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DNA damage levels are raised in Barrett's oesophageal mucosa relative to the squamous epithelium of the oesophagus

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Barrett's oesophagus (BE) is a pre-malignant metaplastic tissue predisposing to oesophageal adenocarcinoma (EC), and gastro-oesophageal reflux is a risk factor for both conditions. Reflux of acid and bile can cause mucosal injury and initiate chronic inflammation. These processes can induce DNA damage, possibly via an oxidative stress mechanism, thus increasing the likelihood of progression from Barrett's metaplasia to dysplasia and finally carcinoma. The comet assay was optimized for the detection of DNA damage (strand breaks and alkali-labile sites) in oesophageal biopsies, including incorporation of the DNA repair enzyme Fapy-DNA glycosylase (Fpg). Fpg allows the detection of 8-hydroxy-2-deoxyguanosine (8-OHdG) sites, a known pro-mutagenic DNA lesion. BE patients were recruited from BE surveillance clinics and oesophageal biopsies collected at endoscopy. Comet analysis revealed significantly increased ($p < 0.001$) DNA damage in Barrett's epithelium compared with matched squamous epithelium, with median % tail DNA values of 25.1% (first to third quartile 21.7–29.6%) and 18.6% (first to third quartile 16.9–21.4%), respectively. The median % tail DNA was up to 70% higher in the matched BE tissue compared with squamous epithelium from the same patient. Fpg sensitive sites were demonstrated in both tissue types at similar levels. The raised level of DNA damage in the premalignant BE may contribute to the accumulation of genetic alterations occurring during progression to EC. Understanding these underlying mechanisms provides a basis for cancer prevention strategies in BE patients.

Keywords: Barrett's epithelium, DNA damage, comet assay, gastro-oesophageal reflux.

Introduction

Barrett's oesophagus (BE) comprises the replacement of normal squamous epithelium by a metaplastic, specialized intestinal epithelium (Jankowski *et al.* 2000, Chen and Yang 2001). BE is recognized as a precancerous condition from which oesophageal adenocarcinoma (EC) can develop along a sequence of metaplasia–dysplasia–adenocarcinoma (Jankowski *et al.* 1999), this process being associated with an accumulation of genetic alterations (Jankowski *et al.* 1999, Chen and Yang 2001, Wijnhoven *et al.* 2001). The risk of both BE and EC are increased in association with chronic reflux disease (Wild and Hardie 2003). Improvements in the management of BE patients may be made by elucidating the underlying mechanisms of carcinogenesis and identifying biomarkers associated with the

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progression of BE to EC. This knowledge, for example the presence of defined genetic alterations in BE (Bani-Hani *et al.* 2000), could provide more specific criteria for the recruitment of patients to endoscopic surveillance programmes, but may also provide a rationale for interventions to interrupt or slow disease progression.

Gastro-oesophageal reflux is a common condition in the Western world, with 30% of adults complaining of heartburn at least once a month (Locke *et al.* 1997). This condition can be further complicated by components of duodenal reflux, including bile, entering the oesophagus in addition to regurgitated acid from the stomach (Jankowski *et al.* 1999, Dixon *et al.* 2001). The severe and prolonged reflux of acid and duodenal contents in BE patients (Vaezi and Richter 1996, Dixon *et al.* 2001) can cause mucosal injury and initiate chronic inflammation and oesophagitis, termed gastro-oesophageal reflux disease (Jankowski *et al.* 2000, Wild and Hardie 2003).

Inflammation in Barrett's tissue can lead to an increase in oxidative stress (Wild and Hardie 2003). If the level of reactive oxygen species exceeds the antioxidant and DNA repair capacity of the tissue, the resultant oxidative stress could lead to pro-mutagenic DNA damage, including DNA adducts, strand breaks and other lesions (Toyokuni *et al.* 1995, Epe 2002, Halliwell 2002). For example, 8-hydroxy-2-deoxyguanosine (8-OHdG) is a major form of pro-mutagenic oxidative DNA damage that is found at raised levels in a number of disease states where inflammation and oxidative stress are considered to play a key role (Shimoda *et al.* 1994, Baik *et al.* 1996, Farinati *et al.* 1998, D'Odorico *et al.* 2001).

