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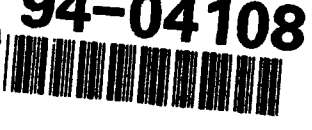

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We have reached a point where substantial reductions have been made in the amount of tissue required for GC-MS analysis. In the future, we will analyze the tissues from a number of normal and exposed organisms using the "micro" method to hopefully validate the approach. The work on monoclonal antibodies is proceeding favorably. Shortly, we expect to obtain a sufficiently pure 8-OH-adenosine to initiate the antigen phase of the work. The FT-IR effort, if successful, will allow for a rapid assessment of DNA modifications in relation to carcinogenesis using as little as a few micrograms of DNA. Overall, the project is on schedule.

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OUTLINE

INTRODUCTION

A need exists for methods to evaluate the genotoxic effects of chemicals on biological systems. Most desirable are approaches that reveal modifications (oxidative, free radical) in DNA of test organisms resulting from exposure to a broad spectrum of environmental chemicals. Ideally, the methods should be readily adaptable to field and laboratory investigations, relatively easy to undertake, and require the use of small amounts of sample.

For the most part, the identification of the effects of complex mixtures of environmental chemicals on biological samples and the establishment of toxicity is based on data relating to single contaminants. In addition, risk assessment is often based on the additivity of effects of known components of the mixture. For the most part, these approaches do not take into account interactive toxicities (e.g., synergistic or antagonistic effects) or the influence of contaminants which are not routinely determined.

A major step toward obtaining a suitable basis for risk assessment would be the development of biomarker (i.e., DNA) protocols that reflect the influence of a variety of contaminants on the biological system of concern. In this regard, small fish species are valuable in the assessment of effects from environmental chemicals because they are less expensive than comparable rodent models and take less time to complete. The choice of a small fish model for testing the toxicity of ground waters, for example, can be rationalized on the basis of a number of considerations, most notably the fact that comparable rodent models are expensive, and related experiments often require two or more years to complete. Moreover, various fish species from around the United States have shown high prevalences of cancer and other pathologic conditions and toxic chemicals have been implicated as causative agents. In addition, a number of studies have shown that small fish species, such as Medaka (O. latipes), develop neoplastic and other types of lesions in various organs (e.g., liver, kidney, eye) when exposed to oncogenic chemicals in the laboratory (2, 12-14, 17 and 22). The liver of this species weighs only a few milligrams, so a requirement exists to develop a DNA biomarker analysis that can be performed on small samples.

In the context of the analytical requirements previously mentioned, we are developing a means of analyzing just a few milligrams of biological sample using a modified gas chromatographic mass spectrophotometric (GC-MS) method we applied to tissue samples and described in previous reports. The "micro" approach employs a non-phenolic DNA extraction designed for the isolation of DNA from milligram amounts of tissue and a substantial increase in

the sensitivity of the GC-MS system used for analysis. Although further testing is required, we have been able to reduce the requirement for tissue (e.g., from Medaka livers) from about 50 mg to 2 mg. This generally allows for the analysis of DNA in duplicate from a single, mature Medaka. The GC-MS method, not including the "microtization," has also been successfully applied to the analysis of a wide variety of tissues, notably those from the normal and cancerous breast. Additionally, we are exploring the use of direct infrared spectral measurement of DNA *that requires only micrograms* of sample. These important new findings are presented and discussed in this mid-term report.

BACKGROUND ON THE SIGNIFICANCE OF RADICAL-INDUCED CHANGES IN DNA

Radicals, formed in the body as a consequence of aerobic metabolism, can produce oxidative damage to somatic cells (reviewed in 1, 26 and 30). These oxidative changes are believed to be an important factor in the etiology of cancer. DNA is a critical target in cellular oxidation because of the pivotal role that this macromolecule plays in information transfer between generations of somatic cells.

In our studies, a variety of DNA lesions have been identified and associated with the interaction of the hydroxy radical(•0H) with the nucleotide bases and subsequent carcinogenesis. These include 8-hydroxyguanine (8-OH-Gua) and 8-hydroxyadenine (8-OH-Ade). Moreover, a clear supportive advance in understanding the association between •OH-induced modifications in DNA and carcinogenesis was obtained when it was shown that 8-OH-Gua was misread in a DNA synthesis system in vitro with E. coli (20). In fact, the presence of the 8-OH-Gua in DNA was viewed as "...an important cause of mutation and carcinogenesis (20)". The examples from our work and that of others represent a growing body of evidence implicating the •OH in DNA damage and carcinogenesis.

Formation of the hydroxyl radical

The reduction of molecular oxygen in all aerobic eukaryotic cells results in the formation of intermediates that are highly toxic. These include the superoxide ion (O_2^-), H_2O_2 and •OH. While O_2^- and H_2O_2 individually may not be particularly damaging, their combined action leads to

to the formation of the highly reactive •OH:

$$O_2^{\cdot} + H_2O_2 \rightarrow {\bullet}OH + OH^{-} + O_2$$

This reaction can be relatively slow; however, when catalyzed by metal ions [e.g., Fe^{+2}], the reaction is substantially accelerated and becomes especially relevant in the initiation of biological damage (16). H_2O_2 itself is converted to •OH through the iron (Fe^{+2}) catalyzed Fenton reaction. The proliferation of •OH may then result in an attack on most molecules in living cells with deleterious consequences. The primary defense against such radical-induced damage is provided by enzymes that catalytically scavenge the intermediates of oxygen reduction. For example, $O_2^{\cdot}$ is eliminated by superoxide dismutase (SOD) which catalyses a dismutation reaction leading to the formation of O_2 and H_2O. In addition, the latter structures are destroyed by catalases and glutathione peroxidase (30). Clearly, circumstances resulting in a failure to control the highly reactive •OH are likely to lead to oxidative damage to DNA and other biological systems.

Using the gas chromatography-mass spectrometry/single ion monitoring (GC-MS/SIM) protocols developed on this project, it is now possible to determine a number of oxidative modifications in DNA extracted from milligram amounts of normal and neoplastic tissues. These include, for example, 8-hydroxyguanine, 8-hydroxyadenine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua).

