Analysis of DNA Adducts in Putative Premalignant Hepatic Nodules and Nontarget Tissues of Rats during 2-Acetylaminofluorene Carcinogenesis

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ABSTRACT

Exposure of rats to a standard four-cycle feeding regimen of 0.06% 2-acetylaminofluorene (AAF) results in the formation of putatively premalignant hepatic nodules, but the types and magnitude of DNA adducts formed in these nodules has not been previously examined. By using a sensitive 32P-adduct assay (R. C. Gupta, Cancer Res., 45: 5656-5662, 1985), we analyzed the DNA adduct lesions in individual hepatic nodules at various times during and after exposure to AAF. Kidney, spleen, and testis were included as nontarget tissues. No qualitative difference was observed in the DNA adducts found in hepatic nodules and nontarget tissues, however, quantitative differences occurred. At least one unknown and two known (dG-C8-AF and dG-N2-AAF) DNA adducts were detected, with dG-C8-AF being predominantly (96-98%) formed, in all tissues examined. At the end of the first three weeks of AAF feeding, the concentration of the deacetylated adduct dG-C8-AF in liver (223 fmol/µg DNA) was found to be about 2, 6, and 5 times higher than in kidney, spleen, and testis, respectively. The concentration of the N2-acetylated adduct in liver (4.5 fmol/µg DNA) was 4-fold higher than in kidney and strikingly higher (51- and 42-fold, respectively) than in spleen and testis. At the end of the fourth feeding cycle, total DNA adducts measured in the hepatic nodules ranged from 30-100 fmol/µg DNA, while the “surrounding liver,” kidney, spleen, and testis showed 235, 218, 62, and 28 fmol/µg DNA, respectively. Sixty days following the cessation of AAF, the binding in both the persistent nodules and liver had decreased to 7% of their respective levels measured at the end of the fourth cycle, while adducts in kidney, spleen, and testis were 32%, 18% and 19%. After 88 days, the binding levels in the nontarget tissues declined further, but no additional adduct removal occurred in the nodules. Our data indicate that (a) although the metabolic apparatus for activation of AAF is diminished in the hepatic nodules, a significant level of adduct formation occurs in the cells of this putative, premalignant lesion, and (b) unlike in the nontarget tissues, repair processes in the premalignant nodules may not be operative several weeks after the cessation of AAF exposure.

INTRODUCTION

A critical phase in the development of hepatocellular carcinoma is the appearance of focal collections of altered hepatocytes, termed nodules, during the preneoplastic period. The majority of the nodules “disappear” by regressing or remodeling to normal-appearing liver by a process of redifferentiation (1) while a minority persist rather than show this remodeling (2, 3). The persistent nodules are considered to represent premalignant lesions.

The covalent binding of the reactive carcinogen or metabolite with DNA is generally believed to be the primary event in the initiation of chemically induced carcinogenesis. The degree of DNA damage and subsequent repair of individual DNA lesions, especially in target cells therefore serve as measures of carcinogenic potential. The experimental hepatocarcinogen AAF or its N-hydroxyl derivative reacts almost exclusively with the guanine moiety in DNA, forming two acetylated adducts, dG-C8-AAF and dG-N2-AAF and one deacetylated adduct, dG-C8-AF, all of which exhibit variable persistence (4-9).

The formation and removal of AAF-DNA adducts have been examined previously after multiple dosing or chronic exposure in liver and kidney (5, 6, 8, 10), but DNA of a putatively premalignant focal lesion has not been analyzed for adducts, primarily owing to methodological limitations. The present study was undertaken to measure by a 32P-postlabeling assay (9, 11) specific DNA adducts in the rat hepatic nodules and the nontarget tissues during and after cessation of chronic AAF exposure.

MATERIALS AND METHODS

Animals and Diets. Male Sprague-Dawley rats (100-g body weight) were obtained from Charles River Breeding Laboratories (Wilmington, MA). They were maintained on a standard four-cycle carcinogenic regimen (2, 3): AAF was mixed with a semipurified diet at 0.06%. A feeding cycle consisted of 3 weeks of AAF diet, followed by 1 week of control diet. Numerous nodules, ranging from 3 to 15 mm in diameter, were observed in the livers at the end of the fourth cycle. These nodules were considered to be putatively premalignant lesions: livers bearing such nodules eventually have a high incidence of hepatocellular carcinomas (2, 3). Liver, nodules, kidney, spleen, and testis were excised to examine the formation and persistence of DNA adducts. The nodules were carefully dissected free of nonnodular tissue; the larger nodules (50-400 mg) were analyzed individually, while smaller nodules (20-50 mg), where specific, were analyzed after pooling. To serve as untreated controls, a group of rats received only control diet.