Clinical studies support a possible role for oxidative stress in oesophageal carcinogenesis (Wild and Hardie 2003). For example, glutathione levels are reduced and myeloperoxidase levels increased in oesophageal biopsies from patients with oesophagitis and BE compared with patients without reflux symptoms or any endoscopic evidence of oesophageal pathology (Sihvo *et al.* 2002). In a comparison of BE and oesophagitis patients, increased levels of free radicals and lipid peroxidation products as well as reduced levels of the antioxidant enzyme superoxide dismutase were observed in the former compared with the latter patient group (Wetscher *et al.* 1995a). Finally, increased levels of inducible nitric oxide synthase were reported in BE tissue (Wilson *et al.* 1998), as were potentially toxic and mutagenic levels of nitric oxide at the gastro-oesophageal junction after ingestion of dietary nitrate (Iijima *et al.* 2002). Overall, these studies are consistent with a link between chronic reflux disease and free radical-induced DNA damage in the oesophageal mucosa.

In this study we optimized the alkaline single cell gel electrophoresis assay (comet assay) (Singh *et al.* 1988) to measure DNA strand breaks and alkali-labile sites in individual cells isolated from oesophageal biopsies from BE patients. The assay was also conducted following incorporation of the Fapy-DNA glycosylase (Fpg) enzyme, which recognizes 8-OHdG and ring-opened purines (Collins 1999) to reveal additional DNA damage. The study tested the hypothesis that the metaplastic cells in BE contain higher levels of DNA damage than the squamous epithelium. This effect could contribute to the accumulation of genetic alterations occurring in BE and the progression to malignancy. Furthermore, indications that

oxidative lesions contribute to the overall level of DNA damage would provide an opportunity for intervention strategies in BE patients.

Materials and methods

Materials

The tissue culture reagents Dulbecco's modified Eagle medium (DMEM), fetal calf serum and trypsin–ethylene diamine tetra-acetic acid (EDTA) were obtained from Invitrogen (Paisley, UK). Comet assay reagents including collagenase (type IV, derived from *Clostridium histolyticum*) and pronase (derived from *Streptomyces griseus*) enzymes were obtained from Sigma Chemical (Poole, UK). The A549 cell line was obtained from the European Collection of Cell Cultures and the Fpg protein was a gift from Professor Andrew Collins of the Institute of Nutrition Research, University of Oslo, Norway. The Fpg protein was supplied as a crude extract with no reported non-specific nuclease activity at the concentrations used in this study.

Tissue collection

Following hospital ethics committee approval and informed consent, 20 patients with a histological diagnosis of BE with specialized intestinal metaplasia on the last surveillance endoscopy were recruited from Barrett's surveillance clinics at Leeds General Infirmary, Leeds, UK. The patient group comprised 13 male and seven female Caucasians with an age range of 30–74 years. Seventeen patients were taking proton pump inhibitor (PPI) medication at the time of endoscopy; the remaining three patients were not taking any prescribed anti-reflux medication.

Mucosal biopsies were collected from the middle of the BE segment and from squamous oesophageal tissue sites (at least 1 cm clear of the Barrett's segment). BE was defined as specialized intestinal metaplasia with the presence of goblet cells, visualized by alcian blue staining. Single biopsy specimens used for comet analysis were collected adjacent to the biopsies submitted for histology. These latter biopsies confirmed that all patients did have BE at the time of the current study; no dysplasia was found. Biopsies were stored in DMEM and 10% dimethyl sulphoxide (DMSO) at 4°C overnight, before comet analysis the following day.

Comet assay

Single biopsies from a given tissue type for each patient were digested in 800 μl DMEM, 100 μl 0.3% collagenase and 100 μl 0.5% pronase at 37°C for 45 min to create a single cell suspension. Standard comet assay conditions used in our laboratory for the analysis of cultured cells and human lymphocytes based on the method of Lebailly *et al*. (1998), with minor modifications, were employed.