EXPERIMENTAL METHODS

Isolation of DNA

Liver samples (e.g., from Medaka) were used for the isolation of pure DNA from as little as 2 to 5 mg of starting material. Our previous "macro" methods involved pretreatments of the sample with Proteinase K and RNase A, after which recovery of the nucleic acids from the cellular lysate was accomplished by employing phenol and chloroform to partition the nucleic acids into an aqueous phase and the other cellular components, including proteins, into an organic phase.

Our laboratory now employs a simpler and much more efficient "micro" method for isolation of the DNA (See Table 1 below). The Microprobe IsoQuick® Nucleic Acid Extraction Kit utilizes the properties of guanidine thiocyanate (GuSCN) to both disrupt the cellular integrity of the sample and, at the same time, inhibit the DNase and RNase activity. The GuSCN is then mixed with a non-corrosive reagent containing a nuclease-binding matrix. The aqueous and organic phases are separated by centrifugation and the DNA is precipitated with alcohol. Four

milligrams of purified Chelex® 100 Resin (Bio Rad) is added to the DNA to remove any Fe^{++} that might be present. As a consequence, we have also found that the addition of Chelex® 100 Resin results in greater purification of DNA, yielding a higher A260/A280 spectral ratio. The DNA is quantitated in aqueous solution by its UV absorption at 260 nm using the relationship 1 absorbance unit = 50 μg/ml. Table 1 shows the yields of DNA obtained from various weights of Medaka, together with the A260/A280 spectral ratio:

Table 1. Improved DNA Extraction Methodology

Sample ID	Liver Weight (mg)	AMT. DNA (ug)	A260/A280
EE3-93-027-17-5	6.4	92.5	1.82
EE3-93-027-17-11	3.0	40.5	1.90
EE3-93-027-18-24	3.0	63.8	1.91
EE3-93-027-18-27	3.4	68.0	1.84
EE3-93-027-18-33	4.1	93.8	1.86
EE3-93-027-17-26	3.6	71.0	1.91
EE3-93-027-17-20	2.2	40.5	1.80
EE3-93-027-17-28	3.6	65.1	1.78

Preparation of trimethylsilyl derivatives.

The procedure employed was a modification of that used previously (7). For example, samples of purified DNA are now made usually with 30 - 50 μg and done in either duplicate or triplicate, depending upon the availability of DNA. All are treated in evacuated sealed tubes at 140°C for 30 minutes with 0.25 ml of concentrated formic acid (60%). The treatment with formic acid does not alter the structure of the nucleotide bases being studied. After hydrolysis, the samples are dried in a desiccator under vacuum. The trimethylsilyl derivatives are produced in a 0.1 ml of mixture of bis(trimethylsilyl)trifluor-acetamide (BSTFA) and acetonitrile (4:1) in polytetrafluorethylene-capped hypovials (Pierce Chemical Company) upon heating for 30 minutes at 140°C.

Synthesis of oxidized nucleotide bases

Several of the standards required for the GC/MS-SIM procedure were obtained from commercial sources; others had to be synthesized in our laboratories. Examples of syntheses conducted are given below:

8-Hydroxyadenine was synthesized, using 5-bromocytosine and 8-bromoadenine, respectively. These compounds were allowed to react with 95% concentrated formic acid at 140° C for 45 minutes. Excess unreacted formic acid was removed by nitrogen purge and the product was purified by recrystalization from water.

2,6-Diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) was synthesized from 2,5,6 triamino-4-hydroxypyrimidine sulfate and 80% formic acid at 60°C for one hour. Excess formic acid was removed with nitrogen. The product of the reaction was purified by recrystalization from water and purity established by GC-MS.

Gas chromatography-mass spectrometry/selected ion monitoring.

The analyses for oxidized nucleotide bases was conducted with a Hewlett-Packard Model 5890 microprocessor-controlled gas chromatograph interfaced to a Hewlett-Packard model 5970B Mass Selective Detector. The injector port and interface were both maintained at 260°C. The column was a fused silica capillary column (12.0 m, 0.2 mm inner diameter) coated with cross-linked 5% phenylmethylsilicone gum phase (film thickness, 0.33 μm). The column temperature was programmed from 120°to 235°C at 10°C/min. after 2 min. at 120°C. Helium was used as the carrier gas with a linear velocity of 57.3 cm/s through the column. The amount of TMS hydrolysate injected onto the column was about 0.5 μg. Quantitation of TMS-nucleotide bases was done on the basis of the principal ion and confirmation of structure was undertaken using two qualifier ions.

Several major improvements have been made in the GC-MS methodology with the objective of "microtizing" the DNA analysis. A Hewlett-Packard Merlin Microseal® has been installed which decreases septum leakage and, at the same time, eliminates the presence of septum particulates in the injection liner which can cause activation of the liner. The MS detector has been changed to a Hewlett-Packard K-M® model, thereby increasing the sensitivity of the MS by about five-fold. The automatic injector has been altered so that the syringe pumps each sample a total of 12 times with a viscosity delay of 7 seconds. The BSTFA:ACN solvent is viscous enough to allow air bubbles to enter the syringe if a viscosity delay is not used. Though minute, these air bubbles can cause a 20-40% error in reproducibility.

A most important advance made in the GC-MS method is the automation of the quantitation procedure. The quantitation files for the base lesions have been integrated into one file, allowing each sample to be quantitated for all five lesions at once, rather than by 5 separate files. The results are individually checked for proper peak integration and then transferred to a MS Excel database in which the conversion from pg/μl to nmol/mg DNA (including all recovery and reproducibility factors) is automatically figured. The result is in tabular form, and the data is readily converted to a bar graph or other suitable depiction.

Data collection and analysis.

Using the GC-MS/SIM methodology, characteristic ions (one principal ion and two qualifier ions) were employed to characterize the oxidized bases; however, as indicated, the principal ion was used for quantitation. All spectra were compared with spectra obtained from commercially obtained standards and authentic samples of TMS derivatives synthesized in our laboratories. The data obtained included SIM plots and derived mass spectra. On the basis of the GC-MS/SIM data, oxidized base concentrations in hepatic DNA were calculated and recorded as nmol/mg.