Isolation of DNA and Analysis of Adducts. Total DNA was isolated from frozen tissues by a rapid solvent-extraction procedure as described (12), except that RNase T1 and A concentrations were increased to 100 units/ml and 150 µg/ml, respectively, for more complete removal of RNA from the liver and nodule DNA. For nuclear DNA, nuclei were prepared (13) from fresh tissues and then DNA was isolated as above.

For adduct analysis, DNA was enzymatically digested to deoxyribose 3'-monophosphates, and adducts were isolated from 5 µg of DNA digest by extraction with 1-butanol and 5'-32P-labeled with carrier-free [32P]ATP (150 µCi; specific activity > 3000 Ci/mmol) by T4 polynucleotide kinase phosphorylation (11). The 32P-labeled digest (equivalent of 4.3 µg of DNA) was analyzed by multidirectional PEl-cellulose TLC (9), with modifications (11). After dilution with water, an aliquot of the DNA digest (5 µg) was directly 32P labeled using the same [γ-32P]ATP preparation as used for labeling of the isolated adducts, and total nucleotides were analyzed by one-directional TLC of an aliquot (0.1 ng DNA) of the labeled digest (11). Radioactivity in adduct and total nucleotides was measured by Cerenkov assay (without the addition of scintillator) and the adduct concentrations were evaluated as follows: relative adduct labeling = cpm in adduct nucleotide(s)/cpm in total nucleotides × (1/dilution factor), which was then translated into fmol of adducts per µg DNA, considering 1 µg of DNA = 0.3 × 105 fmol nucleotides (7, 10). Detailed conditions of the assay have been described elsewhere (11).

1 The abbreviations used are: AAF, 2-acetylaminofluorene; dG-C8-AAF, N-(deoxyguanosin-8-yl)-2-acetylaminofluorene; dG-N2-AAF, 3-(deoxyguanosin-N2-yl)-2-acetylaminofluorene dG-C8-AF, N-(deoxyguanosin-8-yl)-2-acetylaminofluorene; PEI, polyethyleneimine; TLC, thin-layer chromatography.

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5270

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RESULTS

The cyclic feeding regimen induces grossly evident liver nodules at the end of the second cycle, that increase in number and size and demonstrate somewhat altered appearance through the third and fourth cycles. Nodules that persist after cessation of carcinogen feeding are evident only after the fourth cycle.

AAF-induced DNA adducts were measured at the end of the first and fourth carcinogen periods (before return to normal diet) and also 60 and 88 days after the fourth AAF-feeding cycle. At the end of 3 weeks of the AAF regimen, when no focal lesions were detectable in the liver, the target (liver) and non-target (kidney, spleen, and testis) tissues were excised and stored at -80°C until DNA isolation. At the end of the fourth feeding cycle and thereafter, hepatic nodules were excised, and these and the non-target tissues were collected and frozen. DNA isolated from the various tissues was found to be nearly free (97%) from RNA contamination, except for the liver and nodule DNA preparations which showed 8–20% RNA contamination.

RNA was analyzed by one- or two-directional PEI-cellulose TLC (9, 11) of the 32P-labeled DNA digest. Under these conditions, major RNA nucleotides migrate somewhat slower than the deoxynucleotide counterparts. Values of the total DNA nucleotide radioactivity were therefore corrected for RNA contamination when calculating the adduct concentrations.

To determine if adduct concentrations in the total DNA and nuclear DNA were comparable, several hepatic nodules (about 1 g), obtained after the fourth AAF feeding regimen, were pooled. One half of the tissue was used to prepare total DNA, the other half to prepare nuclear DNA. No significant difference was found in the adduct levels in the total DNA (81 ± 1 fmol/μg DNA) as compared to nuclear DNA (79 ± 5 fmol/μg DNA). All subsequent analyses for hepatic nodules and other tissues were therefore done with genomic DNA.

At the end of the first AAF feeding cycle, 32P maps of DNA from the rat liver, kidney, spleen, and testis (Fig. 1, a–g) revealed one predominant (96–98%) (Spot 4) and two minor adducts (Spots 1 and 3) when compared with the map obtained from untreated rat liver DNA (Fig. 1h). Cochromatography with calf thymus DNA modified in vitro with N-acetoxy-AAF or N-OH-AAF (7, 9) demonstrated that the predominant rat DNA adduct was a C8-aminofluorene-substituted guanine adduct (dG-C8-AAF), while one of the minor adducts was a N2-acetylamino-fluorene-substituted guanine derivative (dG-N2-AAF). Rechromatography of Spots 1, 3, and 4 by magnet transfer and development in three solvents, namely, isopropanol: 4 M ammonia, 1:1, 0.7 M sodium phosphate/7 M urea, pH 6.0 and 0.2 M sodium bicarbonate/6 M urea, pH 8.3 did not reveal the presence of any additional spot (not shown). These results indicate that spots 1, 3, and 4 may not contain any unresolved adducts.

