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Increased Etheno-DNA Adducts in Affected Tissues of Patients Suffering from Crohn's Disease, Ulcerative Colitis, and Chronic Pancreatitis

JAGADEESAN NAIR,[1] FRANK GANSAUGE,[2] HANS BEGER,[2] PIERO DOLARA,[3] GÜNTHER WINDE,[4] and HELMUT BARTSCH[1]

ABSTRACT

Chronic inflammatory processes induce oxidative stress and lipid peroxidation (LPO), hereby generating DNA-reactive aldehydes such as *trans*-4-hydroxy-2-nonenal (HNE). Etheno-modified DNA bases are *inter alia* generated by reaction of DNA with HNE. Using an immunoaffinity-^{32}P-postlabeling method, the authors have investigated etheno-DNA adduct levels 1,N^6-ethenodeoxyadenosine (ϵdA) and of 3,N^4-ethenodeoxycytidine (ϵdC) in the pancreas of chronic pancreatitis patients and in the colon of patients with inflammatory bowel disease. Both ϵdA and ϵdC levels were found to be significantly, 3 and 28 times, respectively, elevated in the inflamed pancreatic tissue. In contrast, only ϵdC was found to be increased in affected colonic mucosa of Crohn's disease (19 times) and of ulcerative colitis patients (4 times) when compared to asymptomatic tissues. In all three cancer-prone diseases, the mean ϵdC-levels in tissues were five- to ninefold higher than those of ϵdA. Differential or impaired DNA repair pathways of these adducts, known to occur by two different glycosylases are implicated. K-*ras* in pancreatic tumors and K-*ras* and *p53* in colon mucosa in long-standing inflammatory bowel disease are known to be highly mutated. The conclusion is that promutagenic etheno-DNA adducts are generated as a consequence of chronic inflammation, acting as a driving force to malignancy in cancer-prone inflammatory diseases. *Antioxid. Redox Signal.* 8, 1003–1010.

INTRODUCTION

The concept that persistent oxidative and nitrosative stress and lipid peroxidation (LPO)-derived DNA damage are one of the likely causes of mutations and genomic instability that drives inflammation induced-carcinogenesis (9, 43) has now received wider acceptance. Pathological processes and mechanisms involved in disease causation have been extensively reviewed (3, 25). In support of this paradigm (Fig. 1), our previous work revealed that oxidative stress-related miscoding DNA-adducts, such as etheno (ϵ)-adducts, increase with time in chronically inflamed target organs and preneoplastic lesions of cancer-prone patients (5). These modified DNA base adducts in human tissues are generated by reactions of DNA with two major LPO-endproducts: *trans*-4-hydroxy-2-nonenal (HNE) and malondialdehyde. HNE yields 1,N^6-ethenodeoxyadenosine (ϵdA), 3,N^4-ethenodeoxycytidine (ϵdC), and N^2,3-ethenodeoxyguanosine, which have been detected *in vivo* (11, 12, 52). Nitric oxide *via* peroxynitrite-induced stress can also produce LPO-derived ϵ-DNA-adducts, as demonstrated in a mouse model: NO overproduction *in vivo* led to a concomitant increase in ϵ-adduct levels in tissue DNA (38). These initial results suggested that these promutagenic, chemically stable ϵ-DNA adducts appear

[1]Division of Toxicology and Cancer Risk Factors, German Cancer Research Center, Heidelberg, Germany.
[2]Department of Surgery, University of Ulm, Ulm, Germany.
[3]Department of Pharmacology, University of Florence, Florence, Italy.
[4]Department of General Surgery, Klinikum Kreis Herford, Herford, Germany.