EXPERIMENTAL FINDINGS

Studies on Medaka using reduced tissue sample weights

The USABRDL provided samples of medaka liver exposed to TCE together with controls. The DNA is now being extracted from a number of these liver samples. Preliminary findings indicate that sufficient DNA is obtained from a single medaka liver to allow analysis by GC-MS/SIM in duplicate (See Table 1). The modifications made in the DNA extraction procedure, the increased sensitivity of the Hewlett Packard instrument and other modifications in technique have made the "microtization" a reality. We expect to complete analysis of the medaka DNA soon. Our progress was hampered for a number weeks, mostly related to technical problems affecting the performance of the GC-MS instrument.

Application of the GC-MS/SIM technique to the analysis of human normal and cancerous breast tissues

As a consequence of the work done on this project, substantial hydroxyl radical (•OH)-induced base lesions were found in the DNA of invasive ductal carcinoma of the female breast.

However, virtually no information was available regarding relationships between the different base lesions in the normal and cancerous breast. Such information is essential in understanding initial stages in the development of breast cancer and the potential of the base lesions as early predictors of cancer risk.

The •OH-induced DNA base lesions in normal reduction mammoplasty tissue (RMT) were compared to those from invasive ductal carcinoma (IDC) and nearby microscopically normal tissue (MNT). Comparisons were then undertaken on relationships between the base lesion profiles in the normal and cancerous breast using twenty-two statistical models.

DNA from the RMT was characterized by a high ratio of ring-opening products (e.g., 4,6-diamino-5-formamidopyrimidine) to hydroxy-adducts of adenine and guanine. A dramatic shift in this relationship in favor of carcinogenic hydroxy-adducts (e.g., 8-hydroxyguanine) was found in the cancerous breast. Statistical models with a high sensitivity (91%) and specificity (97%) provided a consistent means of classifying tissues (e.g., 96% correct).

The dramatic shift in the DNA base lesion relationships in oncogenesis is attributed to alterations in the redox potential of the breast favoring oxidative conditions and cancer formation. These findings suggest that base lesion profiles are potential sentinels for cancer risk assessment. Further, intervention in controlling the tissue redox potential may provide benefit in delaying or preventing early oncogenic changes and the ultimate manifestation of cancer.

Specifically, reduction mammoplasty tissue (RMT) was obtained from 15 patients. The tissue from 10 patients was sequentially cut into 1 cm sagittal sections, two cm apart. Two to 13 sections were obtained from each patient for a total of 70 samples. In addition, tumor (IDC) and nearby microscopically normal tissue (MNT) were obtained from the cancerous breasts of 15 surgical patients. This group comprised 22 samples, 7 of which were matched pairs (IDC-MNT); the remainder were single biopsy specimens from either IDC tissue or MNT. The RMT from the non-cancer patients was also microscopically normal with the exception of occasional incidences of non-neoplastic changes (e.g., fibrocystic).

After excision, each tissue was immediately frozen in liquid nitrogen and maintained at -70°C. The frozen tissue (~ 350 mg) was minced with a scalpel, placed in 3 ml of phosphate buffer solution and homogenized for one minute over ice. Then 2.0 ml of 2X Lysis buffer (Applied Biosystems, Inc., Foster City, CA) and 300 μL of RNase A (Boehringer-Mannheim, Corp., Indianapolis, IN) were added and the sample was incubated at 60°C for one hour. Proteinase K (Applied Biosystems, Inc.) was added and incubation was allowed to proceed overnight at 60°C. DNA was then extracted as previously described (9). The DNA was

hydrolyzed as described above. Trimethylsilyl (TMS) derivatives of the previously determined purine bases and 5-hydroxymethyluracil (HMUra) were analyzed by gas chromatography-mass spectrometry with selected ion monitoring (GC-MS/SIM) as previously described in this report. Quantitation of the DNA lesions was undertaken on the basis of the principal ion and confirmation of structure was undertaken by using qualifier ions. For example, the primary ion for the TMS derivative of Fapy-A was $m/z = 354$ and the main qualifier ion was $m/z = 369$. All analyses were performed in duplicate or triplicate, depending upon the amount of tissue available and the lipid content. About 350 mg of breast tissue, yielding an average of 150 μg of DNA, were usually sufficient for a base lesion analysis of a single sample in triplicate. Reproducibility between determinations was greater than 90%. Calf thymus DNA was used as a negative control and showed minimal DNA base lesions (concentrations close to those at the threshold of detection for the GC-MS/SIM procedure).

Statistical models were established for predicting the origin of the tissue sections (cancer or non-cancer) and to determine the sensitivity and specificity of this classification. Sensitivity and specificity were defined in the usual way: sensitivity is the percentage of cancer tissue samples that were correctly classified (true positives), using the models, and specificity is the percentage of non-cancer tissue samples that were correctly classified (true negatives). The value $p < 0.05$ was used to designate statistically significant differences and associations.

Graphical analysis showed the logarithm of values to be more closely related to cancer vs. non-cancer origin of tissue sections and more normally distributed than values on the natural scale. Thus, we used $\log_{10}$ concentrations and $\log_{10}$ ratios of concentrations in all analyses. The concentration of HMUra was below the detection limit of 0.0002 nmol/mg DNA for 14 sections and these sections were assigned a value of 0.0001 nmol/mg DNA. The mean values for cancer and non-cancer tissue of the $\log_{10}$ concentrations and ratios and the statistical significance of differences were calculated using methods developed by Laird and Ware that, in our case, take account of the dependence of multiple sections from individual patients (21). The method is similar to ordinary multiple linear regression in other regards.

In order to build a model for predicting the origin of the tissue sections (cancer vs. non-cancer), we used an extension of these methods by Stiratelli et al., developed for binary variables (28). In our context, this is a model for the probability that a specific tissue derives from a cancer or a non-cancer patient. The probability is expressed as a function of $\log_{10}$ concentrations or ratios of concentrations. To use it as a predictive model, a cut-off probability, P_C, is selected (e.g., $P_C = 0.5$) and tissue samples with an estimated probability above this value are labeled as

cancer-derived. We calculated the sensitivity and the specificity of the classification, based on trial cut-off values from $P_C = 0.1$ to $P_C = 0.9$ in 0.1 increments, and chose the value of P_C that gave the highest combined values (expressed as a sum) of sensitivity and specificity.

We determined if mean concentrations or ratios of concentrations differed between MNT and IDC tissue from cancer patients, using the Laird-Ware model with the $\log_{10}$ values as dependent variables, MNT vs. IDC as a dichotomous independent variable, and patient as a random effect.

