere (29), using an ELISA kit specific for these complexes (MERCK, Darmstadt, Germany, 2h version). Absorbance at 405 nm was recorded spectrophotometrically in an ELISA reader from Bio-Rad, Hercules, CA, and compared with values from known standards. The coefficient of variation of repeated measurements was 8.6%.

Plasma retinol concentrations were determined by HPLC (27). Indexes of inflammation (α_1-acid glycoprotein, white blood cell count, bands) and nutritional status (albumin and cholesterol concentrations) were determined by routine methods. The proinflammatory cytokine TNF-α levels were determined with a TNF-α-ELISA kit from Endogen, Boston, MA.

Statistical analysis. Due to nonconformity of the data with the normality assumption, Wilcoxon matched pairs signed ranks tests were used to compare study variables in patients at baseline and 16 mo, and Mann-Whitney U tests for comparisons between patients and controls. After log transformation, Pearson correlation was applied to study the relationship between different variables. Statgraphics Version 6 (STSC, Inc., Rockville, MD) was used for all statistical procedures. Differ-

ences were considered significant at $p < 0.05$. All results are expressed as mean ± SD unless otherwise stated.

RESULTS

Plasma antioxidants. Baseline plasma β-carotene concentrations were significantly lower in patients compared with healthy controls; they were frequently close to the detection limit. After 16 mo of β-carotene supplementation they increased significantly ($p < 0.0001$) and did no longer differ from controls (Table 1). Plasma β-carotene concentrations did not increase in one patient due to liver involvement and in five additional patients due to poor compliance. Baseline plasma α-tocopherol concentrations and plasma α-tocopherol to cholesterol ratios were well within the normal range due to long-term oral vitamin E supplementation before study entry. During the observation period, a further increase in plasma α-tocopherol concentrations ($p = 0.02$) was observed (Table 1), even though neither the preparation nor the dosage prescribed for vitamin E supplementation had been changed. Possible explanations for this increase include improvement of compliance or a sparing effect of β-carotene on α-tocopherol. Plasma α-tocopherol to cholesterol ratios increased accordingly (7.27 ± 2.29 to 8.69 ± 2.17 mmol/mol; $p = 0.009$); at 16 mo they were higher in patients than in controls (5.73 ± 0.63; $p < 0.0001$). Plasma lycopene concentrations were very low in patients (0.07 ± 0.07 μmol/L) throughout the study (controls, 0.57 ± 0.19 μmol/L). Plasma albumin concentrations at study entry (38.7 ± 3.7 g/L) were well within the normal range and did not change.

Lipid peroxides. Baseline plasma MDA concentrations were elevated in patients compared with controls (Table 1). They decreased significantly ($p < 0.0001$) and were lower in patients compared with controls at 16 mo ($p = 0.004$). Multiple regression analysis revealed that changes in MDA concentrations (−0.57 ± 0.37 μmol/L) were related ($R = 0.56$) to changes in plasma α-tocopherol to cholesterol ratios ($p = 0.003$), but not to changes in plasma β-carotene concentrations ($p = 0.10$).

NE/α_1-PI complexes. Baseline plasma NE/α_1-PI complex levels were more than twice as high compared with controls ($p < 0.0001$) (Table 1). At baseline, multiple regression analysis showed a negative correlation ($R = 0.42$) between plasma NE/α_1-PI complex levels (log-transformed) and both plasma α-tocopherol ($p = 0.05$) and retinol concentrations ($p = 0.03$), but not with β-carotene concentrations (log-transformed) ($p = 0.06$). There was a tendency of higher plasma NE/α_1-PI complex levels in patients with lower Shwachman scores, but the correlation did not reach statistical significance ($r = -0.31$, $p = 0.08$). During β-carotene supplementation, plasma NE/α_1-PI complex levels decreased significantly ($p = 0.02$), but values remained elevated compared with those of healthy controls ($p = 0.0003$) (Table 1). Multiple regression analysis did not show a correlation between the changes from baseline to 16 mo in NE/α_1-PI complex levels and the changes in plasma β-carotene, α-tocopherol, and retinol concentrations.