Significant quantitative differences were found in the formation of individual adducts in the different tissues (Fig. 2A), as calculated by measurement of the adduct radioactivity. The level of dG-C8-AAF adduct in liver (223 fmol/μg DNA) was about 2, 6, and 5 times higher than in kidney, spleen, and testis, respectively. The N2-acetylated adduct concentration in liver (4.8 fmol/μg DNA) was about 4 times higher than in kidney DNA, but strikingly higher than in spleen and testis DNA (51- and 42-fold, respectively). The level of the unknown adduct (Fig. 1, Spot 3) in the liver DNA (4.7 fmol/μg DNA) was also higher by about 2 times than in kidney and 6 times each than in spleen and testis.

At the end of the fourth AAF-feeding cycle, numerous hepatic nodules were detected. Four or more nodules were excised from each rat liver. DNA isolated from nodules contained mostly the same adducts as found in the “surrounding liver” and the nontarget tissues, particularly kidney, i.e., the predominant (95–96%) dG-C8-AAF and the two minor lesions (dG-N2-AAF and adduct 3) (Fig. 1). Significant (up to threefold) internodule differences were observed in the DNA adduct levels (Fig. 2B).

No qualitative differences were found between the adduct profiles of individual nodules or pools or among the adduct profiles of nodules excised from the left lobe and right lobe (data not shown).

Fig. 1. 32P-Adduct maps of DNA isolated from the target (individual hepatic nodules, a–f) and nontarget tissues (e–g) of male Sprague-Dawley rats at the end of the fourth AAF-feeding cycle; h, map of hepatic DNA of rats fed control diet alone. Adducts were isolated from DNA digest, 32P-labeled, and separated by multi-directional PEI-cellulose TLC (see text). After removal of the radioactive nonadduct components by development in 1.0 M sodium phosphate, pH 6.0 (top to bottom), the adducts retained at the origin were resolved by development in 3 M lithium formate, 7 M urea, pH 3.5 (bottom to top) and 0.6 M lithium chloride, 0.5 M Tris-HCl, 7 M urea, pH 8.0 (left to right). The residual radioactive contaminants were removed by a final development in 0.35 M MgCl2 (left to right). Intensifying screen-enhanced autoradiographic exposure was at ~80°C for 4 h. Spot 1, dGp-N2-AAF; Spot 4, dGp-C8-AAF; Spot 2, undigested dinucleotides of adduct 4; and Spot 3, unknown. Spots encircled were detectable after a three- to fourfold longer exposure.
When compared with the first AAF feeding cycle, the binding levels in the liver after the fourth feeding cycle remained unchanged (Fig. 2B). At this stage, the liver was apparently contaminated with the nodule cell population. Therefore, the binding levels in the liver after the fourth feeding cycle remained separate tissues, while for nodules, mean was from 8-14 tissues. The nodules were not detectable at this stage.

Adduct levels measured here may not reflect true binding values than indicated in Fig. I, legend. Under these conditions, both spots were clearly resolved.

Table I: Ratios of the major adduct (C8-AF) to the minor adducts (N2-AAF and adduct 3) during and after the cessation of AAF feeding

<table>
<thead>
<tr>
<th>AAF regimen</th>
<th>Time (days)</th>
<th>Liver</th>
<th>Hepatic nodules</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Testis</th>
</tr>
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<tbody>
<tr>
<td>1 x 3 AAF</td>
<td>50 ± 2*</td>
<td>98 ± 18</td>
<td>419 ± 98</td>
<td>411 ± 362</td>
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<td></td>
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<tr>
<td></td>
<td>(51 ± 6)</td>
<td></td>
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<tr>
<td>4 x 3 AAF</td>
<td>81 ± 39</td>
<td>38 ± 0</td>
<td>47 ± 4</td>
<td>59 ± 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(60 ± 5)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>4 x 3 AAF</td>
<td>88 ± 26</td>
<td>53 ± 11</td>
<td>72 ± 33</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>(82 ± 5)</td>
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* Values of C8-AF/adduct 3 are given in parentheses.

The data presented in Table 1 also indicate that (A) there was a greater reduction in the ratio of C8-AF/N2-AAF than that of C8-AF/adduct 3, in all tissues, suggesting that the unknown adduct 3 is more persistent than the N2-AAF adduct and (B) the ratio of C8-AF/N2-AAF in liver was less than in kidney and much less than in spleen or testis, indicating the lowest levels of acetylated adducts in the spleen and testis.