The GC-MS/SIM analyses revealed dramatic differences in the concentrations of the DNA base lesions between the cancerous breast and the RMT. Both the IDC and the MNT were characterized by relatively high proportions of OH-adducts produced via the oxidation of the nucleotide bases. The base lesions were 8-OH-Ade, 8-OH-Gua and HMUra. Fapy derivatives, which are produced through reductive pathways from the initially formed 8-oxyl derivatives, were present in relatively small concentrations in the cancerous breast. However, the relationship between the concentrations of the OH-adduct and Fapy derivatives was dramatically different in the RMT. Overall, a clear distinction was evident between the ratios of Fapy:8-hydroxy base lesion concentrations in the cancerous tissue and those of the normal tissue.

None of the tissues examined showed evidence of inflammatory responses during histologic examination. Thus, there is no evidence for any contribution from infiltrating cells in the proportions of reported DNA lesions. Each of the IDC and MNT specimens had a mirror-image "control" histologic section prepared and examined in the absence of any knowledge of the DNA base lesion data.

Fapy-A concentrations predominated in the RMT sections compared to 8-OH-Gua by a factor of ~ 4- to 10-fold; as depicted in Figure 1 below:

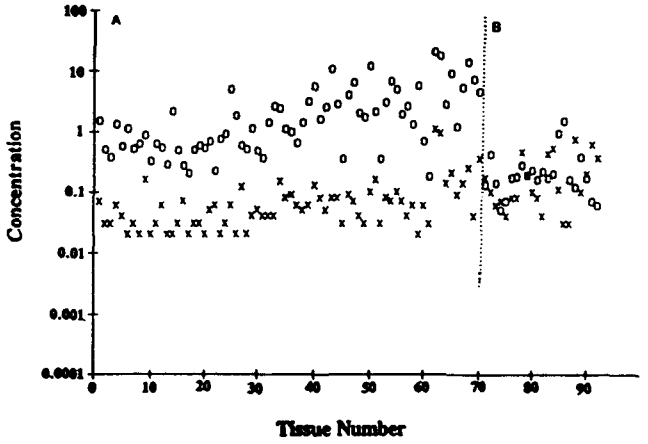


Figure 1. A scatterplot depicting the relationship between the $\log_{10}$ concentration of the base lesions (nmol/mg DNA) versus tissues analyzed. Panel A: RMT; panel B: IDC and MNT. Circles represent Fapy-A and X represents 8-OH-Gua.

Remarkably high concentrations of Fapy-A were found in the RMT (mean ± S.E.= 2.9 ± 0.49 nmol/mg DNA; one base lesion in 320 normal bases). Surprisingly, for example, one patient had a RMT section that contained 21.0 nmol Fapy-A/mg DNA, or one base lesion in 46 normal bases. Overall, high concentrations of Fapy derivatives in the RMT did not prevent the formation of significant concentrations of OH-adducts in some tissues. The tissue section from the patient mentioned above had a relatively high 8-OH-Gua concentration of 1.1 nmol/mg DNA (one base lesion in 540 normal bases). Thus, two aspects relevant to •OH-induced carcinogenesis are the redox status of the tissue and the absolute concentrations of mutagenic OH-adducts (e.g., 8-OH-Gua). Both of these parameters would be pivotal in the assessment of carcinogenic risk factors.

In contrast, the IDC and MNT sections were characterized overall by elevations in 8-OH-Gua compared to RMT, coupled with a marked depletion of Fapy-A residues. Thus, the results further indicate that fundamental differences exist in the nature of the •OH-induced base damage in relation to cancerous and non-cancerous tissues. This is evident from the histogram shown below in Figure 2, which depicts the concentrations (mean ± S.E.) of the base lesions in the RMT, MNT and IDC tissues.

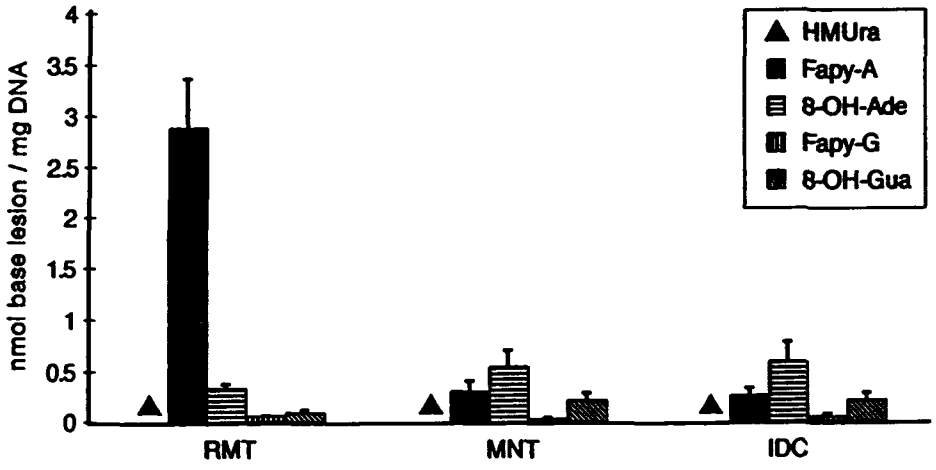


Figure 2. A histogram of DNA base lesion values (mean ± standard error) for the cancerous (MNT and IDC) and normal female breast (RMT). The relatively low base lesion values for HMUra are designated by a triangle (▲): RMT = 0.0007 ± 0.0001; MNT = 0.0021 ± 0.0004; and IDC = 0.0021 ± 0.0005.