The cell pellet from each biopsy or from the lung epithelial cell line (A549), acting as an external control, was mixed with 300 μl of 0.8% low melting point agarose in phosphate buffered saline (pH 7.4) at 37°C. Then 70 μl of this suspension was placed on a microscope slide precoated with 1% agarose and allowed to solidify. Slides containing embedded cells were then submerged in a detergent solution (2.5 M NaCl, 1 mM EDTA, 10 mM Tris, 10% DMSO and 1% Triton-X; pH 10) for at least 1 h to disrupt the cell membranes and remove the proteins. Slides were washed three times in enzyme buffer (40 mM Hepes, 0.1 M KCl, 0.5 mM EDTA, 30% bovine serum albumin; pH 8) for 5 min before the addition of 50 μl of Fpg enzyme (diluted in enzyme buffer) or enzyme buffer alone to each slide. A coverslip was placed on top and slides were incubated at 37°C for 30 min. The coverslips were then removed and the slides immediately placed in an alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA; pH > 13) for 40 min to equilibrate. Slides were transferred to an electrophoresis chamber and placed at 23 V (variable amplitude) for 20 min. After rinsing with neutralizing buffer for 5 min (0.4 M Tris base; pH 7.5), nuclear DNA was stained with ethidium bromide (25 μg ml^{-1}). Comets were viewed using fluorescent microscopy and cells quantified as a percentage of the total DNA in the comet tail using Komet 4.0 software (Kinetic Imaging Ltd).

Comet assay validation for oesophageal biopsies

Enzyme digestion. The potential effect of the enzyme digestion process (0.03% collagenase and 0.05% pronase; 45 min at 37°C) on DNA damage was assessed using a cell suspension of A549 cells. Given that, by definition, a single cell suspension is required for analysis in the comet assay, it is not possible to compare directly the effect of digestion enzymes on biopsies; we therefore used this cell line as a surrogate in this validation step. However, the comet assay has been recommended as applicable to *in vivo* tissues following any cell dissociation method as long as it is not associated with inappropriate background levels of DNA damage (Hartmann *et al.* 2003).

Storage of biopsies overnight. The comet assay takes between 5–6 h to complete and fresh tissue must be used. Validation was therefore required to assess whether biopsies could be stored at 4°C for later analysis without either inducing a significant amount of DNA damage or reducing the sensitivity of the assay to detect DNA damage, perhaps as a result of DNA repair processes *ex vivo*. Similar delays in processing have not led to increased DNA damage in gastric biopsies in earlier studies (Everett *et al.* 2000). Initially the effect of storage was assessed using the A549 cell line because of the limited number of biopsies that can be acceptably obtained from a given patient. Cells were harvested and then comet analysis performed either immediately or after storage overnight in DMEM and 10% DMSO. The effect of storage was then assessed on a limited number of biopsies to confirm the data from the cell line. Adjacent biopsies were taken from the same patient and comet analysis was performed immediately on one of each of the paired biopsies. The remaining matched biopsy from each area was stored in DMEM and 10% DMSO overnight, refrigerated at 4°C prior to analysis (Everett *et al.* 2000). The variability between the cell and biopsy preparations analysed under the two conditions was determined.

Optimization of the Fpg assay. Fpg enzyme was titrated from 1/100 to 1/30 000 to determine a suitable working concentration. Individual cells from the A549 cell line were analysed by comet assay to assess the effect of each concentration compared with enzyme buffer alone, which served as a negative control. To ensure that changes in DNA damage following Fpg incubation were enzyme specific, the enzyme was heat inactivated (60°C for at least 1 h) prior to addition to the nuclei on the slides. Levels of DNA damage were then compared with those on slides that had been treated with active enzyme (positive control) or buffer (negative control).

Intra- and inter-assay variability. To assess intra-assay variability, DNA damage was compared in replicate slides of the same A549 cell suspension. DNA damage was also assessed in preparations of the same cell line prepared on separate days to act as an external control and to assess inter-assay variability.