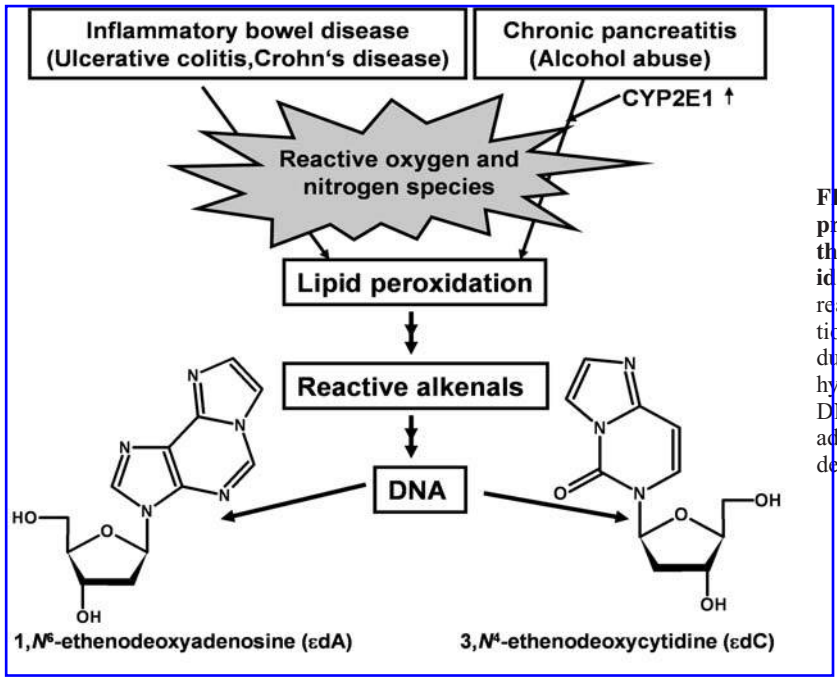

FIG. 1. Illustration of how inflammatory processes in cancer prone organs such as the inflamed pancreas and bowel cause oxidative stress. This involves the release of reactive oxygen and nitrogen species, activation of lipid peroxidation (LPO), and the production of reactive byproducts such as 4-hydroxy-2-nonenal. Upon reaction with DNA-bases, it forms *inter alia* etheno adducts (ϵdA, ϵdC) for which ultrasensitive detection methods have been developed.

to be useful markers for assessing oxidative stress-and LPO-derived DNA damage in early stages of carcinogenesis and that they could play a major role in the development of human cancers, especially those that have an inflammatory component in their etiopathogenesis (6). The biological importance of strongly miscoding ϵ-adducts is further supported as they are also formed from the carcinogen vinyl chloride (4) *via* the reactive oxirane intermediate, and are considered as initiating carcinogenic DNA lesions. HNE has been recognized as a bioactive marker of many pathophysiological processes (55). For example, increased formation of HNE-protein adducts were found in acinar cells of chronic alcoholic pancreatitis patients (8).

Over two-thirds of chronic pancreatitis (CP) cases are caused by alcohol abuse and the risk of pancreatic cancer is significantly elevated in subjects (7, 37) with CP. Some studies have observed an 18- to 28-fold increase in cancer incidence among patients with CP compared to controls, whereas others have revealed more moderate associations (33). Increased oxidative stress and LPO together with depletion of glutathione have been reported to occur in affected tissue of CP patients (8, 51).

Patients with inflammatory bowel diseases (IBD), ulcerative colitis (UC), and Crohn's (CD) have an elevated risk for developing colon cancer (14, 18). In the mucosa of these patients, large quantities of ROS were found to be produced that correlated with disease severity. Furthermore, NO generation and iNOS activity were increased in the affected colonic tissue, together with a depletion of antioxidant defense, rendering DNA in colonic epithelium susceptible to ROS/RNS injury (16). Colon mucosa in long-standing UC showed a high frequency of K-*ras* mutations (22), and an increased *p53* mutation load was observed in regions of UC patients affected by chronic inflammatory conditions than in nonlesional regions of the colon (26).

In this study we have investigated steady-state levels of ϵdA and ϵdC in tissues affected by the cancer-prone chronic inflammatory diseases, CP, CD, and UC, where oxidative stress and LPO are implicated in the disease progression. We have quantified ϵdA and ϵdC by an ultrasensitive immunoaffinity-^{32}P-postlabeling method developed in our laboratory (39). In this report our results on LPO-derived DNA damage, arising from inflammatory processes in human pancreatic and colonic epithelial tissues obtained from patients with CP and IBD at surgery are presented.

We discuss putative mechanisms related to DNA repair so to explain the accumulation of specific etheno-DNA adducts, notably ϵdC, in inflamed cancer-prone organs. We propose that these ϵ-adducts may serve as molecular markers for disease progression and for evaluating the efficacy of disease preventive agents in human intervention trials.