A statistical analysis of the data was conducted yielding the mean values of the various indicators ($\log_{10}$ concentrations or $\log_{10}$ ratios of concentrations) for cancer and non-cancer tissue and the statistical significance of differences using the Laird-Ware regression model. The sensitivity and specificity were calculated using the predictive logistic regression model. Most

significance levels are strikingly small, indicating prominent differences between cancer and non-cancer tissue with respect to a wide array of predictors. No correlation between patient age and predictors was observed. Consequently, age was not included in the analyses, neither in the cancer dataset nor in the non-cancer dataset, and can be ruled out as a cause of the strong association between the base lesions and the origins of tissue sections. These data are presented in Table 2 below:

Table 2. Mean $\log_{10}$ concentrations and $\log_{10}$ ratios of concentrations of DNA-base lesions from reductive mamoplasty tissue (RMT) and cancerous breast tissue (IDC and MNT) and sensitivity and specificity based on statistical models.*

Indicator		Non-cancer patients		Cancer patients		Predictive Model (Logistic regression)		
$\log_{10}$ (concentrations)	P-value*	Mean	S.E.	Mean	S.E.	Sensitivity (%)	Specificity (%)	P-value**
HMUra	.0000	-3.3	.1	-2.8	.1	91	69	.0001
Fapy-A	.0000	.2	.1	-.7	.1	82	93	.0000
8-OH-Ade	.2	-.6	.1	-.5	.1	100	30	.04
Fapy-G	.01	-1.4	.1	-1.7	.1	55	90	.01
8-OH-Gua	.04	-1.2	.1	-.9	.1	59	80	.004
Fapy-A + Fapy-G	.0000	.3	.1	-.7	.1	77	96	.0000
8-OH-Ade + 8-OH-Gua	.2	-.5	.1	-.3	.1	100	36	.02
8-OH-Ade + 8-OH-Gua + HMUra	.2	-.5	.1	-.3	.1	100	36	.02
$\log_{10}$ (ratio of concentrations)								
Fapy-A/HMUra	.0000	3.6	.1	2.0	.1	91	97	.0001
8-OH-Ade/HMUra	.001	2.7	.1	2.3	.1	91	44	.004
Fapy-G/HMUra	.0000	1.9	.1	1.0	.1	73	97	.0000
8-OH-Gua/HMUra	.02	2.1	.1	1.9	.1	95	30	.04
Fapy-A-8/OH-Ade	.0000	.9	.1	-.3	.1	91	96	.0000
Fapy-A/Fapy-G	.0000	1.7	.1	1.0	.1	95	79	.0000
Fapy-A/8-OH-Gua	.0000	1.4	.1	.2	.1	91	94	.0001
8-OH-Ade/Fapy-G	.0004	.8	.1	1.3	.1	68	91	.0003
8-OH-Ade/8-OH-Gua	.06	.6	.03	.4	.1	64	63	.09
Fapy-G/8-OH-Gua	.0000	-.2	.1	-.8	.1	68	91	.0002
Fapy-A/(8-OH-Ade + 8-OH-Gua)	.0000	.8	.1	-.4	.1	91	97	.0001
Fapy-A/(8-OH-Ade + 8-OH-Gua + HMUra)	.0000	.8	.1	-.4	.1	91	97	.0001
(Fapy-A + Fapy-G)/ (8-OH-Ade + 8-OH-Gua)	.0000	.8	.1	-.4	.1	91	97	.0000
(Fapy-A + Fapy-G)/ (8-OH-Ade + 8-OH-Gua + HMUra)	.0000	.8	.1	-.4	.1	91	97	.0000

*Based on *linear* regression random-effects model, testing the null hypothesis of equality of means for cancer and non-cancer (RMT) patients.

**Based on logistic regression random-effects model, testing the null hypothesis that the $\log_{10}$ value is not associated with cancer vs. non-cancer (RMT) classification of tissue section.

Due to the number of comparisons made (22 predictors were assessed; 9 of high sensitivity and specificity are given as examples in the tabular data above), it is likely that one or

two would be statistically significant by chance alone. However, if all the p-values determined are multiplied by 22, which is the conservative Bonferroni adjustment, almost all of the p-values would still be statistically significant, including those for the $\log_{10}$ ratio that is considered further below.

We used the model for the $\log_{10}$ ratio of summed Fapy derivatives to summed OH-adducts plus HMUra because it is based on reductive vs. oxidative conversion pathways of the initial 8-oxyl derivative and is one of the best models for predicting the cancer vs. non-cancer origin of tissue. However, as the above table clearly shows, there are other models with high sensitivity and specificity and very small significance levels. The size of this dataset does not allow definitive selection among the several good models. The predictive equation is :

$$\log_e [P/(1-P)] = 0.76 - 6.34 \times \log_{10} (\text{ratio})$$

where P is the probability that a tissue sample derives from a cancer patient and "ratio" refers to the ratio of the sum of the two Fapy derivatives to the sum of the two OH-adducts plus HMUra. The standard errors of the constant term and for the multiplier of the $\log_{10}$ ratio in the model above are 0.58 and 1.53, respectively. Using the model and the cut-off $P_C = 0.5$, tissue samples with an estimated probability $P > 0.5$ were classified as cancer-derived, and those with $P \leq 0.5$ were classified as non-cancer derived. The corresponding ratio of concentrations that best divides cancer from non-cancer samples is 1.32. As can be seen in Table 2, the sensitivity (91%) and specificity (97%) are both very high.

In addition to the classification based on (Fapy-A + Fapy-G)/(8-OH-Ade + 8-OH-Gua + HMUra), we also show the classification based on a model of high sensitivity and specificity using the ratio (Fapy-A/(8-OH-Gua). In the latter model, the predictive equation is:

$$\log_e [P/(1-P)] = 3.71 - 5.51 \log_{10} (\text{ratio}).$$

The standard errors of the intercept and multiplier of $\log_{10}$ are 1.20 and 1.38, respectively. The cut-point for the predictive probability used in classifying a cancer-derived tissue is $P_C > 0.4$, which corresponds to a ratio of concentrations of 5.6 or less.

The comparison of $\log_{10}$ concentrations and ratios between IDC and MNT showed no statistical differences, thus indicating that the observed DNA base modifications were pervasive in both the IDC and MNT. However, due to the small sample size (N = 22 sections), large

differences in concentrations or ratios between IDC and MNT cannot be ruled out.

