DNA Adducts in Human Breast Tissue: Association with N-Acetyltransferase-2 (NAT2) and NAT1 Genotypes

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Abstract
The etiology of human breast cancer is poorly understood, but circumstantial evidence points toward exogenous genotoxins as causative agents; they are believed to exert their carcinogenic action by binding to DNA. Because this binding is often preceded by metabolic activation, it is dependent on the expression and activities of metabolic enzymes of the host. Human mammary tissue samples from 42 women undergoing surgery for breast cancer or reduction mammoplasty were analyzed for DNA adducts by 32P-postlabeling analysis. With the butanol extraction method of DNA adduct enrichment, adduct levels were determined to be 0–414.6 adducts per 109 nucleotides, with considerable interindividual variation. To characterize the DNA adducts, we reanalyzed the adduct spots by reversed-phase high-performance liquid chromatography. Of two major adduct spots detected on TLC that accounted for up to 70% of the DNA modification, one eluted as a single peak on high-performance liquid chromatography, whereas the other was resolved into two distinct peaks of radioactivity. These major adducts were highly lipophilic in character. The N-acetyltransferase-1 (NAT1) and NAT2 genes were analyzed for common mutations using random RFLP analysis. An association between NAT2 acetylator status and adduct levels was observed; significantly elevated adduct levels occurred in the mammary DNA from women who were designated slow acetylators for NAT2 [median adduct level = 83.0 adducts per 109 nucleotides (range, 9.0–414.6)], as compared with the levels in individuals designated rapid acetylators for NAT2 [median adduct level = 39.7 adducts per 109 nucleotides (range, 0–91.0; P = 0.0053)]. On the other hand, NAT1 genotypes were not significantly associated with adduct levels. Although the agents responsible for the DNA modifications in the human breast are not known, this pilot study supports the hypothesis that DNA adduct formation in the human breast may be influenced by the NAT2 genotype.

Introduction
Exposure to exogenous genotoxic compounds is regarded as a critical factor for human cancer risk. The contribution of chemical carcinogens to the etiology of breast cancer has been proposed, but no specific chemicals have been definitely identified (1, 2). The individual cancer risk is likely to be determined both by exposure to xenobiotics and by host factors, such as DNA repair and metabolic capacity. Biomonitoring the burden of chemical carcinogens by analysis of covalent DNA adducts formed by their metabolically activated intermediates determines the dose at the target molecule DNA and, thus, reflects both exposure levels and host factors (3). Although a number of epidemiological studies did not find an association between tobacco smoking and breast cancer (4–7), more recent studies have indicated an elevated breast cancer risk for some cigarette smokers (8–10). For example, Ambrosone et al. (9) detected an association of breast cancer risk with N-acetylator status: postmenopausal women with slow NAT2 activity and a history of cigarette smoking showed an increased risk. The highly sensitive 32P-postlabeling technique has been proven to be useful in the study of DNA adduct formation, especially when exposure to complex mixtures and unknown carcinogens occurs (11). Perera et al. (12) reported a correlation between DNA adduct patterns in human mammary tissue and smoking habits of the patients. Furthermore, Li et al. (13) reported a significant elevation of DNA adduct levels in breast epithelial DNA in cancer patients compared with controls. Only a limited amount of information can be gained with 32P-postlabeling regarding the chemical structure of the DNA adducts being detected. To characterize the adducts observed in low amounts in the DNA of human tissues more fully, we developed a HPLC method that allows high-resolution analysis of 32P-labeled nucleotide adducts (14).

The spectrum of p53 gene mutations detected in human breast tumors supports the involvement of chemical agents as causative agents (2, 15). Environmental polycyclic aromatic amines or their corresponding nitro derivatives are potent inducers of mammary tumors in rodents, and these compounds have been suggested to be initiators of human mammary carcinogenesis (1). To exert their genotoxic effect, the aromatic amines require metabolic activation to hydroxylamines, mediated by cytochrome P450 isoenzymes, of which cytochrome...
Bladder epithelium: DNA adduct levels were significantly elevated in the lower urinary tract, and individuals can be designated as phenotypically polymorphic due to point mutations in the coding region. Although detected in human breast tissue (20), their intrinsic conjugating activity (19, 20). Although no NAT2 activity was detectable, NAT1, a closely related enzyme with a substrate spectrum different from that of NAT2, was detected in these cells (20). mRNA transcripts of both genes were, however, detected in human breast tissue (20).