Data analysis

Fifty comet cells were visualized and scored for each biopsy. The percentage of tail DNA was recorded, and for each tissue a median was calculated for the total number of cells examined, together with the first and third quartiles. As the percentage of DNA in the comet tails of the cells examined from each biopsy was not normally distributed, non-parametric statistical methods were employed. For comparisons between groups, the median % DNA damage for each tissue biopsy for a given individual was used. Data are therefore presented as medians; the Wilcoxon rank test was used for paired comparisons. A p value of < 0.05 was considered to infer statistical significance. Data were analysed using Microsoft Excel and SPSS 11.0 programs.

Results

An initial series of experiments was conducted to validate the comet assay, with and without Fpg enzyme, for use on human oesophageal biopsies. Once the methodology had been established, a comparison was made between the DNA damage levels in BE and squamous oesophageal epithelium.

Comet assay validation

Enzyme digestion. A cell suspension of A549 cells was subjected to the conditions of enzyme digestion used for biopsies (final concentrations 0.03% collagenase and 0.05% pronase). No increase in DNA damage was observed in two independent sets of slides when comparing levels before digestion (median % tail DNA 8.9% [first to third quartile 2.5–19.5%] and 9.4% [1.8–23.9%]) and after incubation with digestion enzymes (median % tail DNA 9.2% [first to third quartile 3.7–16.8%] and 8.7% [2.3–16.1%]).

Storage of biopsies. Due to the timing of biopsy collection at endoscopy clinics and the duration of the comet assay, biopsies often had to be stored at 4°C for

various periods prior to analysis. It was important therefore to examine the effects of this storage on DNA damage to ensure no significant alterations were occurring *ex vivo*. Initially, the tail DNA content was measured in A549 cells processed immediately or after storage (25 h) to mimic the analysis of the oesophageal biopsies.

This analysis showed that storage did not significantly increase the level of DNA damage, with median % tail DNA values of 6.0% (first to third quartile 5.7–8.0%) with immediate processing compared with 7.5% (5.0–9.5%) for stored cells. Given these results, the comet assay was subsequently measured in adjacent biopsies from the same patient either processed immediately or stored for up to 25 h at 4°C before comet analysis (table 1). It should be noted that this comparison cannot exclude true variability in DNA damage between adjacent biopsies. Notwithstanding this caveat, no significant increase in DNA damage was observed with storage. In fact, a trend towards a slightly lower level of damage was seen in all four comparisons, although this only reached the level of statistical significance in one biopsy pair. In addition, in the analysis of the biopsies from the 20 patients in the study (see below), no correlation was observed between the storage time of biopsies and DNA damage (data not shown). Hence storage under the conditions specified does not significantly change the level of DNA damage from that present *in vivo*.

Optimization of the Fpg assay. Using untreated A549 cells, inclusion of the Fpg enzyme into the comet assay significantly elevated the level of tail DNA damage compared with buffer alone at dilutions of 1:3000 or less (figure 1a). However, there did not appear to be any further increase in detectable DNA damage when enzyme at higher concentrations (1:1000 and 1:100) was used. When the Fpg enzyme at 1:3000 was heat inactivated, the increase in DNA damage due to the enzyme was no longer observed, confirming that this increase is an effect of functionally active enzyme (figure 1b). A working dilution of Fpg at 1:3000 was therefore selected for application to the oesophageal biopsies.

Intra- and inter-assay variability. Slides were prepared in triplicate from the A549 lung epithelial cell line for comet analysis in order to evaluate intra-assay reproducibility (table 2). Triplicate slides were prepared under three different experimental conditions: with enzyme buffer alone, with active Fpg enzyme, and with heat-inactivated Fpg. No significant difference was observed between replicate slide preparations under the same conditions (table 2). Similarly, inter-assay

Table 1. The effect of overnight storage on DNA damage in matched adjacent biopsies.

	Median % tail DNA		
Biopsy no.	Biopsy processed immediately	Biopsy stored overnight	*p* value[a]
1	12.0	8.6	0.86
2	10.7	10.5	0.32
3	11.5	9.9	0.15
4	12.0	9.7	0.05

[a] Represents a comparison of the level of DNA damage between the adjacent biopsies.

