DNA damage in the stomach after vagotomy measured by ^{32}P -postlabelling

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Abstract

This study analysed gastric mucosal DNA by ^{32}P -postlabelling in a series of patients who have had previous vagotomy for benign peptic ulcer disease. DNA adduct levels were found to be significantly higher in patients who had had previous truncal vagotomy than in those who had had previous highly selective vagotomy ($p < 0.001$). Intragastric bile concentrations were also considerably higher in patients after truncal vagotomy but there was no correlation between intragastric bile concentrations and DNA adduct levels. These results suggest that, although duodenogastric reflux may be a cause of gastric mucosal DNA damage in the stomach after vagotomy, measurement of total intragastric bile does not accurately reflect genotoxic insult.

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Although the incidence of gastric cancer is falling in all countries for which reliable data are available¹ there are still certain groups of subjects believed to be at increased risk of developing this disease. Large cohort studies have indicated that one such group consists of patients who have had previous vagotomy for benign peptic ulcer disease.^{2,3} The reason for this increased risk is still the subject for debate, although two theories predominate. One suggests that the change in the intragastric environment after vagotomy leads to an increased concentration of potentially carcinogenic N-nitroso compounds within the stomach.^{4,5} The other favours reflux of either bile or duodenal content as the important carcinogenic factor.⁶

Although the exact agents responsible are not known, it is probable that initiation is induced by a chemical carcinogen. There is considerable evidence to show that many chemical carcinogens act by forming stable covalent bonds with cellular macromolecules such as DNA.^{7,8} In animal models the presence of carcinogen-DNA adducts has been shown to lead to specific mutations within DNA^{9,10} including the activation of known oncogenes^{10,11} and the persistence of DNA adducts in a tissue may be correlated with an increased risk of developing cancer in that tissue.^{12,13} The identification of DNA adducts in human tissues may therefore provide a means of studying the processes participating in human carcinogenesis.

Until recently, however, detection of DNA adducts in human tissue has proved difficult as adduct levels are extremely low (commonly less than 1 adduct/ 10^8 nucleotide bases). Conventional methods of adduct detection such as high pressure liquid chromatography (HPLC) or antibody techniques have proved to be too insensi-

tive unless large quantities of DNA are analysed. The technique of ^{32}P -postlabelling¹⁴ is more sensitive and is able to detect DNA adducts without any previous knowledge of their chemical structure. With modifications of either nuclease P1 enhancement¹⁵ or butanol extraction¹⁶ the assay is sensitive enough to detect adducts in microgram quantities of native human DNA and has been used to detect and quantify adducts in a number of human tissues.^{17,18} Although some DNA adducts are lost in the course of DNA extraction during the enhancement procedures and in the chromatography stage of this assay, and this means that true adduct levels tend to be underestimated, valuable information on relative adduct levels is emerging.

Using this technique, Spigelman *et al*¹⁹ have examined DNA extracted from the foregut of patients with familial adenomatous polyposis. In these patients they found higher adduct levels in the duodenum than in the stomach and they suggest that pancreaticobiliary secretions may play a part in foregut carcinogenesis in patients with familial adenomatous polyposis.

This finding is of great interest with regard to the postvagotomy stomach. If bile reflux is an important aetiological factor in gastric carcinogenesis in this situation, it might be expected that gastric mucosal DNA should also show evidence of bile related adduction. To test this hypothesis we have examined gastric mucosal DNA for the presence of DNA adducts. Using the ^{32}P -postlabelling method with butanol extraction as an enhancement procedure we have compared adduct levels in a group of patients who have had either a previous truncal vagotomy and drainage where bile reflux might be expected or a previous highly selective vagotomy where bile reflux is less common. Intragastric bile acids were also measured in these patients and adduct levels correlated with exposure to bile.

Materials and methods

ENDOSCOPY AND COLLECTION OF SAMPLES

Upper gastrointestinal endoscopy was performed under light sedation after an overnight fast. Immediately upon entering the stomach, a sample of gastric juice was aspirated into a sterile Wallace bronchoscopy suction bottle for pH and bile estimations.

Ten gastric mucosal biopsy specimens were required for DNA extraction. After careful examination of the stomach, duodenum, and any stoma that might be present, 10 gastric mucosal biopsy specimens were taken from different parts of the stomach. These specimens were

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pooled and immediately frozen. They were stored at -70°C until analysed.

The pH of the gastric juice was measured with a Philips digital pH meter and the juice samples stored frozen at -70°C until analysed for the presence of bile acids.

DNA EXTRACTION AND ANALYSIS

The pooled endoscopic biopsy specimens were thawed, homogenised, and incubated in sodium dodecyl sulphate and proteinase K (Boehringer, East Sussex, UK). DNA was isolated using a standard solvent extraction procedure²⁰ after treatment with ribonuclease A and ribonuclease T1 (Sigma, Poole, Dorset, UK). DNA was dissolved in aqueous solution in a total volume of 500 μl . The purity and concentration of the DNA was estimated by ultraviolet spectrophotometry and re-extraction performed until the A260/280 ratio was close to 1.8.

