INEFFICIENT REPAIR OF TAMOXIFEN-DNA ADDUCTS IN RATS AND MICE

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ABSTRACT:

A long-term treatment with tamoxifen (TAM) to women increases the risk of developing endometrial cancer. The cancer may result from genotoxic damage induced by this drug. In fact, TAM-DNA adducts were detected in the liver of rats treated with TAM and initiated to develop hepatocellular carcinomas. To explore the distribution and repair rate of TAM-DNA adducts, the level of TAM-DNA adducts in all tissues of rats and mice was monitored for 28 days and 7 days, respectively, after the termination of TAM treatment, using 32P-postlabeling/polyacrylamide gel electrophoresis and 32P-postlabeling/HPLC analyses. TAM-DNA adducts were formed specifically in the liver of rodents. In rats, the level of hepatic TAM-DNA adducts was decreased only to 43% in 28 days, indicating that the half-life of adducts was approximately 25 days. Among trans [fraction (fr)-1 and fr-2]- and cis (fr-3 and fr-4)-isoforms of TAM-DNA adducts, a trans-form (fr-1) was removed much more slowly than other adducts, indicating that the repair rate of TAM-DNA adducts varied depending on the structure of isofoms. The repair rate of TAM-DNA adducts was also compared between nucleotide excision repair-deficient (Xpc knockout) and wild mice. Although the level of hepatic TAM-DNA adducts observed with Xpc knockout mice was slightly higher than that of the wild type, the removal of TAM-DNA adducts in both mice was only 20% in 7 days. Thus, TAM-DNA adducts are not efficiently repaired from the targeted tissue, leading to the development of cancer.

Tamoxifen (TAM) is used in standard endocrine therapy for breast cancer patients and as a chemopreventive agent for healthy women at high risk of this disease (Fischer et al., 1998; Osborne, 1998). Besides the significant benefit, long-term treatment with TAM to women increases the risk of developing endometrial cancer (van Leeuwen et al., 1996) initiates the development of hepatocellular carcinomas. To explore the distribution and repair rate of TAM-DNA adducts, the level of TAM-DNA adducts formed in the liver (Han and Liehr, 1992; Osborne et al., 1996) initiates the development of hepatocellular carcinomas (Greaves et al., 1993; Hard et al., 1993). The formation of TAM-DNA adducts was observed in various tissues, including reproductive organs of monkeys treated with TAM (Schild et al., 2003; Shibutani et al., 2003). In humans, there is a controversy about detecting TAM-DNA adducts. Some research groups, including ours, have detected TAM-DNA adducts in the endometrium of women treated with TAM (Shibutani et al., 2000a; Martin et al., 2003), whereas other groups did not observe TAM-DNA adducts (Carmichael et al., 1996; Beland et al., 2004).

TAM is metabolized by phase I enzymes to N-desmethyltamoxifen (N-desTAM), 4-hydroxytamoxifen, and tamoxifen N-oxide (TAM N-oxide) (reviewed by Kim et al., 2004). α-Hydroxylated TAM metabolites are produced as minor products from TAM and its metabolites in reactions catalyzed by rat CYP 3A2 and human CYP 3A4 (Kim et al., 2004), are O-sulfonated by hydroxysteroid sulfotransferase (Shibutani et al., 1998a,b), and react with dG residues in cellular DNA, resulting in the formation of two trans- and two cis-isofoms of α-(N2-deoxyguanosinyl)tamoxifen (dG-N2-TAM) adducts (Fig. 1) (Osborne et al., 1996; Dasaradhi and Shibutani, 1997). dG-N2-TAM and α-(N2-deoxyguanosinyl)-N-desmethyltamoxifen (dG-N2-N-desTAM) were major adducts in the liver of rats and mice treated with TAM (Rajaniemi et al., 1999; Umemoto et al., 2001) and in several tissues including reproductive organs of monkeys treated with TAM (Schild et al., 2003; Shibutani et al., 2003). α-(N2-Deoxyguanosinyl)tamoxifen N-oxide (dG-N2-TAM N-oxide) was detected as a minor adduct in mouse liver. dG-N2-TAM adducts were also detected in the endometrium of women treated with TAM (Shibutani et al., 2000a). Site-specific mutagenesis studies showed that dG-N2-TAM adducts have highly mutagenic potential, generating mainly G → T transversions, accompanied by fewer G → A transitions in mammalian cells (Terashima et al., 1999). Similar mutagenic specificity was observed at both lac I and cII genes in the liver of the Allact transgenic rats treated with TAM (Davies et al., 1999). In breast cancer patients treated with TAM, a high frequency of G → T and G → A mutations was detected at codon 12 of the K-ras proto-oncogene in the endometrium (Hachisuga et al., 2005). The mutational spectrum was consistent with that observed in our mutagenesis study (Terashima et al., 1999), suggesting that the mutations that occurred at the K-ras gene are due to the genotoxic effect of TAM.