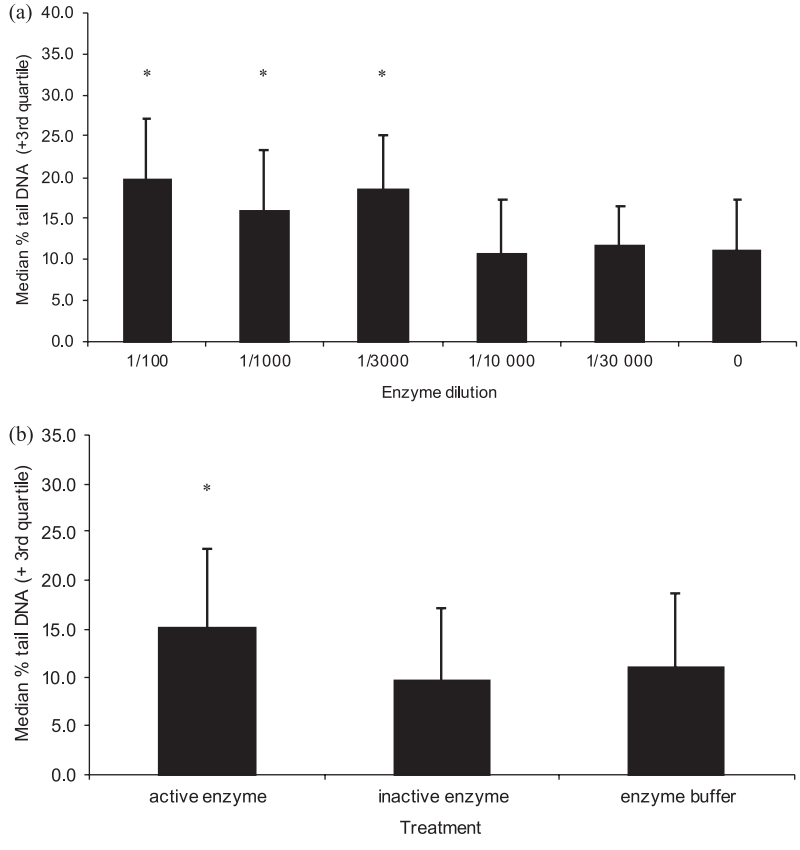

Figure 1. Optimization of Fpg enzyme to detect Fpg-sensitive sites in A549 cells.(a) The effect of Fpg enzyme concentration on the detection of Fpg-sensitive sites. Different dilutions of Fpg extract were incubated for 30 min with A549 cells as described in Materials and methods. Levels of DNA damage were measured using the comet assay and were compared for each enzyme dilution to the control cells incubated with enzyme buffer alone (column 0). * $p <$ 0.05 compared with enzyme buffer control. (b) The effect of Fpg enzyme inactivation on the detection of Fpg-sensitive sites. A549 cells were incubated with a 1:3000 dilution of Fpg enzyme extract, or with the same concentration of enzyme after heat inactivation at 60°C for at least 1 h or with buffer alone prior to measurement of DNA damage in the comet assay. * $p < 0.05$ compared with enzyme buffer control.

variability was assessed by monitoring levels of DNA damage in the control A549 cells at the same time as the biopsies were analysed (15 separate assays runs over 3 months). The median % tail DNA in the 15 experiments using A549 cells was 9% (first and third quartile 8.1–10.8%). These consistent levels of baseline damage observed between experiments over time (figure 2) imply that induction of experimentally induced DNA damage is highly unlikely during comet analysis.

DNA damage in BE and squamous epithelium of the oesophagus

The median levels of DNA damage measured by the comet assay for each patient and tissue type are shown in table 3. In 19 of the 20 patients, DNA damage (comet without Fpg incorporation) in BE exceeded that in the matched squamous epithelium. In several cases the median levels were 60–70% higher in the BE biopsy

Table 2. Intra-assay variation during Fpg enzyme incorporation into the comet assay using A549 cells.