On the other hand, most of these aromatic amines are detoxified by acetylation of the exocyclic amino group catalyzed by NAT2 (16). This enzyme has long been known to exhibit polymorphism due to point mutations in the coding region, and individuals can be designated as phenotypically slow or rapid metabolizers by genotyping (21). These polymorphisms have an impact on DNA adduct formation in human bladder epithelium: DNA adduct levels were significantly elevated in individuals of the slow NAT2 acetylator genotype (22).

Recently, NAT1 has also been shown to exhibit polymorphism. Badawi et al. (22) observed a correlation of mutations in the NAT1 gene for mutations in the bladder cancer and the NAT1-type N-acetylating enzyme. We have, by means of PCR-RFLP analysis, examined the NAT1 gene for mutations in the polyadenylation sequence and the coding region.

This pilot study was undertaken to examine the possible role of NAT1 and NAT2 polymorphisms on DNA adduct formation in human breast tissue and examine the DNA adducts detected.

Materials and Methods

Tissue Sample Collection. Samples of human mammary tissue, removed at surgery for breast cancer or reduction mammoplasty, were obtained through the Department of Histopathology of the Gynecological Clinic, Hamburg University, and the Krankenhaus Alteneichen (both in Hamburg, Germany) and the Institute of Cancer Research (Sutton, United Kingdom). The use of human tissue samples was approved by the local ethics committees. Patients were aged 18–75 years, and the samples analyzed included normal, tumor-adjacent tissue from 11 cancer patients (age range, 29–75 years; mean age = 53.2 years) and normal tissue from 31 reduction mammoplasty patients (age range, 17–58 years; mean age = 31.4 years; Table 1).

DNA was isolated from tissue samples according to published phenol extraction procedures (24). DNA adduct analysis was performed by 32P-postlabeling analysis using the butanol extraction procedure (24). Each sample was analyzed two times.

Chromatography of 32P-labeled DNA Adducts. According to published procedures (11, 24, 25), the labeling mix was applied to polyethyleneimine-cellulose plates (10 × 10 cm) with a wick (10 × 7 cm) of Whatman no. 17 paper stapled to the top. Chromatography in the D1 direction was performed overnight with sodium phosphate buffer (1 M, pH 6.0), development in D2 [5.3 m lithium formate-8.5 m urea (pH 3.5); in the opposite direction to D1 (bottom to top in Fig. 1)] and D3 [1.2 m lithium chloride, 0.5 m Tris-HCl, and 8.5 m urea (pH 8.0); perpendicular to D2, from left to right]. Finally, the plates were developed with 1.7 m sodium phosphate (pH 6.0). Autoradiography was performed at −80°C using intensifying screens. Quantitation of adduct levels was accomplished by Cerenkov counting of excised adduct spots or areas and published calculation procedures (24), taking into account the specific activity of the [γ-32P]ATP batch used in the experiment, which was determined by labeling 2′-deoxyadenosine-3′-phosphate, as described (26). Values varied between 1200 and 4200 Ci/mmol.

HPLC analysis of 32P-labeled adducts was performed according to the method of Pfau and Phillips (14) with modifications and with an apparatus described elsewhere (25). Briefly, adduct spots were excised from polyethyleneimine plates after four-directional chromatography, and the adducts were eluted with pyridinium formate (4.6 mm inner diameter; particle size, 5 μm) and analyzed at a flow rate of 1.0 ml/min with a linear gradient of B (water-
acetonitrile, 35:65 v/v) in buffer A (0.5 M NaH₂PO₄/H₃PO₄, pH 2.0) as follows: 10% B at 0–3 min, 13% B at 10 min, 31% B at 60 min, 90% B at 90 min, and 10% B at 95 min.

**NAT1 Genotype Analysis.** To date, at least nine NAT1 alleles have been reported (23). Due to the complexity of the NAT1 gene, several PCR-based methods were used to determine the NAT1 acetylator status of an individual. In this study, the wild-type allele (NAT1*4) and five variants (NAT1*3, NAT1*10, NAT1*11, NAT1*14, and NAT1*15) were investigated.

