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Ascorbic acid may protect against human gastric cancer by scavenging mucosal oxygen radicals

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High dietary ascorbic acid intake appears to protect against gastric cancer. This may be due to its action as a scavenger of reactive radical species formed in the gastric mucosa, resulting in a reduced level of radical-mediated DNA damage. We have studied 82 patients, of whom 37 had *Helicobacter pylori*-associated gastritis, a condition which predisposes to gastric cancer. Using electron paramagnetic resonance (EPR) spectroscopy we have demonstrated, for the first time, that ascorbyl radicals are generated in human gastric mucosa, presumably as a result of scavenging of free radicals by ascorbic acid. Quantification of ascorbyl radicals demonstrates that there is a higher concentration in those patients with *H.pylori* gastritis compared with subjects with normal histology ($P < 0.01$). We also found gastric mucosal luminol-enhanced chemiluminescence and malondialdehyde concentrations (which are believed to be markers of radical generation and tissue damage) to be higher in patients with *H.pylori* gastritis compared with those with normal histology ($P < 0.001$ and $P < 0.01$ respectively). The observed concentrations of the ascorbyl radical correlate with the level of luminol-enhanced chemiluminescence ($r = 0.41, P < 0.001$), but not with malondialdehyde concentrations ($r = 0.08$, $P = 0.47$). Mucosal ascorbic acid and total vitamin C concentrations did not vary between histological groups, nor did they correlate with mucosal levels of the ascorbyl radical, chemiluminescence or malondialdehyde. These data suggest that ascorbic acid is acting as a scavenger of free radicals generated in human gastric mucosa. The experiments therefore provide direct supportive evidence for the hypothesis that ascorbic acid protects against gastric cancer by scavenging reactive radical species which would otherwise react with DNA, with resultant genetic damage.

Introduction

Helicobacter pylori infection is an established risk factor for gastric cancer (1–3) and the organism has recently been classified as a group I carcinogen by an IARC working group (4). The mechanism by which this bacterium predisposes to gastric cancer is unclear, but may involve the reaction of reactive oxygen species (ROS*) generated in the inflammatory response with DNA and consequent genetic damage. ROS may also promote carcinogenesis by inducing proto-oncogene expression (5,6), by causing generation of genotoxic products like 8-hydroxynonenal (7) or malondialdehyde (8) or by converting procarcinogens to carcinogens (9,10). ROS production is increased in association with *H.pylori* both *in vitro* (11,12) and *in vivo* (13,14) and ROS are known to be capable of causing a variety of DNA lesions (15,16,17), as well as mutations (18,19,20) and malignant transformation of cultured cell lines (21).

Epidemiological studies have demonstrated that dietary ascorbic acid appears to protect against gastric cancer (22,23) and it has been suggested that this may be due to the action of this molecule as a highly effective scavenger of ROS (24,25). The role of ascorbic acid as an anti-oxidant is a complex one, however, and under some conditions it may even act as a pro-oxidant. This may occur when ferric ions are present, since ascorbic acid can reduce Fe(III) to Fe(II), which can in turn promote oxidation through a Fenton reaction. *In vitro* work has shown that ascorbic acid and ferric ions together can produce intense oxidation of polyunsaturated fatty acids (26). The relationship between ascorbic acid and ROS therefore deserves further study.

Many ROS are free radicals and when ascorbic acid scavenges such moieties the ascorbyl radical is produced. In this study we have investigated the free radical scavenging role of ascorbic acid in *H.pylori*-associated gastritis by measuring mucosal levels of the ascorbyl radical in human subjects using electron paramagnetic resonance (EPR) spectroscopy. The concentrations of this species have been compared in subjects with normal histology and those with reactive gastritis. We have also attempted to relate the concentrations of the ascorbyl radical to mucosal chemiluminescence and malondialdehyde levels, two commonly used, but relatively non-specific, parameters of ROS activity and damage, as well as to mucosal concentrations of ascorbic acid and total vitamin C (ascorbic acid + dehydroascorbic acid).

Materials and methods

Subjects were recruited from patients undergoing upper gastrointestinal tract endoscopy for dyspepsia. Local ethics committee approval was granted and written informed consent obtained in all cases.

At endoscopy two antral and two corpus biopsies were taken for histological examination using haematoxylin and eosin staining, as well as a modified Giemsa stain for *H.pylori*. Two antral biopsies were also taken for *H.pylori* culture and urease testing. Further antral biopsies were taken for malondialdehyde equivalent estimation, luminol-enhanced chemiluminescence, ascorbic acid and total vitamin C estimation and ascorbyl radical measurements.

