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Fourier-transform infrared spectroscopy and gas chromatography–mass spectrometry reveal a remarkable degree of structural damage in the DNA of wild fish exposed to toxic chemicals

(cancer etiology/cancer biomarkers/DNA base damage/free radicals/hydroxyl radical)

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ABSTRACT **The use of gas chromatography–mass spectrometry with selected ion monitoring (GC–MS/SIM) and Fourier-transform infrared (FT-IR) spectroscopy revealed a remarkable degree of damage in the hepatic DNA of fish exposed to toxic environmental chemicals, compared with controls. The exposed fish, which were neoplasm-free, were part of a population with a high incidence of liver cancer. GC–MS/SIM showed markedly high concentrations of hydroxyl radical-induced ring-opening products (e.g., 2,6-diamino-4-hydroxy-5-formamidopyrimidine) and 8-hydroxy adducts of adenine and guanine (e.g., 8-hydroxyguanine) in the DNA. FT-IR spectroscopy revealed substantial changes in spectral areas, such as those assigned to NH vibrations of nucleotide bases and CO vibrations of deoxyribose. This diverse and extensive damage to DNA provides a perspective of premalignant changes resulting from xenobiotic exposure and a promising basis for predicting cancer risk in animals and humans.**

Modifications in DNA arising from cytochrome P-450-mediated two-electron oxidation reactions have been a prime focus of attention in attempts to understand mutagenic changes in cellular DNA (1, 2). These reactions, which give rise to bulky adducts of base structures (2), were recently shown to block DNA replication (3) and mRNA transcription (4). However, the cytochrome P-450 reactions also yield H_2O_2 (5) which may be converted to the highly reactive hydroxyl radical (·OH) via the Fe^{2+}-catalyzed Fenton reaction (6).

There is increasing evidence suggesting that ·OH attacks the DNA bases in living systems and that this is a critical factor in mutagenesis and carcinogenesis (7). For example, the ·OH-mediated introduction of a single oxygen into guanine, producing 8-hydroxyguanine (8-OH-Gua), results in a significant, 1–2% base misreading (3, 8, 9) and profoundly alters the methylation of cytosines that control gene expression (10). In fact, ·OH produces a variety of OH adducts and ring-opening products in the DNA of a number of cancerous and noncancerous tissues (7, 11). Thus, clarification of the role played by ·OH in carcinogenesis is of keen interest.

Previous reports (12–14) have shown that high concentrations of ·OH-induced base lesions occur in the livers of fish exposed to environmental chemicals. These included ring-opening products, such as 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapyguanine; Fapy-G), and OH adducts, such as 8-OH-Gua. Strikingly, the base lesions often represented one modification in several hundred normal bases, which is >2000 times the concentration of bulky adducts reported to occur in fish from chemically contaminated environments (15).

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Thus far, only one study has investigated the ·OH-induced base modifications in apparently healthy fish living in a chemically contaminated environment, and relatively high concentrations of base lesions were found in the liver (14). Thus, the question remained whether the results can be generalized to other contaminated environments and, importantly, whether they portend a wider degree of structural damage to the whole DNA molecule. To investigate this possibility, we compared the hepatic DNA of healthy control fish from a relatively pristine area with the hepatic DNA of tumor-free fish from a heavily contaminated waterway where the fish population had a high incidence of hepatic tumors (16). Structural determinations on the DNA employed the powerful complementary techniques of gas chromatography–mass spectrometry with selected ion monitoring (GC–MS/SIM) and Fourier-transform infrared (FT-IR) spectroscopy. The latter technique is ideally suited to the identification of a broad spectrum of structural change that is beyond the scope of the GC–MS/SIM technology (17). The present findings have a wide application to a variety of biological systems and provide important insight into xenobiotic-induced DNA damage and its relation to cancer etiology. Moreover, a promising basis is established for developing premalignant markers for cancer risk assessment with potential application to human and animal populations.

MATERIALS AND METHODS

English sole (1–2 years old) were obtained from the lower reaches of the Duwamish River, Seattle, WA, which is heavily contaminated with various carcinogens and other xenobiotics (18). These include aromatic hydrocarbons (e.g., benzo[*a*]pyrene) and chlorinated hydrocarbons (e.g., polychlorinated biphenyls, PCBs) in the sediments. Controls were obtained from the clean waters off Newport, OR. All livers were excised and immediately frozen in liquid nitrogen.