Bulky DNA adducts, including dG-N2-benzo[α]pyrene (Cerutti et
al., 1997) and dG-C8-acetylaminofluorene (Howard et al., 1981), are generally removed by nucleotide excision repair (NER) enzymes. In an in vitro experimental system using mammalian and human nucleotide excision repair enzymes, we found that TAM-DNA adducts are removed slowly from DNA (Shibutani et al., 2000b). Mutation frequency induced by 3\(^2\)/H9251-acetoxyTAM in NER-deficient cells (XPA) was higher than that observed with NER-proficient cells, indicating that NER plays an important role in removal of TAM-DNA adducts (McLuckie et al., 2005). If TAM-DNA adducts persist in the tissues of animals and women treated with TAM, cancer may be initiated by the cumulative TAM-DNA damage. The repair efficiency of TAM-DNA adducts may thus be a key factor in TAM carcinogenicity.

A few research groups have determined repair of hepatic TAM-DNA adducts in rat using 3\(^2\)P-postlabeling analysis and chemiluminescence assay (White et al., 1992; Divi et al., 1999; da Costa et al., 2001); however, only total amounts of TAM-DNA adducts were determined. Since each TAM-DNA adduct has different mutagenic potential (Terashima et al., 1999) and repair potential (Shibutani et al., 2000b), the repair rate of each TAM-DNA adduct in rats was determined in the present study using sensitive 3\(^2\)P-postlabeling/PAGE and 3\(^2\)P-postlabeling/HPLC analyses. Xpc knockout mice are deficient in both alleles of mouse xeroderma pigmentosa complementation group C, one of the factors involved in nucleotide excision repair (Sands et al., 1995). The repair rate of TAM-DNA adducts was also determined in the Xpc knockout mice and the wild-type mice to explore the contribution of NER to removal of TAM-DNA adducts.

Materials and Methods

Materials. TAM, calf thymus DNA, micrococcal nuclease, and potato apyrase were purchased from Sigma-Aldrich (St. Louis, MO). Spleen phosphodiesterase was obtained from Worthington Biochemical Corp. (Lakewood, NJ). 3\(^2\)-Phosphatase-free T4 PNK and nuclease P1 were obtained from Roche Applied Science (Indianapolis, IN). TAM \(\alpha\)-sulfate, and diastereoisomers of trans-forms [fraction (fr)-1 and fr-2] and cis-forms (fr-3 and fr-4) of dG \(\alpha\)-monophosphate-\(N^2\)-tamoxifen (dG3\(\alpha\)-\(N^2\)-TAM) were prepared as described previously (Dasaradhi and Shibutani, 1997; Shibutani et al., 1998a). [\(\gamma\)-\(32\)P]ATP (specific activity, >6000 Ci/mmol) was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