Additional investigations. Plasma α_1-PI concentrations (2.63 ± 0.60 g/L) were well within the normal range (2–4 g/L). Plasma retinol concentrations increased ($p = 0.0001$) (Table 1), but remained lower than in controls ($p < 0.0001$). Commonly used indexes of inflammation (white blood cells, bands, α_1-acid glycoprotein) did not show any changes (Table 1). TNF-α levels were below or close to the detection limit in 17 patients at baseline and 24 patients at 16 mo; they were >6.3 ng/L, the upper limit of normal, in 10 patients at baseline, decreased to normal in nine of these, and were elevated in four additional patients at 16 mo. These changes did not correlate with changes in NE/α_1-PI complex levels. Patients were in a good nutritional status; they exhibited significant weight gain during the study period ($p = 0.005$) (Table 1).

DISCUSSION

Plasma NE/α_1-PI complex levels have been proposed as an early and sensitive marker for monitoring pulmonary inflammation in CF patients (16, 17). They correlate with bronchoalveolar lavage fluid neutrophil counts (4) and, in infants and small children with CF, also with bronchoalveolar lavage NE/α_1-PI complex levels (16). This study shows a decrease in elevated plasma NE/α_1-PI complex levels during efficient oral β-carotene supplementation in vitamin E-sufficient CF patients.

Table 1. *Changes in NE/α_1-PI complexes, MDA and antioxidant concentrations, and weight gain during β-carotene supplementation*

	Patients ($n = 33$)			Controls ($n = 34$)	
Test	Baseline	16 months	Baseline *vs* 16 mo* *P* value		Patients at 16 mo *vs* controls† *P* value
NE/α_1-PI (μg/L)	102.2 ± 16.0	83.0 ± 10.4	0.02	45.6 ± 3.2	0.0003
MDA (μmol/L)	1.01 ± 0.07	0.46 ± 0.03	<0.0001	0.61 ± 0.04	0.004
α-Tocopherol (μmol/L)	23.8 ± 1.3	28.4 ± 1.8	0.02	28.7 ± 1.1	NS
β-Carotene (μmol/L)	0.09 ± 0.01	1.07 ± 0.19	<0.0001	1.02 ± 0.07	NS
Retinol (μmol/L)	1.05 ± 0.06	1.23 ± 0.07	0.0001	1.99 ± 0.09	<0.0001
Weight (*z* score)	−0.55 ± 0.16	−0.39 ± 0.17	0.005	n.d.	
White blood cells (10^9/L)	8.47 ± 0.52	8.52 ± 0.57	NS	n.d.	
Bands (%)	21.1 ± 1.9	22.1 ± 1.7	NS	n.d.	
α_1-Acid glycoprotein (g/L)	1.06 ± 0.06	1.02 ± 0.06	NS	n.d.	

Values are mean ± SEM. NS = not significant; n.d. = not determined.
* Wilcoxon matched pairs signed ranks tests.
† Mann-Whitney *U* tests.

Plasma β-carotene concentrations reached values comparable to those in healthy subjects, whereas a concomitant increase in plasma α-tocopherol during the study period led to plasma α-tocopherol concentrations similar to, and plasma α-tocopherol to cholesterol ratios higher than, those of healthy controls. In addition, plasma retinol concentrations increased significantly, most likely due to β-carotene conversion to retinol (30), but remained lower in patients compared with controls. This was accompanied by a more than 50% decrease in plasma MDA concentrations, resulting in values comparable to those in healthy subjects; this decrease correlated with the additional increase in plasma α-tocopherol to cholesterol ratios, starting from values that were already normal, but not with the increase in plasma β-carotene concentrations. Simultaneously, a statistically significant decrease in plasma NE/α_1-PI complex levels was observed. As a possible underlying mechanism we propose that β-carotene and α-tocopherol were efficiently detoxifying oxidants released from activated neutrophils in airway secretions to less reactive forms (9, 31). In this context, we hypothesize that they interrupted the chain reaction of ongoing lipid peroxidation and provided enhanced protection of α_1-PI against oxidative inactivation. As a result, NE could have been more efficiently neutralized in ELF by formation of NE/α_1-PI complexes, and NE-mediated propagation of inflammation could have been attentuated. However, because this study focused on plasma only, the mechanisms leading to elevated plasma levels of NE/α_1-PI complexes and to the decrease during the study period remain speculative.