Treatment	Median % tail DNA	*p* value[a]
Enzyme buffer alone	10.4	
	10.9	0.59
	11.5	
Active enzyme	14.5	
	15.1	0.42
	15.8	
Inactive enzyme	10.0	
	8.3	0.12
	10.7	

[a] Represents a comparison of the level of DNA damage between the three slides with the same treatment conditions.

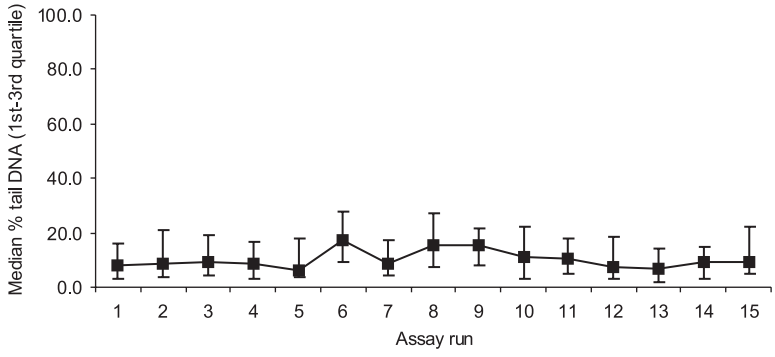

Figure 2. Consistency of measured level of DNA damage in untreated A549 cells. DNA damage was measured in the comet assay (see Materials and methods) on untreated A549 cells run in parallel to human oesophageal biopsies in 15 separate assay runs over 3 months.

compared with the biopsy taken from squamous epithelium from the same patient. Comparison of median DNA damage for all subjects (figure 3) revealed a significantly ($p < 0.001$) higher level of damage in BE (column B) (median % tail DNA 25.1% [first to third quartile 21.7–29.6%]; $n = 20$) when compared with matched squamous mucosa (column D) (18.6% [16.9–21.4%]).

The addition of the Fpg enzyme resulted in an increase in damage in both BE and squamous epithelium (table 3), demonstrating the presence of Fpg-sensitive lesions in the DNA of these tissues. Comparison between the median DNA damage in the groups of matched BE and squamous epithelium from all 20 patients (figure 3) confirmed the increases in both types of epithelium (BE, column A versus column B; squamous, column C versus column D). In addition, the higher level of DNA damage in BE (median % tail DNA 35.4% [first to third quartile 30.6–46.1%]; column A) compared with squamous mucosa (median % tail DNA 27.5% [first to third quartile 24.3–31.7%]; $p = 0.001$; panel C) was still observed after Fpg treatment, indicating a similar level of Fpg-sensitive sites in both tissue types (median % tail DNA due to Fpg-sensitive sites of 10.1% for BE and 9.2% for squamous).

Table 3. DNA damage as measured by the comet assay in patients with BE.

			Median % tail DNA			
			No Fpg		With Fpg	
Patient no.	Sex	Age (years)	Barrett's[a]	Squamous	Barrett's[b]	Squamous
1	Female	68	18.6	17.9	49.4	35.0
2	Female	63	21.7	19.4	31.2	37.2
3	Male	64	17.2	16.3	28.6	27.0
4	Male	63	19.5	14.1	20.4	22.6
5	Male	66	29.5	17.2	40.1	20.7
6	Female	70	24.2	20.6	33.3	25.1
7	Male	73	24.2	17.4	32.8	24.5
8	Male	71	30.4	26.1	42.0	27.5
9	Female	72	29.9	18.5	35.3	28.3
10	Male	40	22.3	23.9	40.0	31.8
11	Female	74	26.1	20.0	33.6	31.6
12	Male	70	27.2	18.8	35.5	24.6
13	Female	68	37.3	28.7	52.4	44.6
14	Male	53	28.1	17.1	51.6	29.2
15	Male	67	21.7	15.6	28.9	27.8
16	Male	52	29.1	19.2	48.9	22.9
17	Male	30	23.5	14.7	28.2	19.8
18	Male	55	34.9	28.4	49.1	39.1
19	Female	66	21.7	14.7	25.8	27.4
20	Male	66	29.8	23.8	45.1	23.6

[a] With no Fpg, 19 out of 20 patients had higher levels of DNA damage in the Barrett's epithelium compared with the matched squamous epithelium.