Malondialdehyde equivalent estimation

Biopsies were stored at −70°C and assayed by a modification of the procedure of Yagi (27) within 2 weeks of collection; previous preliminary experiments (not shown) had shown that malondialdehyde levels were stable for at least 2 weeks under these conditions. After thawing each sample was weighed and added to 4 ml water. One millilitre of thiobarbituric acid solution, made by dissolving 0.167 g thiobarbituric acid in a mixture of 25 ml water, 25 ml glacial acetic acid, was then added. A set of malondialdehyde standards was also freshly prepared and added to 1 ml thiobarbituric acid solution in the

*Abbreviations: ROS, reactive oxygen species; EPR, electron paramagnetic resonance.

same way. After mixing all samples and standards were heated at 100°C for 1 h. Samples and standards were cooled on ice and the malondialdehyde equivalents extracted by adding 5 ml butan-1-ol to each. Each tube was centrifuged at 3000 r.p.m. for 10 min to separate the aqueous and butan-1-ol phases and the fluorescence of the butan-1-ol phase at 555 nm was then recorded using an excitation wavelength of 515 nm; values were compared with those obtained from malondialdehyde standards.

Luminol-enhanced chemiluminescence measurement

Biopsies were stored in phosphate-buffered saline and assayed within 3 h. After weighing each biopsy was added to a pre-counted scintillation vial containing 1 ml 75 μM luminol solution and counted for 5 min in a liquid scintillation counter set in the 'out of coincidence' mode. Three measurements of each sample were made and the mean calculated in each case.

Ascorbic acid and total vitamin C measurements

Biopsies were stored in liquid nitrogen and assayed within 2 weeks. After thawing the samples were blotted dry, then weighed. They were homogenized in 0.5–1.0 ml 2% metaphosphoric acid and divided into two parts. Dithiothreitol was added to one part to a final concentration of 6 mg/ml for total vitamin C estimation. Both parts were centrifuged and the supernatant solution analysed by HPLC using reversed phase ion pair chromatography on a C18 column (28). Ascorbic acid was selectively measured using an electrochemical detector set at 350 mV. Total vitamin C was estimated by incubating the sample containing dithiothreitol at 45°C for 120 min prior to analysis by HPLC.

Ascorbyl radical measurements

Biopsies were placed in liquid nitrogen immediately after endoscopy. They were subsequently thawed, weighed and homogenized in 0.5 ml normal saline using a glass hand-held homogenizer, before being stored in liquid nitrogen until analysed by EPR spectroscopy. Samples for analysis by EPR were thawed at room temperature, then inserted into a standard aqueous EPR sample cell. EPR spectra were recorded at room temperature using a Bruker ESP 300 (X-band) spectrometer equipped with 100 kHz modulation and a Bruker ER035M Gaussmeter for field calibration. Hyperfine coupling constants were measured directly from the field scan. Relative radical concentrations were determined from peak-to-trough line heights of signals from spectra recorded using identical spectrometer settings and conditions.

Statistical analysis

The Mann–Whitney test was used to compare different groups and correlations were calculated using the Spearman–Rank test.

Results

Data were obtained from a total of 82 patients, of whom 37 had *H.pylori*-associated chronic gastritis, 16 reactive (or chemical) gastritis and 29 normal histology. The median age of the patients in each of these three groups was 49, 41 and 31 years respectively.

Malondialdehyde equivalents

There were significantly greater concentrations of malondialdehyde equivalents in the mucosa of patients with *H.pylori*-associated gastritis compared with those with normal histology ($P < 0.01$), but no difference between patients with reactive gastritis and those with normal histology ($P = 0.32$) (Table I).

Luminol-enhanced chemiluminescence

Mucosal levels of luminol-enhanced chemiluminescence were higher in patients with *H.pylori*-associated gastritis compared with those with normal histology ($P < 0.001$), but there was no significant difference between patients with reactive gastritis and those with normal histology ($P = 0.67$) (Table I).

Ascorbic acid and total vitamin C levels

There were no significant differences between concentrations of mucosal ascorbic acid, mucosal total vitamin C or the ratio mucosal ascorbic acid:total vitamin C between any of the three histological groups (Table II). Nor was there any correlation between any of these parameters with either mucosal chemiluminescence levels or malondialdehyde equivalent concentrations.