Histology sections were prepared from the liver and cellular changes were classified as previously reported (16). The reference fish selected as controls proved to be histologically normal, aside from occasional cellular anomalies, such as necrosis. Fish selected from the contaminated environment (the exposed group) were also neoplasm-free but contained lesions previously recognized as toxicopathic (e.g., nuclear pleomorphism) and related to contaminants in sediments (16).

DNA was isolated from the liver (12), dissolved in deionized water, and aliquoted for GC–MS/SIM (≈50 μg) and FT-IR spectroscopy (≈20 μg). Each aliquot was completely

Abbreviations: GC–MS/SIM, gas chromatography–mass spectrometry with selected ion monitoring; FT-IR, Fourier-transform infrared; Fapy-G and Fapy-A, 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 4,6-diamino-5-formamidopyrimidine (ring-opening products of guanine and adenine, respectively).

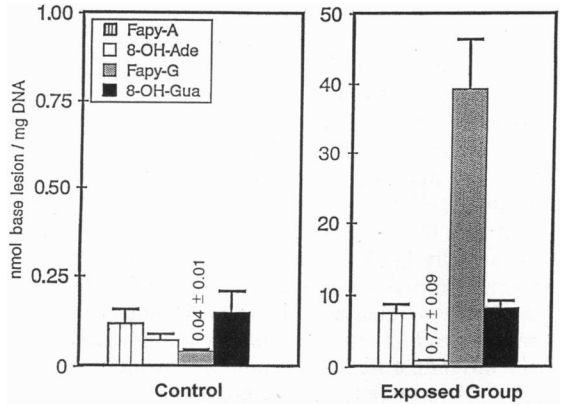

FIG. 1. Concentrations of DNA base lesions in the livers of fish from the control (n = 9) and exposed (n = 8 for Fapy-A and 8-OH-Ade; n = 4 for Fapy-G and 8-OH-Gua) group.

dried by lyophilization, purged with pure nitrogen, and stored in an evacuated, sealed glass vial at −80°C. The ≈50-μg portion was hydrolyzed, derivatized, and analyzed by GC–MS/SIM as described (12). The GC–MS/SIM data were expressed in nmol of base lesion per mg of DNA. The ≈20-μg portion was used directly for FT-IR spectroscopy.

IR spectra were obtained with a Perkin–Elmer System 2000 FT-IR spectrometer equipped with an IR microscope and a wide-range mercury–cadmium–telluride detector. The DNA was placed on a BaF_2 plate in an atmosphere with a relative humidity of less than ≈60% and flattened to make a transparent film. Analyses were performed in triplicate on 3–5 μg and the spectra were computer averaged. Two hundred and fifty-six scans at 4-cm^{-1} resolution were performed for each analysis. Typically, it took 3–5 min to obtain an IR spectrum. None of the spectra showed a band at 1703 cm^{-1}, which is indicative of specific base pairing. This indicated that the samples were essentially water-free and had acquired a disordered form, the D-configuration (19).

IR spectra were obtained in transmission units and converted to absorbance units for data processing. Perkin–Elmer Infrared Data Manager (IRDM) software controlled the spectrometer for data acquisition and performed the deconvolution of spectra. GRAMS/2000 software (Galactic Industries, Salem, NH) performed post-run spectrographic data analysis. Mean IR absorbances were plotted against wavenumbers in the range 1500 to 700 cm^{-1}. The area of the spectrum above ≈1500 cm^{-1} contains a large, poorly differentiated absorption band and is susceptible to absorbances associated with traces of water vapor. This area was also generally unsatisfactory for establishing a suitable valley-to-valley baseline normalization for statistical analysis. Thus, this area was not studied in detail. Spectral differences were evaluated and P values were determined for *every* wavenumber between ≈1500 and 700 cm^{-1}. Normalization was accomplished by converting all absorbancies to a constant mean absorbance.

RESULTS

The GC–MS/SIM analyses showed that the mean values for the base lesions (Fapy-A, 8-OH-Ade, Fapy-G, and 8-OH-Gua) in the control group were <0.20 nmol/mg of DNA (Fig. 1). This is consistent with previous baseline data from the liver DNA of healthy English sole (14). The values from the exposed group were dramatically higher, ranging from 0.77 nmol of 8-OH-Ade per mg to 39.2 nmol of Fapy-G per mg. This remarkable degree of base modification is also evident from the fact that the Fapy-G and 8-OH-Gua concentrations represented one base modification in 15 and 73 normal guanines, respectively. These base lesion relationships for the control and the exposed group are presented in Table 1.