[b] With Fpg, 17 out of 20 patients had higher levels of DNA damage in the Barrett's epithelium compared with the matched squamous epithelium.

Discussion

The primary aims of this study were to validate the comet assay for the analysis of DNA damage in oesophageal biopsy specimens and to test the hypothesis that levels of DNA damage are higher in precancerous BE compared with normal squamous epithelium. This hypothesis is based on the premise that BE is more

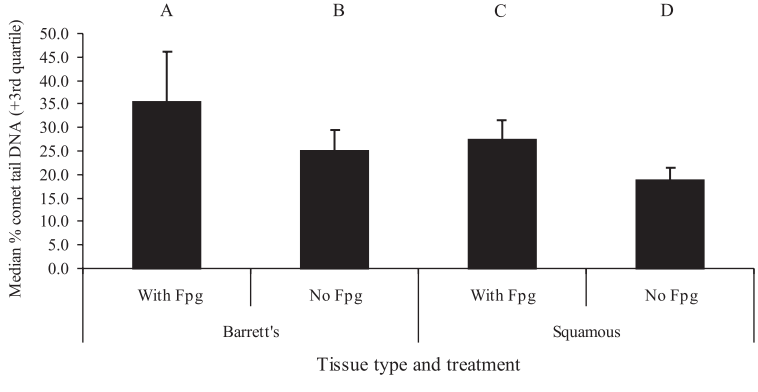

Figure 3. DNA damage in 20 patients with Barrett's oesophagus. DNA damage was measured in the comet assay under the conditions described in the Materials and methods. For both BE (columns A and B) and squamous epithelium (columns C and D), the median % comet tail DNA (+third quartile) is shown with (columns A and C) and without (columns B and D) incorporation of Fpg enzyme at a dilution of 1:3000.

susceptible to DNA damage stimulated by components of duodenal and gastro-oesophageal reflux, and is therefore predisposed to accumulation of the genetic alterations observed in the progression of BE to EC.

Collectively the validation data for the comet assay indicate that this is a robust approach to assessing DNA damage in oesophageal biopsies. The data on inter- and intra-assay variation indicate that the assay is reproducible, whilst the logistic necessity of storing biopsies did not lead to significant alterations in DNA damage; it is important to verify this latter point empirically for any assay where lesions could arise during sample processing and storage.

The data are consistent with previous studies using gastric biopsies, where overnight storage had no effect on levels of DNA damage in the comet assay (Everett *et al.* 2000). Similarly, enzyme digestion did not elevate DNA damage. Furthermore, the study validated the incorporation of the Fpg enzyme into the comet assay, permitting an understanding of the identity of some of the DNA lesions present in these biopsies.

Further validation of the comet assay procedure for analysis of oesophageal biopsies has come from our recent studies of methylene blue chromoendoscopy in Barrett's patients (Olliver *et al*. 2003). When photoactivated with white light, methylene blue leads to the generation of oxygen radicals and strand breaks. We demonstrated that when this compound is applied to Barrett's epithelium in BE patients, biopsies collected after chromoendoscopy had markedly higher DNA damage levels in the comet assay (both with and without Fpg incorporation) than matched biopsies collected prior to the procedure. As well as raising questions about the appropriateness of this clinical procedure, the data also provide an important *in vivo* demonstration that the comet assay as applied here can detect DNA damage in oesophageal biopsies following conditions of oxidative stress (Olliver *et al.* 2003).

BE is recognized as the first step in the metaplasia–dysplasia–carcinoma sequence that leads to EC (Jankowski *et al.* 2000). The development of BE, and subsequently EC, arises as a consequence of chronic reflux from the stomach and duodenum into the oesophagus, although as yet no molecular mechanism has been established to explain this process.