For the purposes of postlabelling, a volume of DNA solution containing 5 μg DNA was evaporated to dryness in a Univap rotor evaporator (Uniscience, Cambridge, UK) and digested to mononucleotides with micrococcal nuclease (Sigma, Poole, UK) and spleen phosphodiesterase (Boehringer, Lewes, UK). Adducted nucleotides were concentrated by butanol extraction as described by Gupta,¹⁶ re-evaporated to dryness, and dissolved in 10 μl of distilled water. Each sample was labelled with 100 μCi of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ synthesised by the method of Johnson and Walseth²¹ using two to three U of T4 polynucleotide kinase (NBL, Cramlington, UK) per sample. Excess ATP was destroyed with potato apyrase (Sigma, Poole, UK) and labelled, adducted nucleotides were separated by chromatography on PEI cellulose plates (Camlab, Cambridge, UK) as described by Gupta.²² Adduct spots were visualised and counted as previously described¹⁸ and adduct levels calculated by the method of Gupta.¹⁶ Samples were analysed twice, with a different batch of ATP each time and the results averaged.

BILE ACID ESTIMATION

Gastric juice samples were thawed and bile acid concentrations estimated using the Sterognost 3α spectrophotometric kit (Nyegaard, Oslo).

STATISTICS

Statistics were analysed using the FIRST statistics package (Serious Statistical Software,

Patient details

	Nature of operation	
	HSV	TV and D
No of patients	29	59
No of men	22	43
No of women	7	16
Mean age (y)	61.3	62.5
Age range	44-82	29-87
Years since operation (SEM)	14.8 (2.6)	14.9 (5.4)
No of smokers	15	32
Intragastric pH (SEM)	3.12 (2.34)	4.04 (2.62)

HSV=highly selective vagotomy; TV and D=truncal vagotomy and drainage.

Figure 1: DNA adduct levels in smokers and non-smokers. Adduct levels were higher in smokers (mean = 9.6 adducts/ 10^8 nucleotides) than in non-smokers (mean = 6.0 adducts/ 10^8 nucleotides; $p=0.034$).

Willaston, South Wirral). Statistical differences between groups were estimated with the Mann-Whitney U test and correlation coefficients calculated with the Spearman rank test for non-parametric data.

Results

PATIENTS

Eighty eight patients attending the Gastric Follow-Up clinic at York District Hospital were entered into the study. All patients were volunteers and all had had previous vagotomy for benign duodenal ulcer disease. Smoking and drug histories were taken although no patient was receiving any treatment that would be expected to give rise to DNA damage.

The Table gives details of patients with respect to age, length of time since operation, intragastric pH, and smoking history. The two groups are remarkably similar with respect to age, male/female ratio, length of time since operation, and percentage of smokers.

DNA ADDUCTS

DNA adduct spots were identified in all samples tested. There was no evidence that the nature of previous surgery had any effect upon the pattern of adduct spots seen although occasionally highly distinctive adduct patterns were seen, which were clearly specific for a given patient. DNA extracted from the gastric mucosa of smokers tended to have a dense pattern of adduct spots similar to that reported in other tissues of smokers.¹⁸

Adduct levels ranged from 0.7 adducts/ 10^8

Figure 2: DNA adduct levels (adducts/ 10^8 nucleotides) after either highly selective vagotomy (HSV) or truncal vagotomy (TV and D). Adduct levels were significantly higher after truncal vagotomy ($p < 0.001$).

nucleotides to 29 adducts/ 10^8 nucleotides, which is similar to values reported for other tissues.¹⁸ Adduct levels tended to be higher in smokers than in non-smokers. The mean adduct value found in smokers was 9.6 adducts/ 10^8 nucleotides compared with only 6.0 adducts/ 10^8 nucleotides in non-smokers ($p = 0.034$ Mann-Whitney U test; Fig 1).

Adduct levels were also significantly higher in patients who had had previous truncal vagotomy and drainage (mean adduct level = 10.2 adducts/ 10^8 nucleotides) than in those who had had highly selective vagotomy (mean adduct level =

3.3 adducts/ 10^8 nucleotides; $p < 0.001$ Mann-Whitney U test; Fig 2).

INTRAGASTRIC BILE ACIDS

Intragastric bile acids were measured in 55 of 88 patients. They were significantly higher in patients after truncal vagotomy and drainage ($n = 37$; mean = 3.5 mmol/l) than after highly selective vagotomy ($n = 18$; mean = 0.24 mmol/l; $p < 0.001$ Mann-Whitney U test) (see Fig 3).

Discussion

Evidence from animal studies shows that the presence of carcinogen-DNA adducts may be associated with an increased risk of developing cancer. Although at present there is insufficient evidence to extrapolate these findings to the human situation, the finding of chemically modified DNA in human tissues is clearly worrying.

This study has shown that DNA adducts are readily identifiable in human gastric mucosal DNA. Adduct levels were found to be higher in smokers than non-smokers and adduct levels were significantly higher after truncal vagotomy and drainage than after highly selective vagotomy. Both these findings are in accord with epidemiological data on gastric carcinogenesis.