Based upon the pronounced differences in base lesion profiles and concentrations between the cancer and normal tissue, a graph of predicted probability of the cancerous origin of a tissue vs. $\log_{10}$ of the concentration ratios was constructed. This demonstrates the strong ability of this model to discriminate the nature of each tissue. The data are given below in Figure 3:

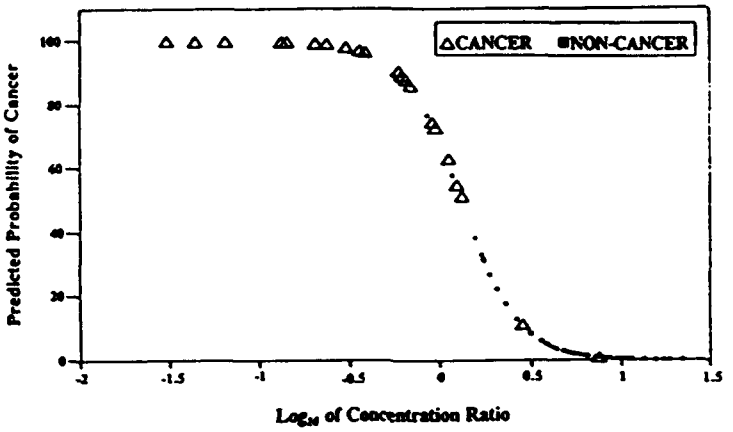


Figure 3. The predicted probability of the cancerous origin of a tissue is plotted with $\log_{10}$ of the concentration ratio (Fapy-A + Fapy-G)/ (8-OH-Ade + 8-OH-Gua + HMUra) for all samples analyzed.

It is known that oxidative stress is linked to cancer formation (30) and that increases in OH- ducts (e.g., 8-OH-Gua) are a likely consequence of oxidative conditions in the cell. Consistent with this is the concept that the oxidative modifications of DNA structure reported in breast cancer are the probable basis for the carcinogenic action of H_2O_2 generation. Although multiple biochemical processes may be involved, it is suggested that the •OH may arise as a consequence of the formation of H_2O_2 from redox cycling of endogenous (e.g., hormones) or exogenous effectors (e.g., polychlorinated biphenyls [PCBs] and chlorinated hydrocarbons), mediated by cytochrome P-450 and cytochrome P-450 reductase (6).

It is noteworthy that breast tissues of women with breast cancer have elevated concentrations of PCBs compared to those with benign breast disease (10). In this regard, the previously reported (8) relationship between fat intake and HMUra in DNA of peripheral nucleated blood cells of women with breast cancer may reflect, at least in part, the influence of organic xenobiotics enriched in the dietary fat.

The H_2O_2, which is readily transported across the nuclear membrane, is likely converted to

the •OH via the Fe^{++}-catalyzed Fenton reaction. The subsequent attack of the •OH on the nucleotide bases results in the formation of the 8-oxyl derivatives of the purines and the hydroxylation of thymine to form HMUra. At this point, the conversions of the purines can either lead to oxidatively-formed OH-adducts that potentially increase cancer risk or to reductively-formed Fapy derivatives that are putatively non-genotoxic. The synthesis of the ring-opening structures appears to protect the DNA from potentially mutagenic OH-adduct formation and, as such, reflects a unique antioxidant role for the DNA base structure. Strikingly, the nature of these transformations occurring in the cell leading to differing classes of base lesions is entirely consistent with the redox-coupled pathways of •OH-induced purine modifications occurring in aqueous solution as described by Steenken (27). It is particularly noteworthy that oxidants (e.g., O_2) in aqueous solution quantitatively suppress Fapy derivatives and increase the yield of 8-OH-adducts (see citations in 27). In view of this, we were not surprised that the most effective predictive models shown in Table 1 [e.g., (Fapy-A + Fapy-G/8-OH-Ade + 8-OH-Gua + HMUra)] were completely consistent with the above-mentioned pathways. The proposed pathway for the synthesis of the OH-adducts and Fapy derivatives is given below in Figure 4:

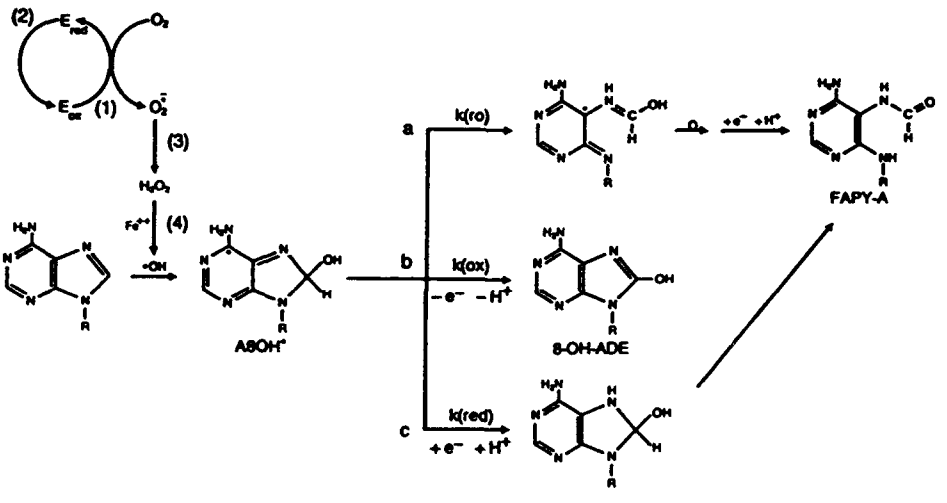


Figure 4. A proposed scheme for the formation of the ring-opening (Fapy) derivatives and 8-OH-adducts in the female breast. As an example, adenine is converted to the 8-oxyl derivative (A8OH·) via the attack of the ·OH. The ·OH is derived from the Fe^{++}-catalyzed conversion of H_2O_2 (pathway 4). The H_2O_2 may arise from multiple metabolic processes occurring in the breast epithelial cells, one of which may include the redox cycling of an endogenous or exogenous effector molecule (E) via cytochrome P-450 oxidase (pathway 2) and cytochrome P-450 reductase (pathway 1). The A8OH· can be converted oxidatively to 8-OH-Ade (pathway b) or reductively to Fapy-A (pathway a or c). The redox balance in the breast cells would dictate the ratio, for example, of 8-OH-Gua:Fapy-A formed with increases in cellular oxidants favoring pathway b and potential cancer formation. The cytochrome P-450 pathways are essentially as described by Deodatta et al.[19] and the aqueous solution redox chemistry and transformation reactions are based on those described by Steenken.[10]

We conclude that the •OH-induced oxidative base damage likely represents an event of considerable importance in the early development of breast cancer. For example, the DNA from several sections of the normal breast contained greater than one 8-OH-Gua base lesion in 1,000 normal bases. The presence of elevated levels of 8-OH-Gua in the DNA of a relatively small number of normal breast sections is perhaps to be anticipated considering the fact that one out of eight women develop breast cancer on a lifetime basis. In this context, the attack of the •OH on the base structure of the breast DNA would be expected to result in the activation or augmentation of nuclear oncogenes and the deregulation of tumor suppresser genes, such as p53 (15). Other genotoxic changes are likely and the greater the intensity of the radical attack, the greater the expectation of mutagenic events occurring.