Animal Study. Fisher 344 rats (female, 8 weeks old), Xpc knockout mice, and B6129F1 mice (female, 8 weeks old) were purchased from Taconic Farms (Germantown, NY). The use of animals was in compliance with the guidelines established by the National Institutes of Health Office of Laboratory Animal Welfare. Animals were acclimated in temperature (22 ± 2°C)- and humidity (55 ± 5%)-controlled rooms with a 12-h light/dark cycle for at least 1 week before use. Regular laboratory chow and tap water were allowed ad libitum. Rats were treated orally with TAM (20 mg/kg/day) for 7 days. Xpc knockout mice and B6129F1 mice were given TAM (20 mg/kg/day or 120 mg/kg/day) for 7 days by gavage. Control rats and mice were treated with an identical volume of corn oil. The rats and mice were euthanized by CO\(_2\) asphyxiation at 5 h, or 7 and/or 28 days after the final treatment, an open thoracotomy. All tissues were removed quickly, frozen, and stored at -80°C until DNA extraction.

Digestion of DNA Samples. The tissue DNA was extracted using a QIAGEN DNA isolation kit (QIAGEN, Valencia, CA) following the manufacturer’s protocol. The concentration of DNA was determined by UV spectrophotometry as 50 \(\mu\)g/ml = OD\(_{260}\) nm \(\times\) 1.0. The DNA sample (1.0–5.0 \(\mu\)g) was enzymatically digested at 37°C overnight in 100 \(\mu\)l of 17 mM sodium succinate buffer (pH 6.0) containing 8 mM CaCl\(_2\), using micrococcal nuclease (30 units) and spleen phosphodiesterase (0.15 unit) (Terashima et al., 2002). The reaction mixture was incubated for another hour with nuclease P1 (1 unit). After the incubation, 150 \(\mu\)l of water was added. The reaction samples were then extracted twice with 200 \(\mu\)l of butanol. The butanol fractions were combined, back-extracted with 50 \(\mu\)l of distilled water, and evaporated to dryness.

\(32\)P-Postlabeling/PAGE Analysis. The DNA digests were incubated at
37°C for 40 min with 10 μCi of [γ-32P]ATP and 3'-phosphatase-free T4 polynucleotide kinase (10 units), and then incubated with aprysase (50 milliunits) for another 30 min, as described previously (Terashima et al., 2002). Known amounts (0.152 pmol mol, 0.0152 pmol, 0.00152 pmol, or 0.000152 pmol) of dG-N2-TAM-modified oligodeoxynucleotide, prepared by a phosphoramidite chemical procedure (Santosh Laxmi et al., 2002), were mixed with 5 μg of calf thymus DNA (15200 pmol) and served as a standard (1 adduct/10^6 nucleotides, 1 adduct/10^7 nucleotides, 1 adduct/10^9 nucleotides, or 1 adduct/10^10 nucleotides). The amount of TAM-DNA adducts detected increased linearly depending on the amounts of oligodeoxynucleotide used. A part of the 32P-labeled sample was electrophoresed for 4 to 5 h on a nondenaturing 30% polyacrylamide gel (35 × 42 × 0.04 cm) with 1400 to 1600 V/20 to 40 mA. The position of 32P-labeled adducts was established by a phosphorimager analysis (GE Healthcare). To quantify the level of 32P-labeled products, integrated values were measured using a β-PhosphorImager and compared with the standards. The detection limit for 5 μg of DNA was approximately 7 adducts/10^6 nucleotides.

32P-Postlabeling/HPLC Analysis. After the 32P-labeled products were developed on the gel as described above, the bands of 32P-labeled products were cut from the gel and put into 1 ml of distilled water overnight at room temperature. The 32P-labeled products extracted from the gel were evaporated to dryness. Recovery of 32P-labeled products was −95%. The 32P-labeled products were dissolved in 0.2 ml of distilled water and subjected to a Hypersil C18 analytical column (0.46 × 25 cm, 5 μm; Thermo Electron Corporation, Waltham, MA), eluted at a flow rate of 1.0 ml/min with a linear gradient of 0.2 M ammonium formate, and 20 mM H3PO4, pH 4.0, containing 20 to 30% acetonitrile for 40 min, 30 to 50% acetonitrile for 5 min, followed by an isocratic condition of 50% acetonitrile for 15 min. The radioactivity was monitored using a radioisotope detector (Berthold LB506 C-1; ICON Scientific Inc., North Potomac, MD) linked to a Waters 990 HPLC instrument (Waters, Milford, MA). As described above, known amounts (0.152–0.000152 pmol) of dG-N2-TAM-modified oligodeoxynucleotide prepared by a phosphoramidite chemical procedure (Terashima et al., 2002), were mixed with 5 μg of calf thymus DNA (15200 pmol) and served as a standard. As described previously (Terashima et al., 2002), the amount of TAM-DNA adducts detected increased linearly depending on the amounts of oligodeoxynucleotide used. The detection limit of this assay was −2 adducts/10^9 nucleotides for 5 μg of DNA.