MATERIALS AND METHODS

Tissues and DNA isolation

Archival frozen samples from surgical interventions for the diseases, viz, CP (mostly alcohol induced), CD, and UC were used to isolate DNA. DNA was extracted directly from the homogenized pancreatic tissues, while colonic epithelium was isolated from colon samples using collagen treatment, a procedure described earlier (50). DNA was isolated using Qiagen® columns (Qiagen, Hilden, Germany) according to the manufacturer's protocol with the following modification of the elution buffer in the kit: pH was set to 7.4 and the NaCl concentration was increased to 1.4 *M*. DNA was precipitated by the addition of 0.7 volume isopropanol: it was collected by centrifugation, washed twice with 70% ethanol and dried *in vacuo*. Before analysis, DNA was redissolved in water and quantified by spectrophotometry at 260 nm. From some IBD patients, both affected and unaffected colon mucosa was available for comparison.

DNA adduct analysis

ϵdA and ϵdC were analyzed in DNA by an immunoaffinity/^{32}P-postlabeling method (39). In brief, ~25 μg of DNA was hydrolyzed to nucleotide-3′-monophosphates, using micrococcal nuclease and spleen phosphodiesterase. Normal nucleotides were quantitated by HPLC, and the ϵ-adducts enriched on immunoaffinity columns prepared from the monoclonal antibodies EM-A-1 for (ϵdA) and EM-C-1 for (ϵdC). The antibodies were obtained through a collaborative study with M. Rajewsky (University of Essen, Essen, Germany). The adducts and the internal standard deoxyuridine-3′-monophosphate were labeled with ^{32}P[γ]-ATP (>5000 Ci/mmol) and T4-polynucleotide kinase. The adducts were resolved on polyethyleneimine-TLC plates, using two-directional chromatography, and the relative adduct levels per parent nucleotides are calculated (39).

Statistical analysis

The ϵ-adduct levels in tissues with disease manifestation were compared with those found in asymptomatic normal tissue: pancreas samples were obtained from organ procurement for transplant or research (29), and colon specimens (tissues from accident victims, and distal to carcinoma tissues from operated cancer patients) (50) analyzed earlier in our laboratory. The Mann–Whitney rank sum-test was used for comparisons.

RESULTS

Typical autoradiograms of standards and of ϵ-adducts detected in affected tissue of CP, CD, and UC-patients are depicted in Fig. 2. All samples had measurable adduct levels.

Etheno-DNA adducts in human pancreas DNA

Figure 3 shows the box–whisker plot analysis of pancreatic tissue from asymptomatic (NP) and CP patients. The means (± SD) of ϵdA and ϵdC in CP were 6.3 ± 5.6/10^8 dA and 35.4 ± 25.1/10^8 dC, respectively, and were higher than in the NP (ϵdA 1.9 ± 0.1/10^8 dA and ϵdC 1.1 ± 0.9/10^8 dC). Both etheno-DNA adducts were significantly increased ($p < 0.001$) in CP *versus* NP, ϵdC being 28-times and ϵdA 3-times higher in the inflamed organ. The ϵdC/ϵdA ratio in CP tissue was 9 compared to ~1 in NP. Highly variable ratios of ϵdA/ϵdC levels have been observed in other human organs, likely attributable to differential for impaired DNA repair rate of the two ϵ-adducts (see below).

Etheno-DNA-adducts in colonic epithelial tissues from CD- and UC-patients

Box–whisker plots of etheno-DNA adduct levels in the three groups [asymptomatic controls (N), CD, and UC patients], are shown in Fig. 4. The means (± SD) of ϵdA/10^8 dA in N, CD, and UC were 2.8 ± 2.3, 4.1 ± 4.0, and 1.2 ± 1.0; for ϵdC/10^8dC they were 1.7 ± 1.3, 32.5 ± 34.6, and 6.9 ± 3.6, respectively. Increase of mean ϵdC-levels in the affected tissues was most pronounced in CD, being 19-times higher followed by 4-times in UC ($p < 0.001$). ϵdA levels were about 1.5-times in CD but the difference was not statistically significant. Interestingly, about 60% lower mean ϵdA-levels ($p < 0.005$) were detected in UC compared to normal colon tissue.