In considering the proposed role played by cellular redox conditions and base lesion formation in the etiology of breast cancer, it was recognized that DNA repair may potentially play a significant part in processes that govern these circumstances. Enzymes capable of repairing Fapy and 8-hydroxypurine derivatives are known to be constitutively expressed in E. coli and mammals (4). Moreover, growing evidence indicates that one of these enzymes, the FGP protein, is involved in the repair of both Fapy and 8-hydroxy base lesions (29). Although the 8-hydroxy-dG derivative may result in some inhibition of DNA replication, more specifically it is known to be mutagenic, resulting in miscoding lesions due to a 1-to-2% level of misrepair (11, 18). However, there is no current evidence supporting a mutagenic property for the ring-opening lesions. Instead, the Fapy residues have been shown to block DNA synthesis (3). Thus, unrepaired Fapy residues, which are abundant in the DNA from the normal breast, would not be expected to be genotoxic, although they may be cytotoxic. For differential DNA repair to explain the present findings, the transition between high ratios of Fapy : hydroxy derivatives in the RMT to low ratios in the IDC and MNT would be expected, for example, to involve preferential repair of the Fapy-A residue while the 8-hydroxy derivatives increased. This circumstance does not conform to the known behavior of the DNA repair mechanisms involved. In fact, support for our hypothesis for oxidation-driven base lesion changes during oncogenesis in breast cancer includes evidence for decreased DNA repair in cancers of the breast, colon and lung (24), the presently demonstrated increased concentrations of OH-adducts in the cancerous breast, and the finding that trans-tamoxifen exerts an antioxidant effect (i.e., a decrease in tumor promoter-induced H_2O_2 formation in human neutrophils) that correlates with diminished concentrations of oxidatively-formed HMUra. Of additional significance is the fact that patients with a single breast cancer are at increased risk of having a second primary tumor in the breast (25). Our findings showing that $\log_{10}$ base concentrations and ratios between

IDC tissue and MNT were not statistically different is consistent with this finding. That is, significant oxidative base damage in the DNA would be expected to still be present in the MNT after the tumor is removed, thus potentially increasing the risk of a second tumor occurring.

Regarding the statistical models, the sensitivity and specificity calculated from our specific dataset (Table 2) can be expected to be somewhat high (non-conservative) compared to the specificity and sensitivity that would be calculated from a trial of the predictive equation with a new population of tissue samples. This bias occurs because the sensitivity and specificity have been optimized within this specific dataset. The statistical significance calculations, however, are unbiased for inference about a similar mix of RMT and cancer patients as observed here. Thus, the promise of this area is very strong (based on significance levels), but specific screening models should be based on a larger dataset with more cancer patients and normal individuals.

The sensitivity and specificity in this study have been calculated for classification of tissue samples and not for classification of individual patients. However, multiple tissue samples from patients are very likely to be classified consistently. For example, classification of tissue sections based on $\log_{10}$(Fapy-A + Fapy-G)/(8-OH-Ade + 8-OH-Gua + HMUra) and $\log_{10}$(Fapy-A/8-OH-Gua) showed only 4/92 and 6/92 incorrect classifications of cancer vs. non-cancer tissue, respectively. Thus, the method is most promising for use in classification of patients based on individual samples of tissue.

The models considered were based on retrospective analysis of the origin of the tissue. The curve displaying the *probability of cancer vs. the $\log_{10}$ of base lesion concentration* ratios clearly affirms the difference in the results between the cancer and non-cancer patients (See Figure 3). Given the biological implications of the differing classes of base lesions, which are formed as a function of the cellular redox potential as discussed above, it is reasonable to conclude that this may represent a basis for prospective cancer risk to be estimated through log transformations of base lesion concentrations in the DNA of breast tissues. In this regard, it is noteworthy that the probability model classifies certain of the tissues examined as having base lesion concentrations that may reflect transitional states between those of normal and cancerous tissue. Evaluation of the potential risk of an individual developing breast cancer at an early stage represents an important potential of this analysis.

The model that predicts cancer vs. non-cancer status may thus also predict future risk as well. Evaluation of the present methodology for clinical application would require a prospective study of women at variable predicted risks for developing breast cancer, based on models such as we have developed here. Such a study would naturally include an evaluation of relationships

between diet, ethnic differences, reproductive history, familial history, and other relevant factors. If prospective studies confirm our results, individuals identified to have a heightened predicted cancer risk would be expected to benefit from close monitoring and possible intervention with antioxidants or other agents. The close association between cancer chemoprotection and compounds with antioxidant activity (19, 23, 31 and 32) is consistent with this potential and the results presented in this paper.

In conclusion, it is clear that the DNA base lesion profiles reflect intrinsic differences that exist between normal and cancer-derived tissues in a manner regulated by the redox condition of the breast cells. The nature of these base lesions present in the tissues represents a useful sentinel for evaluating the prevailing redox conditions. Further, potential mutagenic damage to the DNA base structure can be assessed. On this basis, it is a logical assumption that a shift in the base profiles characteristic of normal breast tissue to profiles characteristic of cancer tissue is early evidence for a heightened risk of cancer formation. In this context, the results presented describe a potentially powerful method for defining characteristic changes in the DNA of female breast tissue during oncogenesis. Given the fact that analyses can be performed readily on small amounts of biopsied tissue, this method could ultimately have wide application for determining individuals at risk in the population.

Monoclonal antibody studies

Research has been conducted on the development of monoclonal antibodies specific for oxidative modifications of DNA bases. The intention was to use appropriate specific antibodies as a means to detect and quantitate oxidative lesions in DNA derived from biological specimens. The ultimate goal is to simplify the process for analysis of these lesions.