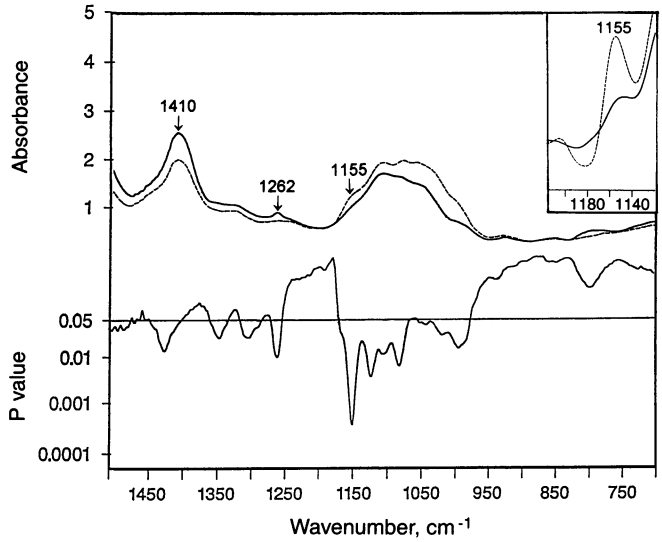

FIG. 2. FT-IR spectra and P-value profiles for comparison of DNA from the control (n = 5) and exposed (n = 16) groups. P values were determined by using an unpaired t test for each wavenumber. The solid and dashed lines represent mean spectra of the control and exposed group, respectively. (*Inset*) Mean spectra in the region of 1150 cm^{-1} after deconvolution (width and smooth factors for deconvolution were 42.0 and 30.0, respectively), an area which is attributed to the nearly degenerate stretching vibrations of the CO group of deoxyribose (17).

In interpreting the FT-IR spectral profiles, associations between frequencies and structural components were made on the basis of previous findings on the IR spectroscopy of DNA (17). About 40% of the wavenumbers between 1500 and 700 cm^{-1} are significantly different at $P \leq 0.05$. Substantial damage to major structural units of the DNA from the exposed fish is particularly evident from the differences occurring mainly in two areas of the spectrum, 1430 to 1250 cm^{-1} and 1160 to 980 cm^{-1} (Fig. 2). The complexity of the DNA structure, particularly after chemical-induced modification, precludes the assignment of specific structural vibrations to many of the areas of spectral difference. However, the significant difference (P = 0.05) in the prominent peak at 1410 cm^{-1}, which we attribute to NH and CH in-plane deformations of the nucleotide bases (17), is most likely associated with the ·OH-induced base modifications shown by GC–MS/SIM (Fig. 1). The spectral difference at 1262 cm^{-1}, which appears as a peak in the control, is provisionally

Table 1. Relationships of base lesions between the control and exposure group

Base lesion	No. studied		P value	Ratio of base lesion to normal base	
	Control	Exposed		Control	Exposed
Fapy-A	9	8	0.000	1:8200	1:120
8-OH-Ade	9	8	0.000	1:12,700	1:1200
Fapy-G	9	4	0.001	1:13,200	1:15
8-OH-Gua	9	4	0.000	1:3600	1:73

P values were determined by an unpaired t test.

assigned to the PO_2 antisymmetric stretching vibrations of the phosphodiester backbone. In mammalian DNA this peak usually appears between 1244 and 1225 cm^{-1}, depending on the type of tissue from which it was derived (17, 20). A highly significant difference ($P = 0.004$) is apparent at 1155 cm^{-1}, which appears as a shoulder in the mean spectrum of the exposed group. This spectral band is attributed to the nearly degenerate stretching vibrations of the CO group of deoxyribose on the basis of prior studies with mammalian DNA (17). To more clearly illustrate the difference at 1155 cm^{-1}, this spectral region was deconvoluted (Fig. 2 *Inset*). The broad area of difference between about 1140 and 980 cm^{-1} in the original convoluted spectra probably includes the PO and PO_2 stretching vibrations of the phosphodiester backbone and stretching vibrations associated with the CO group of deoxyribose (17).