NAT1 PCR-RFLP (27) was performed to distinguish between NAT1*4, NAT1*11, and a group of alleles consisting of NAT1*10, NAT1*3, NAT1*14, and NAT1*15. A single PCR was carried out by adding 100 ng of genomic DNA to a PCR mix containing 15 pmol of each of primers T3975 (5'-ctattta-gaatgagagaga) and T3976 (5'-acagcccatctttagga; Osasel DNA services, Southampton, United Kingdom). Two hundred mmol of dNTPs, 1 unit of Taq polymerase, and PCR buffer supplemented with 80 µg of BSA, and 2 mm MgCl₂ and made up to a final volume of 30 µl. The PCR was denatured at 94°C for 4 min and then subjected to 35 cycles of 94°C for 30 s, 42°C for 30 s, and 72°C for 45 s, followed by a final 72°C extension for 5 min using a Hybrid Omnigene PCR machine. A 20-µl aliquot of the PCR products was removed, and restriction enzyme digests for four specific variant alleles using KpnI (NAT2*5A, M1 allele), TaqI (NAT2*6A, M2 allele), BamHI (NAT2*7A, M3 allele), and MspI/Alul (NAT2*14A, M4 allele) were performed. The KpnI, BamHI, and MspI/Alul digestions were performed at 37°C for 2–3 h and the TaqI digest at 65°C for 2–3 h. Agarose gel electrophoresis was then carried out using 0.5 Tris-borate-EDTA with 0.25 mg/ml ethidium bromide buffer at a voltage of 7 V/cm. The gels were photographed under UV light.

The wild-type allele is cut by each of the restriction enzymes, whereas the variant allele remains uncut due to the loss of the corresponding restriction site. The NAT2 genotype is designated slow if two variant alleles are present. If one or both alleles are wild-type (NAT2*4), the genotype is designated rapid.

**Data Analysis.** Mean values (± SD) of adduct levels are expressed as relative adduct labeling levels as adducts per 10⁷ normal nucleotides. Comparisons of adduct levels were analyzed by a two-tailed Mann-Whitney test. Regression analysis was performed for determination of age dependency of adduct levels.

**Results**

DNA samples isolated from breast tissue of cancer patients or patients undergoing reduction mammoplasty were analyzed using the 32P-postlabeling technique. Adduct spots as well as diagonal radioactive zones could be detected on ion-exchange TLC plates (Fig. 1). Analyzing tumor tissue and tumor-adjacent tissue of the same patient resulted in a similar pattern of adduct spots with lower adduct levels in tumor DNA (data not shown). Butanol extraction of lipophilic DNA adduct-3'-nucleotides prior to the labeling reaction resulted in adduct levels of 0–414.2 adducts per 10⁷ nucleotides, with considerable interindividual variation.

Using the butanol extraction enhancement procedure, we observed that adduct spots with low mobility in both D2 and D3 chromatographic directions accounted for up to 70% of total radioactivity detected on the TLC plates (Fig. 1). To gain further insight into the chemical nature of these adducts, we eluted the radioactivity from these spots and applied these 32P-labeled adducts to reversed-phase HPLC analysis. This resulted in a single peak with a retention time of 72.4 min and two peaks at 72.1 min and 78.4 min, respectively (Fig. 2, A and B, respectively). These were clearly different from a number of DNA adduct standards derived from reaction of dial epoxides of polycyclic aromatic hydrocarbons with DNA or DNA adduct standards of heterocyclic amines or polycyclic aromatic amines.
in either of the two chromatographic systems, ion-exchange TLC or reversed-phase HPLC (14, 25).

The same DNA samples that were analyzed for DNA adducts as described above were examined by PCR-RFLP analysis for mutations in the NAT genes NAT1 and NAT2.

Of the 42 samples, 27 individuals (64.3%) were homozygous for NAT1*4, 11 (26.2%) were heterozygous NAT1*4/NAT1*10, 2 (4.6%) were heterozygous NAT1*4/NAT1*14, 1 (2.3%) was homozygous NAT1*10/NAT1*10, and 1 was heterozygous NAT1*4/NAT1*11 (Table 1). The rare alleles NAT1*3 and NAT1*15 were not observed in this set of DNA samples. This distribution of alleles is comparable to earlier reports on Caucasian populations (10, 22, 23, 30).

Statistical analyses on DNA adduct levels were performed comparing tissue samples from individuals who were homozygous for NAT1*4/NAT1*4 and heterozygous for NAT1*4/NAT1*10 (Table 2). An elevated median adduct level was observed for individuals homozygous for NAT1*4/NAT1*4 [66.0 adducts per 10^9 nucleotides (range, 0–339.0)] as compared to those individuals heterozygous for NAT1*4/NAT1*10 [37.0 per 10^9 nucleotides (range, 0–414.6)]. This difference was not significant (P = 0.25). When only samples obtained from reduction mammoplasty were taken into account, again, tissue samples from patients homozygous NAT1*4/NAT1*4 showed a nonsignificantly elevated median adduct level [90 per 10^9 nucleotides (range, 15.0–339.0)], as compared to patients heterozygous for NAT1*4/NAT1*10 [51.4 per 10^9 nucleotides (11.4–414.6)]. The same trend was detected in the small number of DNA samples from tumor patients when these two genotypes were compared.