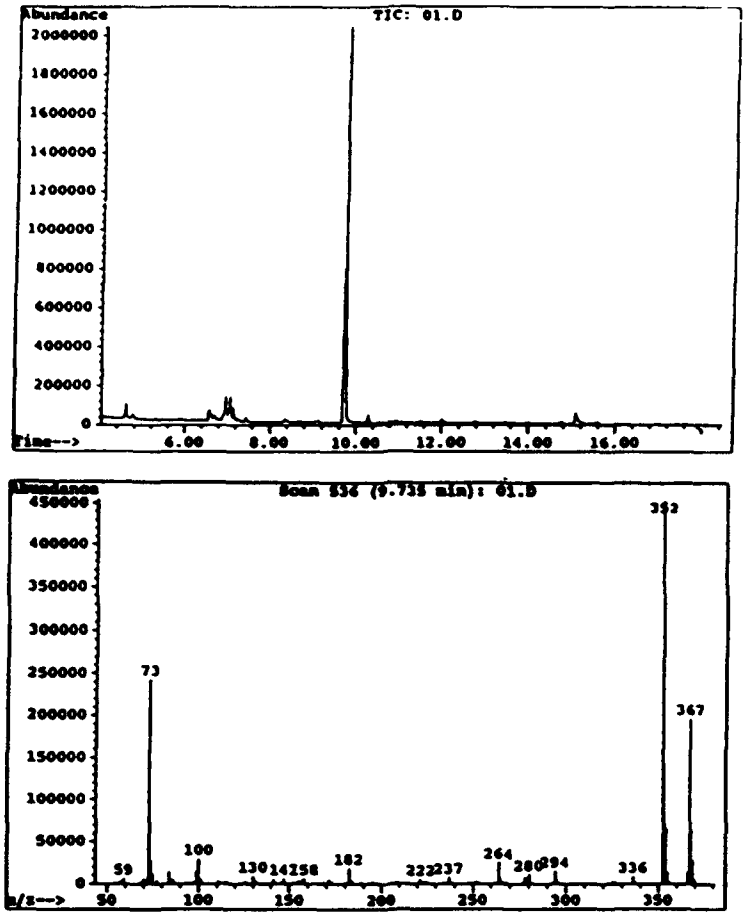
We have initially focused on the oxidative lesions of adenosine, 8-OH-adenosine and the ring-opening Fapy-adenosine derivatives. The initial step is the production of each lesion in adequate amounts for immunization of animals and screening of hybridomas. The strategy we are using is as follows:

8-OH-adenosine

This compound has been prepared from 8-Br-adenosine using the procedure described by Cho and Evans (5). In this procedure 8-Br-adenosine is reacted with benzyl alcohol and Nametal. An 8-benzyloxyl derivative is formed which decomposes to give 8-OH-adenosine upon

acidification of this mixture. This structure was confirmed by GC-MS analysis and is illustrated in Figure 5 below:

Fig. 5.



8-OH-Adenosine is then isolated by chromatography and crystallization. This material is then allowed to react with $NaIO_4$ to oxidize the vicinal hydroxyl groups of the ribose, yielding aldehyde groups capable of forming Schiff bases with primary amines. The antigen for immunization will be prepared by Schiff base formation with lysine groups of keyhole limpets hemocyanin followed by reductionwith $NaCNBH_3$. The resulting antigen will be used for immunization. Keyhole limpets hemocyanin is a conjugation protein of choice for such a coupling reaction since it aids in stimulating an immune response from attached ligands. Because of this, such an antigen will not be useful for hybridoma screening. Instead, the IO_4^- oxidized derivative will be coupled to phosphatidylethanolamine. This will place the specific ligand of interest on a molecule that will be ideal for solid-phase immunoassays for use in the screening process.

Fapy-adenosine

We are using commercial Fapyadenine (Sigma Chemical Co.) as an antigen source. In order to couple it to keyhole limpets, hemocyanin for immunization, or to a phospholipid for screening, a different strategy is required. There is a plane of symmetry dividing the molecule such that the two $-NH_2$ groups are equivalent. Thus, application of a photoactivatable, heterobifunctional cross-linking reagent (eg. SANPAH, Pierce Chemical Co.) is used to couple the Fapy-A group to protein. This cross linker reacts with primary amino groups forming an amide linkage to a spacer arm which contains an azide group on the opposite end. By mixing this with either protein or lipid and exposing it to UV light, the antigen ligand can be photoinserted into the carrier molecule.

At present we have prepared the ligand structures for coupling to carrier molecules. Once completed, immunizations will be conducted immediately. In each case above, animals are immunized multiple times and derived spleen cells fused with myeloma cells. The process of screening involves standard steps commonly used in the laboratory for antibody screening. This portion of the project is the next step.

Infrared statistical analysis of DNA

Our intention is to examine DNA using the Fourir Transform-Infrared (FT-IR) instrument which we recently obtained. Preliminary data, provided courtesy of the Perkin-Elmer Corporation, indicated that substantial differences exist in the spectral properties of DNA from cancerous breast tissue and normal breast tissue. Accordingly, GC-MS data will be compared with that obtained by FT-IR.

CONCLUSIONS AND FUTURE PLANS

We have reached a point where substantial reductions have been made in the amount of tissue required for GC-MS analysis. In the future, we will analyze the tissues from a number of normal and exposed organisms using the "micro" method to hopefully validate the approach. The work

on monoclonal antibodies is proceeding favorably. Shortly, we expect to obtain a sufficiently pure 8-OH-adenosine to initiate the antigen phase of the work. The FT-IR effort, if successful, will allow for a rapid assessment of DNA modifications in relation to carcinogenesis using as little as a few micrograms of DNA. Overall, the project is on schedule.

PUBLICATIONS FROM THIS PROJECT

Malins DC, Holmes EH, Polissar NL and SJ Gunselman. 1993. The etiology of breast cancer: Characteristic alterations in hydroxyl radical-induced DNA base lesions during oncogenesis with potential for evaluating incidence risk. Cancer, 71 (10): 3036-3043.

Malins, DC. 1993. Identification of hydroxyl radical-induced lesions in DNA base structure: Biomarkers with a putative link to cancer development. J. Tox. Environ. Hlth. (In Press).

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