DISCUSSION

Major alterations in the DNA bases of the exposed fish (Fig. 1, Table 1) are attributed to reactions involving ·OH (14), which may arise from H_2O_2 via the Fe^{2+}-catalyzed Fenton reaction (6). The H_2O_2 is possibly produced through the redox cycling (5) of a number of the environmental chemicals to which the fish were continually exposed. The initial attack of ·OH probably first involves the formation of the 8-oxyl derivatives of adenine and guanine (7, 11). At this point, the cellular redox status appears to be critical in determining the degree to which ring-opening products vs. 8-OH adducts are formed (7, 11). Given that recent evidence indicates that the reductively formed Fapy derivatives block DNA replication (3) and mRNA transcription (4), whereas at least 8-OH-Gua leads to significant base misreading (3, 8, 9), the exposed fish would be expected to have a significant (although perhaps temporary) advantage in avoiding cancer by favoring the reductive (Fapy) pathway. In addition, the high concentrations of Fapy derivatives in the DNA of the exposed group (the mean Fapy-G concentration was about 1000-fold greater than that of the control) are likely to slow DNA replication, thereby enhancing the surveillance and repair of OH adducts during cell division (7, 11). Accordingly, 8-OH-Gua may have a diminished genotoxicity in the presence of the elevated concentrations of Fapy derivatives.

The importance of the Fapy derivatives in carcinogenesis is only just being recognized (11, 14, 21). High concentrations of Fapy-G were also found in the cancerous livers of English sole exposed to hydrocarbons (12–14). High ratios of Fapy-A to 8-OH-Gua and 8-OH-Ade were shown to occur in the healthy female breast (11). Fapy-A was substantially depleted, however, in the cancerous breast in favor of the 8-OH adducts. Thus, the putative role played by the Fapy derivatives in protecting tissues against the carcinogenic effects of mutagenic base products (7, 11) may well be phylogenetically conserved (11).

The reaction of ·OH with DNA is known to involve the modification of diverse structural groups. Previous studies with *in vitro* systems (22) have shown that ·OH attacks the deoxyribose moiety, giving rise to a variety of products resulting from hydrogen abstractions of the pentose ring. These lead to ring-opening structures and a carbon-centered radical at the 5′ carbon which links the deoxyribose moiety with the phosphodiester group. A number of radical intermediates are formed which ultimately result in the loss of phosphoric acid and strand breakage (22).

FT-IR spectroscopy of the control DNA and that of the exposed group provided direct evidence in a living system for profound chemically induced damage to the infrastructure of this biopolymer, as exemplified by the fact that a high proportion of the spectral difference between 1500 and 700 cm^{-1} was statistically significant (Fig. 2). It is presently not possible to attribute all of this damage to the action of ·OH; however, at least the substantial alterations in the NH and CO stretching vibrations assigned to the nucleotide bases and the deoxyribose moiety were most likely associated with this highly reactive oxygen species.

The introduction of potentially mutagenic base alterations, such as 8-OH-Gua, into the DNA was substantial and would be likely to increase the cancer risk. This may be partly due to the fact that the replacement of 8-OH-Gua for guanine in DNA profoundly inhibits the methylation of cytosines controlling gene expression (10). Moreover, while the organism has the ability to channel the radical-induced stress into ring-opening products that potentially inhibit carcinogenesis (3, 4), the ultimate outcome is about a 20% incidence of liver cancer (e.g., hepatocellular carcinoma) as a consequence of the continual exposure to high concentrations of xenobiotics (16, 18). The highest incidence of hepatic tumors is found in fish older than those used in this study (i.e., >2 years old) (16, 18). It is notable with respect to the present findings that hepatic tumors of fish exposed to xenobiotics were shown to have higher proportions of 8-OH-Gua compared with Fapy-G (7, 14). This is fully consistent with the redox-associated base transformations found in human breast tissues (11) and the progressive genotoxicity brought about by ·OH damage which ultimately gives rise to a carcinogenic stimulus.

Almost certainly, the xenobiotic-induced DNA damage created a considerable degree of genetic instability. This may well be pivotal in the activation of oncogenes and the deregulation of tumor-suppressor genes, such as p53 (23), and it may at least partially explain the timing of oncogenesis and the ultimate manifestation of liver cancer among the fish population. In this regard, the findings provide an important framework for future studies, with a variety of chemically stressed biological systems, to better understand the nature of the presently reported structural changes in DNA and their implications to carcinogenesis.

In conclusion, the DNA underwent an unprecedented degree of structural modification suggesting that it is considerably more responsive to environmental stresses than previously believed possible. Accordingly, special importance is attached to reactions involving ·OH and possibly other reactive oxygen species in these structural modifications. Further, the diverse structural changes in the DNA, which are readily determined by GC–MS/SIM and FT-IR spectroscopy using small amounts of tissue, provide a promising basis for the development of sensitive biomarkers for assessing genotoxic injury and cancer risk in human and animal populations.

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