Ascorbyl radical concentrations

Examination of thawed and homogenized biopsy samples by EPR spectroscopy demonstrated the presence in many cases of the highly characteristic signal from the ascorbyl radical with parameters (a_H 0.176, a_{2H} 0.019 mT, g 2.00518) identical to those reported in many previous studies (29–32). The relative concentrations of this radical species in the various samples examined were determined by measurement of the signal height (which is directly proportional to the absolute radical concentration) of the spectral lines from this species. The concentration was found to be higher in the mucosa of patients with *H.pylori*-associated gastritis compared with those with normal histology ($P < 0.01$) (Table II). There was no significant difference in the concentration of this radical between patients with reactive gastritis and those with normal histology ($P = 0.66$) (Table II).

If all 82 cases are considered together then mucosal ascorbyl radical concentrations correlate with chemiluminescence level ($r = 0.41$, $P < 0.001$), but not with malondialdehyde equivalent concentrations ($r = 0.08$, $P = 0.47$). There was no relationship between ascorbyl radical levels and the concentration of mucosal ascorbic acid ($r = 0.10$, $P = 0.39$), total vitamin C ($r = 0.08$, $P = 0.48$) or mucosal ascorbic acid:total vitamin C ratios ($r = -0.04$, $P = 0.74$).

Discussion

We believe this is the first time that the ascorbyl radical has been detected in gastric mucosa. Our data suggest that ascorbic acid acts as a radical scavenger in gastric mucosa and that this process results in the generation of ascorbyl radicals. The concentrations of this radical have been found to be significantly increased in the presence of *H.pylori* infection and this increase is believed to be due to a higher level of oxidative stress in these patients. It is believed that at least some of the radicals being scavenged by the ascorbic acid are oxygen-derived, as there is a significant correlation between ascorbyl radical concentration detected and the level of luminol-enhanced chemiluminescence. Chemiluminescence is primarily a measure of the presence of oxidizing species in a particular system and studies using a number of inhibitors have suggested that oxygen-derived reactive species are largely responsible for the mucosal chemiluminescence in inflammatory conditions (14,33).

The levels of malondialdehyde equivalents are thought to reflect ROS-mediated lipid damage and are therefore useful for assessing the membrane damaging role of ROS. The lack of any correlation between ascorbyl radical concentrations and the levels of mucosal malondialdehyde equivalents may arise from the fact that although ascorbic acid scavenges ROS, other factors are also of relevance in determining whether ROS produce membrane damage. Thus it is known that other radical scavengers, such as α-tocopherol and the carotenoids, play a significant role in protecting membranes from oxidative damage and 'free' transition metal ions (especially Fe[II] and Cu[II]) are also thought to be important in catalysing some forms of free radical-mediated damage. In addition, malondialdehyde equivalent concentrations are believed to reflect *lipid* phase ROS-mediated damage, whereas ascorbic acid is only active as a direct radical scavenger in the aqueous phase (although it has been suggested that it may act as the terminal anti-oxidant by repairing lipid-soluble anti-oxidant radicals such

Table I. Mucosal luminol-enhanced chemiluminescence levels and malondialdehyde equivalent concentrations (median and interquartile range)

	Normal (n = 29)	*H.pylori*-associated gastritis (n = 37)	Reactive gastritis (n = 16)
Chemiluminescence (c.p.m./mg wet biopsy wt)	1929 (361–2762)	16102 (3697–47864)	1249 (433–4806)
MDA equivalents (nmol/g wet biopsy wt)	96.0 (70.4–110.8)	114.5 (85.1–142.7)	81.3 (65.9–97.5)

Table II. Mucosal ascorbic acid, total vitamin C and ascorbyl radical concentrations (median and interquartile range)

	Normal (n = 29)	*H.pylori*-associated gastritis (n = 37)	Reactive gastritis (n = 16)
Mucosal ascorbic acid (μg/g wet biopsy wt)	85.9 (53.3–119.9)	95.7 (50.4–122.6)	75.9 (62.6–83.9)
Mucosal total vitamin C (μg/g wet biopsy wt)	106.0 (73.0–130.2)	108.0 (65.7–148.3)	85.5 (80.5–98.6)
Mucosal ascorbyl radical (arbitrary units/mg wet biopsy wt)	0.16 (0.11–0.26)	0.27 (0.19–0.46)	0.15 (0.12–0.21)

as the α-tocopheroxyl species; 34) and this may explain the lack of a direct correlation between these two parameters.