Baseline plasma NE/α_1-PI complex levels were related to plasma vitamin E and retinol concentrations, suggesting an impact on inflammation also of the vitamin A status in the study patients. Vitamin A has long been known to affect immune functions (32), and recent *in vitro* experiments (33) show that retinol exerts an inhibition of superoxide anion release from activated neutrophils. In our study, conversion of β-carotene to retinol, not only in plasma but perhaps also in ELF, might have contributed to a decrease in the oxidant burden and, subsequently, to attenuation of lung inflammation.

The decrease in NE/α_1-PI complex levels occurred in the absence of a dose-response relationship with the increase in plasma β-carotene, α-tocopherol, and retinol concentrations. Data on antioxidant and retinol concentrations in ELF or on the cellular level were not obtained in this study. There is evidence that α-tocopherol levels in ELF can be augmented by oral vitamin E supplementation, and the oxidized metabolite, α-tocopherol quinone, is present in ELF fluid (31, 34). So far, equivalant data on β-carotene and retinol levels are not available. Besides between-subject differences in the amounts of antioxidants reaching the site of inflammation (compared with plasma concentrations), additional and more complex interactions between different antioxidants, availability of lipids as the oxidizable substrate, and between-subject variability in lung inflammation itself may account for this lack of correlation.

During i.v. antibiotic treatment, plasma NE/α_1-PI complex levels in CF patients decrease by up to 50% (4, 17, 18), but remain higher than in healthy subjects (4, 17) and increase rapidly after cessation of antibiotic therapy in clinically stable patients (4). There is evidence of significant inflammation in the airways of CF patients with clinically mild lung disease (35). Thus, long-term intervention to reduce inflammation might be beneficial even in patients without symptoms of acute exacerbations.

In this study, we investigated a relatively homogeneous patient population in stable clinical condition and with almost one third of patients on continuous oral antibiotic therapy. They were outpatients and, with few exceptions, free of acute pulmonary exacerbations. Patients were also efficiently supplemented with oral vitamin E before study entry, leading to plasma α-tocopherol concentrations similar to those in healthy subjects. The plasma NE/α_1-PI complex levels in our study population were considerably lower than in other studies of CF patients (4, 18, 36), but comparable to values for stable CF patients followed at yearly control visits (17). Conceivably, these relatively low values reflect the mild and stable pulmonary disease status of these patients maintained on efficient antioxidant (oral vitamin E) and antibiotic therapy. Even in these patients with relatively mild disease, a significant reduction in NE/α_1-PI complex levels was achieved with additional β-carotene supplementation.

Commonly used indexes of inflammation did not correlate with NE/α_1-PI complex levels, which is in line with other studies (16, 17), probably because NE/α_1-PI complex levels represent a more specific and sensitive marker of pulmonary inflammation. None of the standard indexes of inflammation changed during the study period. TNF-α levels were elevated only in a small proportion of patients, both at baseline and at 16 mo. Perhaps due to normal values in most patients, we were unable to confirm the correlation between plasma NE/α_1-PI complex and TNF-α levels observed in another group of CF patients (36).

In conclusion, elevated NE/α_1-PI complex levels in plasma of clinically stable CF patients decreased during efficient antioxidant supplementation, as did plasma lipid peroxide levels. Because this was an open, uncontrolled observational study that was limited to plasma levels, a controlled clinical trial, focusing on the effects of β-carotene supplementation on free NE, NE/α_1-PI complexes, antioxidants, and lipid peroxides, both in plasma and bronchoalveolar lavage fluid, should now be conducted.

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