There is evidence that both the acidic pH and bile salt components of reflux can induce increased cell proliferation in BE (Fitzgerald *et al.* 1996, Triadafilopoulos 2001) and alter the expression of genes involved in the inflammatory response, such as *COX-2* (Shirvani *et al.* 2000, Zhang *et al.* 2001). However, an additional contribution may come from an increase in oxidative stress and consequent stimulation of DNA damage by these same components of reflux.

In this study we have shown higher levels of DNA damage in BE compared with matched squamous epithelium from the same patient. This was a consistent finding among the majority of patients, both with and without incorporation of Fpg into the assay. One possible explanation for this observation is that the metaplastic cells of BE are inherently more susceptible to DNA damage than the normal squamous epithelium. Thus the replacement of the normal epithelium, in response to injury induced by chronic reflux, may create a cellular environment that is more prone to the process of carcinogenesis. An understanding of the molecular basis for such a

change requires further consideration of both the specific type of DNA damage present and other relevant factors such as DNA repair and cell proliferation in the two tissue types, factors which will influence the risk of mutations subsequent to the initial formation of DNA damage.

Although the majority (17 out of 20) of patients were taking PPI medication, we cannot assume that acid reflux was completely controlled. pH monitoring has shown that patients asymptomatic for reflux or heartburn who are taking a PPI often still experience reflux episodes (Katzka and Castell 1994), and it has been suggested that incomplete acid suppression may in fact be more harmful than no suppression at all (DeMeester and DeMeester 2000). Consequently, the DNA damage we observed could be a consequence of residual acid or additional components of refluxate, including bile acids. It is possible that, since the BE is located closer to the gastro-oesophageal junction, this tissue also receives greater exposure to reflux components than the more proximal, squamous epithelium.

The DNA damage measured by the comet assay comprises strand breaks and alkali-labile sites. The additional Fpg-sensitive sites revealed by addition of the Fpg enzyme include 8-OHdG and other ring-opened purine lesions (Tchou *et al.* 1994). The DNA damage observed in the oesophageal biopsies is therefore consistent with the presence of free radical species generated as a consequence of reflux and an associated inflammatory response (Wetscher *et al.* 1995b, 1997, Oh *et al.* 2001). In an animal model of duodeno-gastro-oesophageal reflux, 8-OHdG levels were significantly increased in the oesophageal epithelium (Chen *et al.* 2000). In addition, it is interesting that the level of DNA damage in Barrett's epithelium, as measured by the percentage tail DNA, was similar to that observed in lymphocytes from individuals with diabetes, another condition where oxidative stress is thought to be implicated, but higher than in lymphocytes from healthy subjects (Collins *et al*. 1998). Despite all these indications of a role for oxidative stress, the comet assay provides no direct evidence of the chemical nature of the damage observed.

The fact that the Fpg treatment increased the detectable level of DNA damage in the comet assay by similar amounts in the squamous and Barrett's epithelium does not suggest that this type of damage is specific to the latter mucosa; types of DNA lesions other than oxidative damage might also be important. In this regard, we have recently demonstrated in oesophageal cell lines that both acid and bile salts can directly induce DNA damage measured by the comet assay (Jolly *et al.* 2002).

Further characterization of the DNA damage present in oesophageal biopsies will require the use of more lesion-specific assays. Analytical approaches, however, will need to overcome the limited amounts of tissue, particularly of squamous epithelium, obtained during the endoscopy procedure; in responding to these requirements the comet assay has merit in application to human biopsy material (Everett *et al.* 2000).

In conclusion, we have successfully validated the comet assay for application to oesophageal biopsies collected at endoscopy. The data from this study show that DNA damage is elevated in BE compared with squamous epithelium in the oesophagus. These observations indicate that oxidative stress may play a role in the generation of DNA lesions, although more specific assays are needed to investigate

this inference and the potential role of other compounds in inducing DNA damage. An understanding of the nature of the damage and the process of formation will be informative in providing advice for the management of BE patients in order to minimize the risk of these patients progressing to EC.

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