Statistical Analysis. Results are expressed as mean ± S.D. Student’s t test was used to evaluate the difference. Values of p ≤ 0.05 were considered statistically significant.

Results

Rats were treated orally with TAM (20 mg/kg/day) for 7 days and sacrificed at 5 h after the final treatment. 32P-postlabeling/PAGE was performed to analyze TAM-DNA adducts in each tissue using a standard (5 adducts/10^6 nucleotides). A high level of TAM-DNA adducts (18.3 adducts/10^6 nucleotides) was detected in the liver of all rats treated with TAM (Fig. 2A). Since the migration of each trans (fr-1 and fr-2)- and cis (fr-3 and fr-4)-isofrom of dG3p-N2-TAM is the same as that of dG3p,N2-N-desTAM (Kim et al., 2005), the major TAM-DNA adduct in the liver was expected to be a mixture of fr-2 of dG3p-N2-TAM and dG3p,N2-N-desTAM. Among the extrahepatic tissues, a low level of TAM-DNA adduct (0.5 adduct/10^7 nucleotides) was detected in one of three stomach DNA samples (Fig. 2A). The migration of this adduct was similar to that of standard fr-1 of trans-dG3p-N2-TAM or dG3p,N2-N-desTAM. When the sample was subjected to HPLC on-line with a radioisotope monitor comparing with the standard, the retention time of this adduct was identical to that of fr-1 of dG3p-N2-TAM (data not shown). No DNA adduct was detected in any other tissues.

To determine the ability to repair TAM-DNA adducts, the level of hepatic TAM-DNA adducts was monitored 7 and 28 days after the final TAM treatment (Fig. 3; Table 1) using 32P-postlabeling/PAGE. At day 7 after the cessation, the total amount of TAM-DNA adduct was reduced to 53 to 65%, indicating that fr-1 resists the repair more effectively, and the half-lives of dG3p,N2-TAM and dG3p,N2-desTAM were removed to 38 to 47% as compared with the values observed at 5 h (Table 1). The half-lives of fr-2 of dG3p,N2-TAM and dG3p,N2-desTAM were 22 and 28 days, respectively, and the half-lives of cis-forms of dG3p,N2-TAM and dG3p,N2-desTAM were 23 and 27 days. Another minor trans-form (fr-1) was reduced to 53 to 65%, indicating that fr-1 resists the repair more than other isofroms; the half-lives of fr-1 of dG3p,N2-TAM and dG3p,N2-desTAM were 40 and 29 days, respectively.

Xpc knockout mice and the wild (B6129F1) mice were also given TAM (20 mg/kg/day or 120 mg/kg/day) for 7 days by gavage. The level of TAM-DNA adducts was determined in all tissues using...
adducts/10^7 nucleotides and 7.1 adducts/10^7 nucleotides, respectively. Compared with the wild-type mice, the values of TAM-DNA adducts in Xpc knockout mice were slightly higher: 132% for fr-1, 176% for fr-2, and 138% for fr-3 and fr-4. However, no significant difference in the level of TAM-DNA adducts was observed between Xpc knockout mice and the wild type. On day 7 after the final treatment, the levels of total TAM-DNA adducts were decreased to 71% (8.2 adducts/10^7 nucleotides) for Xpc knockout mice and 80% (5.7 adducts/10^7 nucleotides) for the wild type. No significant difference was observed in the removal between dG3N^-2-TAM and dG3N^-2-desTAM adducts. Except for cis-forms of Xpc knockout mice, removal of a trans-TAM-DNA (fr-2) and cis-TAM-DNA adducts (fr-3 and fr-4) was slightly faster than that of another trans-TAM-DNA adduct (fr-1).