An increased risk of developing gastric cancer in the smoking population has been highlighted in a number of large scale prospective cohort studies²³⁻²⁵ and there is increasing evidence accumulating from postlabelling studies that DNA adducts can be correlated with smoking history. This has been most clearly shown for lung tissue^{16,26} but smoking related adducts have also been described in a number of other tissues for which smoking has been implicated as a cancer risk factor.¹⁸ We have previously shown that adduct levels in DNA extracted from gastric cancers are higher in smokers than non-smokers²⁷ and this study therefore suggests that smoking related adducts may be found in non-neoplastic as well as neoplastic mucosa.

An increased risk of developing gastric cancer after previous vagotomy has also been reported.^{2,3} Although the nature of the vagotomies surveyed in these reports is not stated, the follow up period is such that they must have been truncal vagotomies. To date there is no evidence to suggest that there is an increased cancer risk in those patients who have had previous highly selective vagotomy and hence our finding of higher adduct levels in the DNA of patients after truncal vagotomy is again in accord with epidemiological data.

While it is easy to comprehend the cause of DNA adducts in the smoking population, the origin of the adducts found after truncal vagotomy is rather more obscure. Although the chromatography eluants used in this study are particularly suited to detecting tobacco related carcinogens – and will miss some small molecular weight adducts altogether – it is unlikely that smoking can account for the increased adduct levels found as the percentage of smokers in the truncal vagotomy group (54%) was very similar to that of the highly selective vagotomy patients

Figure 3: Intragastric bile concentrations (mmol/l) after vagotomy. Concentrations were higher after truncal vagotomy (TV and D) (mean = 3.5 mmol/l) than after highly selective vagotomy (HSV) (mean = 0.24 mmol/l; $p < 0.001$).

Figure 4: Relation between DNA adduct levels (adducts/ 10^8 nucleotides) and intragastric bile concentrations (mmol/l). There is no significant correlation ($r=0.02$; $p=NS$).

(52%). The most obvious explanation is that reflux of duodenal content into the stomach after operation is responsible. Bile or components of bile have been identified as potential carcinogens in other organs such as the colon²⁸ or pancreas²⁹ and it has been suggested that the increased risk of developing gastric cancer after gastric surgery is a function of duodenogastric reflux.⁶ In the rat model, operations that lead to duodenogastric reflux give a higher yield of gastric tumours in animals dosed with carcinogen.³⁰ Conversely, operations that divert bile away from the stomach will ameliorate dysplastic features induced by previous gastric surgery.³¹ This argument is supported by the report of Spigelmann¹⁹ suggesting that exposure of the foregut to bile may give rise to DNA adducts. Our data would support this hypothesis but also suggest that bile does not act as a simple genotoxic carcinogen for, although the intragastric bile concentrations found after truncal vagotomy were higher than those found after highly selective vagotomy (Fig 3), there was no correlation between bile concentrations and DNA adduct levels (Fig 4).

It is feasible that there is some subcomponent of bile that is responsible for DNA adduction. There is evidence to suggest that the operation of vagotomy changes the ratio of primary and secondary bile acids found in the gall bladder³² and that it is the secondary bile acids that are carcinogenic.²⁹ The assay system used in this study measures total bile content and does not distinguish between classes of bile acids hence differences in constitution of bile would go unnoticed. Similarly, if non-bile components of duodenogastric reflux are responsible for adduc-

tion, then measurement of intragastric bile will only act as a crude marker for reflux and not reflect the true carcinogenic potential of the gastric juice. Likewise, the carcinogenic action of bile may be related to the duration of exposure of gastric mucosa to biliary carcinogens. The assay used in this study measures bile concentrations at a single point in time and may well not be an accurate reflection of overall exposure.

Finally, it must be considered that the bile is acting as a non-genotoxic carcinogen. That certain carcinogens exert their effect without binding to DNA is well known. In the rat model for example it has been shown that the compound butyl hydroxy anisole, which is carcinogenic in the forestomach, does not bind directly to DNA. It will however increase the binding of other carcinogens to gastric mucosal DNA³³ and it is conceivable that bile acts in a similar manner. The ³²P-postlabelling assay suffers from the drawback that the nature and origins of the adducts detected is unknown. Thus the adducts detected in this study may not be derived from bile at all but merely have their binding to DNA increased by bile.

Whatever the agent responsible, or its mode of action, this is another factor to be considered when selecting an operation for benign peptic ulcer disease. At present ulcer recurrence rate, postoperative complication rate, and cancer risk are the major factors determining the choice of operation and current opinion is tending to favour the highly selective vagotomy.³⁴⁻³⁶ To date this operation seems to be free of cancer risk although one case of cancer has been reported seven years after operation.³⁷ If the experience with partial gastrectomy and truncal vagotomy is relevant, a latency period of about 20 years is required before any cancer risk becomes apparent.² Highly selective vagotomy was introduced about 20 years ago^{38,39} and hence any cancer risk should become apparent in the next few years. Our results will be reviewed with great interest in this context as they may show that the risk of cancer will be less after highly selective vagotomy than after truncal vagotomy.

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