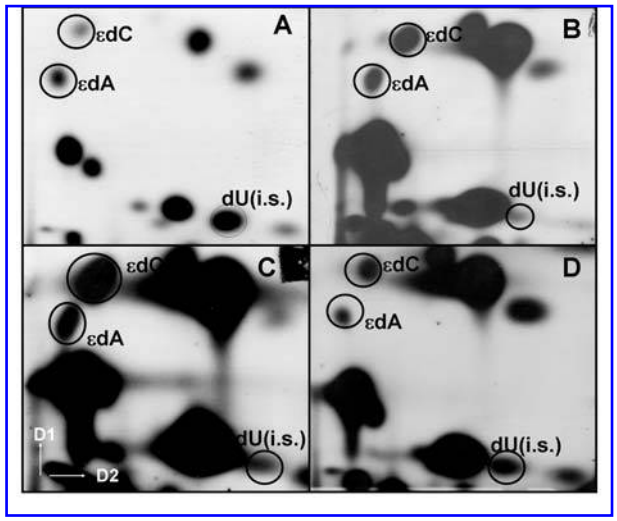

FIG. 2. Representative autoradiograms of TLC of standards (A), from chronic pancreatitis (B), Crohn's disease (C), and ulcerative colitis tissues (D), with adduct spots for ϵdA and ϵdC, using deoxyuridine 3′-monophosphate as internal standard (i.s.). Unmarked spots are residual normal nucleotides and impurities from ATP and kinase (D1 = acetic acid 1 *M*, pH 3.5; D2 = saturated ammonium sulphate, pH 3.5).

The ϵdC/ϵdA-ratios were about 8 and 6 for CD and UC, respectively. ϵ-Adduct analyses of affected versus nonaffected colon mucosa in 5 CD and 4 UC patients are shown in Fig. 5. In 4/5 CD patients, ϵdA and ϵdC-levels were higher in affected mucosa, whereas only ϵdC was found to be elevated in affected mucosa in 3/4 UC patients.

DISCUSSION

Chronic pancreatitis (CP)

This is the first demonstration of LPO-induced DNA damage occurring in affected pancreatic tissue of CP patients (re-

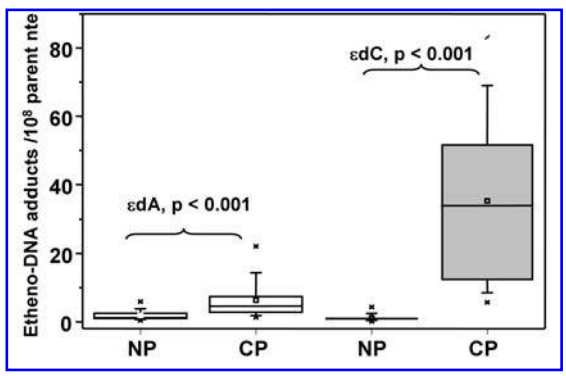

FIG. 3. Etheno-DNA adducts (ϵdA, ϵdC) in human asymptomatic normal pancreas tissue specimens (NP, *n* = 28) and pancreatic epithelium samples from chronic pancreatitis patients (CP, *n* = 20). The box–whisker plot denotes the 25th and 75th percentile values; *horizontal lines* in the box denote the median value. The *error bars* denote the 5th and 95th percentile values. The symbols below the 5th percentile error bar denote the 0th and the symbols above 100th percentiles.