Individuals phenotypically characterized as rapid acetylators for NAT2 have been identified as either heterozygous or homozygous for the wild-type alleles (21, 29). The methods used here can distinguish the wild type (NAT2*4) and the four variant alleles M1 (NAT2*5A), M2 (NAT2*6A), M3 (NAT2*7A), and M4 (NAT2*14A), which are the most prevalent in Caucasians (10, 21). Of the patients included in this study, 2 were found to be homozygous for the wild type, and 15 individuals were heterozygous for the wild type. The remaining (slow acetylators) were either homozygous for M1 (12 individuals) or heterozygous for M1 (11 M1/M2 and 1 M1/M3) or homozygous for M2. No M4 NAT2 mutant allele was detected in this set of DNA samples. Thus, 15 individuals (40%) were designated fast N-acetylators, and 27 individuals (60%) were designated slow acetylators of the NAT2 type. This distribution is in accordance with reported values (9, 10, 29, 31–35).

The median DNA adduct level derived by butanol extraction of lipophilic DNA adducts and 32P-postlabeling analysis of the mammary DNA samples was ~2-fold higher [83.0 adducts per 10^9 nucleotides (range, 9–414.6)] for slow NAT2 acetylators than the adduct levels observed in rapid acetylators [39.7 per 10^9 nucleotides (0–91.0)]; this difference is highly significant (P = 0.0053, Fig. 3). This significant elevated level of adducts for slow NAT2 acetylators was also observed when the analysis was performed with only tissue samples from reduction mammoplasty patients. When only the tumor patients were considered, there was no difference in adduct levels between slow and rapid NAT2 acetylators.

Additionally, adduct levels were compared for the combined genotypes (Table 2). An ~3-fold elevated median adduct level was observed in breast tissue DNA from patients with a slow NAT2 genotype and NAT1*4/NAT1*4 as compared those of NAT2 rapid genotype.

Because cigarette smoking has recently been reconsidered as a factor that is possibly involved in the etiology of breast cancer (7–10) and numerous studies have found an influence of tobacco smoke on DNA adduct formation in other tissues (3, 12, 36), the smoking data of the patients were collected. Among the patients, only 4 were identified as smokers, 19 patients stated that they were nonsmokers, and smoking data were not available for another 19 patients. Thus, it was not possible to compare adduct levels from smokers and nonsmokers on a statistical basis. When we stratified the data on adduct levels for smoking (Table 2), the mean adduct level observed for nonsmoking slow NAT2 acetylators (120.0 per 10^9 nucleotides) remained significantly elevated as compared to rapid NAT2 nonsmoking patients (36.5 per 10^9 nucleotides, P = 0.015).

Because risk of developing breast cancer increases with age, the adduct levels were examined for age dependence. Regression analysis indicated that there was no correlation between adduct levels and age of tissue donor (r = –0.25, P = 0.44).

Discussion

Here, we were able to demonstrate the presence of aromatic DNA adducts in human mammary tissue. To characterize the DNA adducts detected by means of the 32P-postlabeling method, we applied the major adduct spots to reversed-phase HPLC analysis. Two major adduct spots that were observed...
or polycyclic or heterocyclic amines revealed that these adducts are highly lipophilic.

Furthermore, by PCR-RFLP analysis of both the NAT1 and NAT2 genes, the patients were genotyped for common mutations in these genes. It has been shown that certain mutant genotypes are indicative of slow NAT2 N-acetylator phenotypes (21). Although only a small number of individuals were analyzed, we observed significantly elevated DNA adduct levels in the mammary tissue of those individuals who were slow acetylators for NAT2.

Many epidemiological studies have not identified a link between tobacco smoke and breast cancer risk in women (4–7), but recent studies by Morabia et al. (8) and Ambrosone et al. (9) report an elevated risk for smoking women. These findings are consistent with a pilot study by Perera et al. (12) that demonstrated an association between cigarette smoking and DNA adduct formation in breast tissue. Most recently, Li et al. (13) analyzed DNA of from breast cancer patients and reduction mammoplasty patients (controls) and reported an elevated level of DNA adducts in the target tissue of cancer cases. These data and results from similar studies in lung (36) or bladder tissue (22) confirm the hypothesis that DNA adduct levels in the target tissue may be a relevant biomarker.

Here, we were unable to detect an elevated adduct level in the DNA derived from normal tissue adjacent to breast tumors compared to tissue samples from reduction mammoplasty patients, regardless of acetyltransferase genotype (Table 2). This may be due to the small number of tumor patients included in the analysis. This study was not designed to compare adduct levels in cancer with noncancer patients; the two groups are also not matched for age. Another factor may be that earlier studies analyzing DNA adduct formation in human breast tissue used the nuclease P1 enhancement procedure (12, 13, 37). This variant is believed to enhance mainly the detection of DNA adducts derived from polycyclic aromatic hydrocarbons.