The increased chemiluminescence levels and malondialdehyde equivalent concentrations seen in *H.pylori*-associated gastritis are in keeping with the results reported by ourselves and others (13,14,35) and probably reflect ROS generation by the neutrophil polymorph infiltrate seen in this condition.

Despite the key role believed to be played by ascorbic acid in protecting against radical-induced damage, no relationship was found between the levels of mucosal ascorbic acid, the ascorbic acid:total vitamin C ratio and the histological findings; these results are consistent with previous studies (36). Moreover, no correlation was found between these values and either measure of ROS activity (chemiluminescence and malondialdehyde equivalent levels). In contrast, 8-hydroxydeoxyguanosine levels (a measure of oxidative damage to DNA) in human sperm appear to be inversely related to the concentration of ascorbic acid in seminal fluid (37). It may be that there is rapid removal of the ascorbyl radical either via disproportionation of two ascorbyl radicals (which would yield one molecule of ascorbate and one of dehydroascorbate), as a result of its increased concentration (38,39), or by way of further oxidation to dehydroascorbic acid and enzymatic reduction. Both mechanisms might be expected to decrease the ascorbic acid:total vitamin C ratio, but as the steady-state ascorbyl radical concentrations are very low (of the order of a few micromolar) considerable precision in the ascorbate measurements would be required to detect such small changes in the ratio. The total vitamin C measurements may reflect the levels of this material in a number of different compartments (intra- versus extracellular, for example) within the system under study, whereas oxidation of ascorbate may only be occurring to any significant extent at one site, so that any changes may not be seen against a large background of unoxidized material. The infiltration of neutrophils and macrophages seen in *H.pylori*-associated gastritis may, through rapid membrane transport and intracellular reduction, also increase the conversion of dehydroascorbic acid back to ascorbate (40) and thus mask changes in this ratio towards ascorbate oxidation as a result of simultaneous generation of ROS. Whichever of these explanations is correct, these data suggest that further increasing mucosal ascorbic acid concentrations by dietary means may not lead to more effective ROS scavenging (at least in this comparatively well-nourished group) unless depletion is occurring at certain crucial sites which have relatively low normal ascorbate levels. Likewise, supplementation may not decrease the cancer risk attributable to the generation of ROS-mediated DNA damage unless mucosal ascorbic acid levels are either naturally very low or become depleted due to oxidant stress.

These results are in marked contrast to certain other acute cases of oxidant stress, where massive depletion of ascorbic acid has been observed. Thus it has been demonstrated (H.Goode, M.J.Davies and N.R.Webster, unpublished data) that ascorbic acid levels in the plasma of patients with sepsis in intensive care units are very low and that supplementation of these patients results in increased levels of ascorbyl radicals as a result of increased scavenging of oxidant radicals (and hence presumably a reduction in tissue damage); in these cases the levels of ascorbic acid (which are naturally very low in plasma) appear to be sub-optimal and supplementation is justified. A similar depletion of ascorbic acid levels (as evidenced by a decrease in ascorbyl radical concentrations) in response to increasing levels of oxidant stress (induced by exposure to increasing levels of hydroperoxides) has been observed in murine skin; supplementation of these systems with additional anti-oxidant capacity was shown to have a protective effect (32). However, ascorbic acid levels in gastric mucosa appear to be relatively high and our data suggest that further supplementation with the vitamin might not be useful in reducing ROS damage. The protective effect of ascorbic acid against gastric cancer suggested by epidemiological work might be due primarily to mechanisms other than ROS scavenging, e.g. a reduction in formation of carcinogenic *N*-nitroso compounds in gastric juice.

In conclusion, we have demonstrated for the first time that ascorbic acid has a free radical scavenging role in human gastric mucosa which results in generation of the ascorbyl radical. The experimentally determined concentrations of this radical are greater in a group of patients at increased risk of gastric cancer, suggesting that this group is exposed to a greater degree of oxidative stress. This is believed to be

due to the generation and subsequent scavenging of higher concentrations of radicals or other ROS, which have also been shown to be increased in this high risk group, possibly as a result of the leucocytic infiltration and inflammation which is characteristic of this condition. However, our data do not support the hypothesis that an increase in ascorbic acid intake in the population studied, as a means of further increasing ROS scavenging capacity, may reduce the cancer risk in this group unless the subjects already had very low levels of ascorbic acid. The situation with other antioxidants, which may concentrate in other cellular compartments, may, however, be different.

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