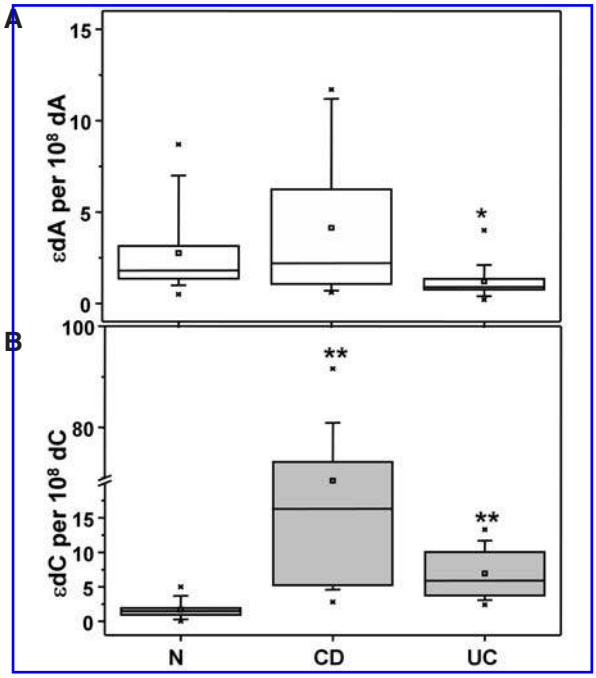

FIG. 4. Etheno-DNA adduct levels (A = ϵdA; B = ϵdC) in normal colon (N), colonic epithelial tissue from patients suffering from Crohn's disease (CD) and ulcerative colitis (UC). (*$p < 0.005$, lower when compared to control N; **$p <$ 0.001, higher when compared to control N). The *box* denotes the 25th and 75th percentile values; *horizontal lines* in the box denote the median value. The *error bars* denote the 5th and 95th percentile values. The symbols below the 5th percentile error bar denote the 0th and the symbols above 100th percentiles.

ported first in an Abstract in 1999 (40)). As almost all cases of CP are associated with alcohol abuse, we conclude that the primary cause of the DNA damage is due to ethanol-induced LPO, most probably *via* CYP 2E1 induction and glutathione depletion, as in the case of alcohol liver disease, where an excess of hepatic ϵ-adducts has been detected (17). Indeed CYP 2E1 induction in the rat pancreas upon chronic alcohol administration (7, 42) and an increase in LPO products upon acute alcohol administration (1) have been demonstrated. In chronic pancreatitis LPO products, conjugated dienes as well as malondialdehyde concentrations in the tissue were significantly elevated. Reduced glutathione was significantly decreased, suggesting glutathione depletion due to oxidative stress (51). In chronic alcoholic pancreatitis, HNE-protein adducts were elevated in acinar cells (8). Taking these data together with our results, there is now strong evidence for a massive increase in LPO and subsequent DNA and protein damage in CP induced by alcohol abuse. We hypothesize that promutagenic ϵ-adducts together with other oxidative DNA-damage increase mutation load and act as a driving force of CP to malignancy. K-*ras* have been found to be highly mutated in hyperplasia and human pancreatic carcinoma (30) that could arise from LPO and oxidative stress-induced DNA damage (35).

Ulcerative colitis (UC) and Crohn's disease (CD)

These inflammatory bowel diseases (IBD) are characterized by chronic intestinal inflammation. Chronic and recurring UC and CD are risk factors for colorectal cancer (33). In the affected colonic mucosa, elevated levels of ROS and RNS were reported that correlated with disease severity; also, nitric oxide generation and iNOS activity were increased, together with a depletion of antioxidant defense, rendering the colonic epithelium susceptible to oxidative injury (16). LPO, as estimated by the malondialdehyde concentration, was elevated in both the inflamed CD and the inflamed UC colon mucosa, and was identified in the luminal epithelium by histochemistry (34). We analyzed the ϵ-adducts in colon epithelium from CD and UC patients, and compared their levels with those found in normal colon. In the cancer-prone colon tissues we demonstrated markedly enhanced levels of promutagenic ϵ-adducts. However, there were adduct-specific dif-