Interestingly, the newly detected adduct (Fig. 1, Spot 3), which composed 2–3% of the total adducts at the end of the first and fourth AAF-feeding cycles, was present in 1–2 times greater amounts than the N2-acetylated adduct in the liver, kidney, and nodules, and 3–9 times more in the spleen and testis (Fig. 2, A and B). The unknown adduct represented 6–10% of the total adducts in various tissues 88 days after the cessation of the AAF regimen (Fig. 3B), suggesting a more efficient removal of this adduct than the C8-AF adduct (also see Table 1). Although the chemical identity of this adduct remains as yet unknown, its close chromatographic resemblance to C8-AF was noted.?
to the C8-AF adduct suggests it is a deacetylated derivative (or a compound of similar hydrophobic nature) rather than an acetylated derivative. The possibility that this adduct is a decomposition product [such as the ring-opened forms (4)] of the C8-AF adduct was ruled out because of the continuous decrease in the ratio of the C8-AF adduct to the adduct in question during and after the cessation of the AAF regimen. Furthermore, the ring-opened forms of C8-AF adduct migrate much faster than adduct 3 (7, 9). The latter adduct was also unrelated to any possible dinucleotides of dG-C8-AF because these adducted dinucleotides migrate in the location marked as Spot 2 (Fig. 1).

**DISCUSSION**

A highly sensitive 32P-postlabeling assay has provided a unique opportunity to measure a spectrum of DNA adducts in target (liver and liver nodules) and nontarget (kidney, spleen, testis) tissues during and after exposure to AAF. Experiments presented here demonstrated the following: (1) a significant level of adduct formation occurs in this putative, premalignant lesion (hepatic nodules) and persists for about 3 months after cessation of AAF exposure; and (2) AAF-DNA adducts are formed in nontarget tissues and persist for several months.

Hepatic nodules have been shown to be defective in their capacity to metabolize carcinogens (14, 15). Although they did not analyze the DNA adducts, Farber et al. (16) showed a two- to fourfold lower binding of radiolabeled AAF to node DNA than to the surrounding liver. The size of this difference was correlated with the decrease in carcinogen uptake or a reduction in its activation (16). Stout and Becker (13) have reported a considerable decrease in the ability of microsomes from these nodules to produce mutagenizing products from AAF. Using immunohistochemical technique, Poirier and coworkers (17) have demonstrated a lack of AAF-adduct formation in enzyme-altered foci. The results shown here indicate a marked reduction in the level of all specific adducts in nodules compared with the surrounding liver, normal liver, and even nontarget tissues, particularly kidney. Since the N2-acetylated adduct results from O-sulfation (18) and the C8-deacetylated adduct probably results from an N,O-acetyltransferase-catalyzed product (19-21) or deacetylation (22), the proportionate reduction in these adducts observed in nodules (Fig. 2B) suggests that the various metabolizing enzymes were equally deficient in the premalignant hepatic lesions. A significant reduction in the level of AAF-DNA adducts in hepatic nodules (59–125 fmol/μg DNA) as compared to the surrounding liver (173–244 fmol/μg DNA) has also been observed in yet another study in which male F-344 rats were fed 0.25% ciprofibrate diet for 60 weeks to induce nodules (23), followed by 0.25% AAF diet for 5 weeks. Furthermore, DNA adduct removal from the nodules and surrounding liver in this study was observed only up to 2 weeks examined after the cessation of AAF exposure.*

Upon feeding radioactive AAF or N-OH-AAF Irving and Veazey (24) and Szafracz and Weisburger (25) found binding levels of dG-C8-AF in the liver in the range of 100–400 fmol/μg DNA. Using radioimmunoassay, Poirier et al. (6) also found the level of dG-C8-AF adduct to be in the range of 100–250 fmol/μg DNA. Given the potential sources of variability in carcinogen feeding and the diverse methods used to measure adducts it is remarkable that our data obtained by another assay fall in the same range (223 fmol/μg DNA, Fig. 24).

Another finding of this study is the observation that, although a large (an average of 93%) reduction occurred in the level of adducts in nodules 60 days after the last feeding cycle, no further adduct losses were apparent over the subsequent 28 days. Adducts were, however, progressively removed from the DNA in the nontarget tissues. These results suggest that repair processes or cell proliferation or both may be nonoperative in the nodule tissues 60 days after carcinogen withdrawal. It may be noted that adduct persistence in the normal hepatic DNA continues to occur even after 7 months in male Sprague-Dawley rats following four i.p. injections of AAF (26). The persistent nodules used in the present study led to hepatocellular carcinomas as early as 60 days after cessation of feeding AAF, and over 60% of the livers develop carcinomas after 12 months (2, 3). Whether the persistent AAF-induced DNA adducts in these hepatic premalignant lesions are present in subpopulations or in specific genomic regions (10, 12) and what consequences these molecular events may have on the process of carcinogenicity remain yet to be seen.

**REFERENCES**

15. Okita, K., Noda, K., Fukumoto, Y., and Takemoto, T. Cytochrome P-450 in specific genomic regions (10, 12) and what consequences these molecular events may have on the process of carcinogenicity remain yet to be seen.


5273
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