In this study, we used the butanol extraction of lipophilic DNA adducts (24) prior to the labeling that been shown to enhance adducts derived from N-substituted polycyclic aromatic compounds, such as amines or nitro compounds. Some of these, including 1-nitropyrene, 1.8-dinitropyrene, and 4-aminobiphenyl, have been shown in animal experiments to be potent inducers of mammary carcinoma (1). Recently, we were able to demonstrate that human mammary epithelial cells in vitro are metabolically competent to activate aromatic amino and nitro compounds to DNA adduct-forming metabolites (17, 18). The metabolism of
these classes of chemical carcinogens has been shown to be influenced by the N-acetylation enzymes (16, 20). Li et al. (13) tentatively identified an adduct spot detected in human breast tissue of breast cancer patients as a benzo(a)pyrene-like adduct by comparing chromatographic mobility on ion-exchange TLC plates. Because resolution in this chromatographic system is poor, characterization of adduct spots should be confirmed by a different chromatographic technique with higher resolving power, such as HPLC analysis (14).

Polymorphic distribution of N-acetylation activity (NAT2) has long been known (21). Several case-control studies have been published that investigated a possible association of NAT2 acetylator phenotype (31-33) or NAT2 genotype (9, 10, 34, 35, 38) and breast cancer risk with conflicting results. Among these, one study (31) reported a greater proportion of rapid acetylators among breast cancer patients, although this was not reproduced in subsequent studies (9, 10, 32-35, 38).

Recently, an effect was observed for one subgroup: smoking postmenopausal women with a slow acetylator NAT2 genotype appeared to be at an elevated risk of developing breast cancer (9); this finding was confirmed by Millikan et al. (16). An increased risk (relative risk = 2.0) for postmenopausal breast cancer was also reported for Chinese women of the slow acetylator genotype (35). In a recently published cohort study, Hunter et al. (38) were unable to confirm these associations.

Here, we observed considerable levels of DNA adducts in the mammary gland determined by 32P-postlabeling analysis. This was apparently unrelated to tobacco smoking because over 80% of the patients for whom this information was available stated that they were nonsmokers. Furthermore, we detected an association of the slow NAT2 acetylator genotype with an elevated DNA adduct level that was also not associated with smoking (Table 2). One might speculate that the unknown chemicals forming DNA adducts in the human breast are apparently detoxified by NAT2 because reduced DNA adduct levels are observed in those patients with rapid acetylation activity. Human mammary lipid has been shown to accumulate lipophilic compounds, including organochlorines and genoxins (39, 40). Previously, we were able to demonstrate the capability of human mammary epithelial cells to metabolically activate heterocyclic aromatic amines and polycyclic nitro compounds (17, 18). NAT1-dependent acetylation of hydroxylamine metabolites might lead to covalent modification of DNA in the human mammary epithelium. Indeed, Swamianathan et al. (19) and Sadrieh et al. (20) demonstrated the presence of NAT activity (NAT1) in human breast epithelial cells, albeit at a lower level as compared to the activity reported in human bladder tissue (22). A correlation of acetylator status and DNA adduct level has been demonstrated in the urinary bladder (22, 41). Badawi et al. (22) observed the highest adduct levels in the DNA of those individuals with a slow NAT2 but a rapid NAT1 acetylator phenotype associated with the NAT1*/NAT1*/10 genotype in the bladder epithelium. Although Bell et al. (27) observed an increased bladder cancer risk for individuals with the NAT1*/NAT1*/10 genotype, Probst-Hensch et al. (30) were not able to confirm this observation in a subsequent case control study. Hughes et al. (28) reported on an association of slow N-acetylation activity of the NAT1 type with mutations in the coding region of the NAT1 gene. We included the RFLP analyses for these latter mutations in our analyses. Due to the rather small sample size of this pilot study, only two individuals were identified with a heterozygous NAT1*/NAT1*/14 genotype, which has been associated with a slow NAT1 acetylator phenotype. Perhaps incidentally, these two patients are among those in the lowest quartile of adduct levels. Further analyses with a larger number of individuals are in progress to evaluate the possible association of NAT1 activity on DNA adduct formation in the human mammary gland.

Here, we present, for the first time, data suggesting that acetylation polymorphism for NAT2 is a host factor associated with the level of DNA adducts in the mammary gland. Even in the absence of exposure information, a significant association was obtained. It appears that individuals with NAT2 rapid acetylator genotype are, to some extent, partially protected from DNA adduct formation in the breast.

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References


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