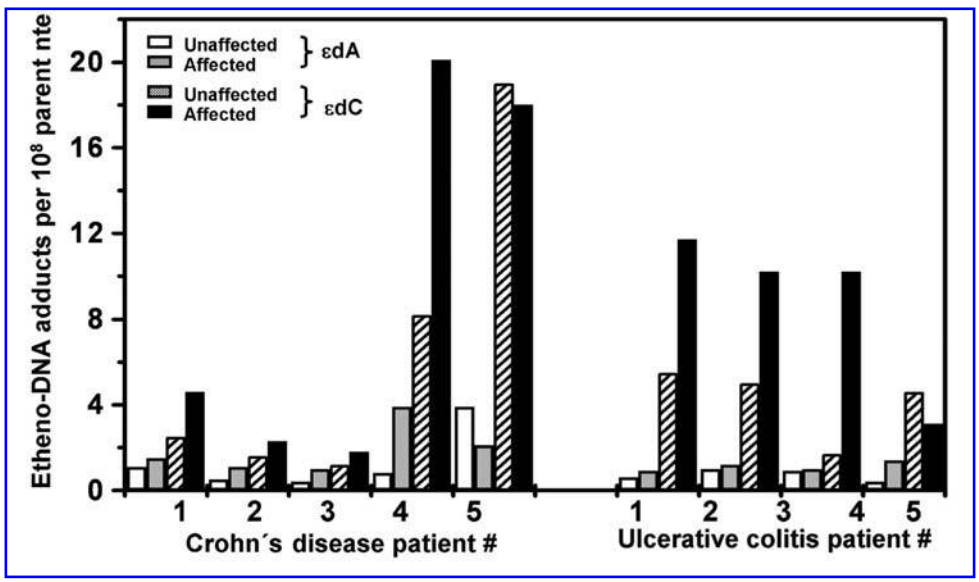

FIG. 5. Comparison of etheno-DNA adducts in affected (inflamed) and unaffected colonic mucosa in individual inflammatory bowel disease patients (n = 4–5).

ferences in these two IBD, both with regard to absolute ϵdA and ϵdC-levels and the adduct level ratios. Hussain *et al*. (26) analyzed UC patients to show that DNA-damage in colon translates into p53 mutations. They found an elevated mutation frequency in dysplastic colon and in colorectal carcinoma. Further, the mutational load was higher in inflamed regions versus nonlesional regions of the same colon. Recently an adaptive imbalance in base excision DNA-repair enzymes (24) was proposed to generate microsatellite instability (MSI) in noncancerous colon of UC patients (see below). These data suggest indeed that an inflammatory microenvironment confers higher susceptibility to colorectal cancer, supporting Rudolph Virchow's original hypothesis (3).

Host-factors that influence steady-state levels of promutagenic oxidative DNA lesions in inflamed cancer prone organs

Host factors that could influence the steady-state levels of ϵ-DNA adducts formed *in vivo* in inflamed cancer prone tissues may include: (a) reduced antioxidant tissue levels; (b) variations in detoxifying reactions of HNE, for example, by glutathione-*S*-transferases that display genetic polymorphisms; (c) a reduced apoptotic rate of damaged cells; (d) differential or impaired DNA repair pathways of ϵ-DNA-adducts in inflamed tissues; and (e) different rates of removal of ϵdA and ϵdC by the substrate-specific repair enzymes (i.e., 3-alkyl-adenine-DNA-glycosylase (ANPG) and mismatch-specific thymine-DNA-glycosylase, respectively) (20, 23, 49). In the latter context the efficient hijacking of the human ANPG by ϵdC-lesions when present in double-stranded or single-stranded DNA was reported (21). Thus, ANPG that normally repairs ϵdA, does not excise but binds to ϵdC, suggesting that ϵdC is not rapidly repaired, accumulates, and could be more genotoxic than ϵdA-lesions *in vivo* following cell division. A recent report (24) has linked inflammation in UC patients with an imbalanced increase in the DNA repair enzymes ANPG and apurinic/apyrimidinic endonuclease (APE1), and paradoxically, with increased MSI. It was positively correlated with the above imbalanced enzymatic repair activities (24). These results were supported by mechanistic studies using yeast (19) and human cell models in which overexpression of ANPG and/or Ape1 was associated with frameshift mutations and MSI. Thus, the adaptive and imbalanced increase in DNA repair enzymes is a novel mechanism contributing to MSI in patients with UC and may extend to chronic inflammatory or other diseases with MSI of unknown etiology.

CP, CD, and UC are chronic inflammatory diseases in which we have shown for the first time that excess steady-state levels of ϵdC over ϵdA exists in DNA of the affected tissues. Moreover, we found that ϵdA-levels were significantly lower in UC compared to normal colon mucosa (Fig. 3). These observations support the above mechanisms that ϵdA is efficiently repaired in UC by upregulation of ANPG but that the repair of ϵdC is hampered by excess ANPG, that binds to ϵdC-lesions. As we observed significantly higher ϵdC-levels in affected tissues of CP and IBD patients, we postulate that an impaired repair of miscoding ϵdC-lesions may be a common feature of chronic inflammatory diseases leading to malignancy.

On the other hand, there is also growing support for the inhibition of some DNA repair pathways and blocking of apoptosis by inflammatory mediators such as NO (54) and by the LPO-endproduct HNE. For example, in a cell line global DNA repair was inhibited when NO was overproduced via iNOS after addition of IL-1β, IFN-γ, and TNF-α (27). A key repair enzyme (Ogg1) responsible for base excision of 8-oxoguanine was inhibited by NO, an inflammatory mediator in a human cell line (28). This inhibition of Ogg1 was also seen in the LEC rat liver (10). This inbred rat strain, a model for human Wilson's disease, accumulates copper, oxidative lesions, and etheno-DNA adducts developing liver tumors at a high rate (41). Another study reported that 8-oxoguanine was only repaired by 50% when a human cell line was treated with IL-6; this interleukin inhibits apoptosis through upregulation of an antiapoptotic gene *mcl*-1, thus retaining more cells with oxidative DNA lesions (36). Recently, Feng *et al*. (15) could show that HNE inhibits nucleotide excision repair in human cells, providing a possible mechanism for LPO-induced carcinogenesis. The repair capacity for benzo[a]pyrene diol-epoxide and UV light-induced DNA damage was greatly compromised in human cells or human cell extracts treated with HNE. Together these results strongly suggest that the LPO-endproduct HNE damages not only DNA but also DNA repair mechanisms. These two detrimental effects of HNE may contribute synergistically to human inflammation-driven carcinogenesis.

Similar conditions (increased levels of inflammatory mediators and of HNE) may prevail in inflamed tissues, possibly leading to the inhibition of global and/or specific repair pathways of oxidative and LPO-derived DNA lesions. Also in premalignant stages the expression of the DNA-dependent protein-kinase, which participates in the repair of DNA double-strand breaks, was significantly decreased when human colon adenomas were compared to normal tissue (48).

Based on these reports and our current results, we assume that inhibition of DNA repair enzymes by inflammatory mediators and/or blockage of proapoptotic pathways may occur, which leads to a differential accumulation of various miscoding DNA lesions that may drive the inflamed tissue cells to full malignancy. Systematic investigations of which of the repair and apoptotic pathways are impaired or imbalanced under inflammatory conditions, whereby NO is often upregulated and HNE overproduced, are now warranted.

Perspectives of using LPO-derived DNA lesions as lead markers for chemoprevention of inflammation-driven malignancies

Work from our laboratory and others have provided evidence that persistent oxidative/nitrosative stress and excess LPO, are induced by inflammatory processes, causing in affected organs accumulation of massive DNA damage from endogenous sources. Together with deregulation of cell homeostasis, these events appear to play an important role in human chronic disease pathogenesis (25). Thus, DNA damage caused by ROS, RNS, and LPO-endproducts that include a variety of promutagenic exocyclic DNA-base adducts (12, 13, 31, 32) provides promising markers for risk prediction and these could also be targets for preventive measures. To facilitate such clinical and field studies in humans, we have de-

veloped (a) a noninvasive urine assay (22) that can quantify excreted etheno-adducted-nucleosides (ϵdA) in a few ml of urine, and (b) biomonitoring methods for simultaneous determination of ϵ-adducts and M_1dG in human tissues and white blood cell DNA (53). Their applicability in human pilot biomonitoring pilot studies have already been shown. Our results on the presence of powerful antioxidants in olive oil (44–47) should encourage trials to explore this antioxidative and cancer-protective potential, particularly in patients with inflammatory bowel and pancreatic diseases.

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ABBREVIATIONS

ANPG, 3-alkyl-adenine-DNA-glycosylase; APE1, apurinic/apyrimidinic endonuclease; CD, Crohn's disease; CP, chronic pancreatitis; ϵdA, 1,N^6-ethenodeoxyadenosine; ϵdC, 3,N^4-ethenodeoxycytidine; HNE, *trans*-4-hydroxy-2-nonenal; IBD, inflammatory bowel disease; LPO, lipid peroxidation; M_1dG, malondialdehyde-deoxyguanosine adduct 3-(2-deoxy-beta-D-erythro-pentofuranosyl)-pyrimido[1,2-alpha]purin-0(3H)one; MSI, microsatellite instability; NO, nitric oxide; NP normal pancreas; Ogg1, 8-oxoguanine glycosylase; RNS, reactive nitrogen species; ROS, reactive nitrogen species; UC, ulcerative colitis.

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Address reprint requests to:
Helmut Bartsch
Division of Toxicology and Cancer Risk Factors
German Cancer Research Center (DKFZ)
Im Neuenheimer Feld 280
69120 Heidelberg, Germany

E-mail: h.bartsch@dkfz.de

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