**Discussion**

Among tissues of rats treated with TAM, TAM-DNA adducts were detected primarily in the liver, although a trace of TAM-DNA adduct was observed in some extrahepatic tissues, as recently reported (Philips et al., 2005). In contrast, in monkeys treated with TAM, TAM-DNA adducts were observed in brain, ovary, and uterus, in addition to liver (Schild et al., 2003; Shibutani et al., 2003). The adduct was also detected in the endometrium of women treated with TAM (Shibutani et al., 2000a). Thus, the formation of TAM-DNA adducts in rats is species-specific and liver-specific, resulting in developing hepatocellular carcinoma (Greaves et al., 1993; Hard et al., 1993).

The repair rate of hepatic TAM-DNA adducts was determined in rats using ^32^-postlabeling/PAGE and ^32^-postlabeling/HPLC analyses. After termination of TAM treatment, the level of TAM-DNA adducts was decreased to 86% at day 7 and 43% at day 28 as compared with the values observed at 5 h after the final treatment. The half-life of total TAM-DNA adducts was approximately 25 days, indicating that TAM-DNA adducts persist for an extended period. Our results were consistent with the previous observations determined using chemiluminescence immunoassay (Divi et al., 1999), ^32^-postlabeling/thin-layer chromatography (White et al., 1992), and ^32^-postlabeling/HPLC (da Costa et al., 2001). Loss of TAM-DNA adducts in the rat liver may be due to repair and/or cell death. Significant cell death was not observed in the livers of F344 rats treated for 26 weeks with a dose of TAM similar to that used in our study (Stanley et al., 2001). Therefore, loss of TAM-DNA adducts probably reflects repair.

Previous papers (White et al., 1992; Divi et al., 1999; da Costa et al., 2001) showed only the fate of total TAM-DNA adducts. In the present study, all isomers of TAM-DNA adducts were monitored. The half-life (40 days) of the minor trans-form (fr-1) of dG3^-N^2^-TAM was much longer than that of another major trans-form (fr-2) (22 days) and minor cis-forms (fr-3 and fr-4) (23 days), indicating that the fr-1 is more resistant against repair than other isoforms. This result was supported by the fact that fr-1 was not efficiently removed from the DNA by mammalian whole-cell extracts as compared with other isoforms (Shibutani et al., 2000b). In contrast, the half-lives (27–29 days) of dG3^-N^2^-desTAM isoforms were not significantly different. Thus, each isoform of TAM-DNA adducts may have different repair susceptibility.

When B6129F1 mice were treated with TAM (20 mg/kg/day), the same dose used for rats, only a trace of TAM-DNA adduct was detected. Mice are more resistant to TAM than rats (White et al., 1992; Umemoto et al., 2001); therefore, no liver tumors were developed at doses that were hepatocarcinogenic in rats (Tucker et al., 1984). When a 6-fold high dose of TAM (120 mg/kg/day) was given to the mice as reported previously (Umemoto et al., 2001), significant amounts of TAM-DNA adducts were detected in the liver; however,
total amounts of TAM-DNA adducts (7.08 adducts/10^7 nucleotides) in B6129F1 mice were 2.6 times lower than that observed in rats treated with TAM (20 mg/kg/day). This result may be due to the fact that TAM and its metabolites in mice are rapidly excreted into the urine and/or feces, and/or that the capability of forming \( \alpha \)-hydroxylated and \( \alpha \)-sulfated TAM metabolites, precursors of forming TAM-DNA adducts, is low. The level of hepatic TAM-DNA adducts at day 7 after the final TAM treatment was only decreased to 80% as compared with that observed at 5 h. In contrast to the previous report showing that TAM-DNA adducts were removed in a couple of days from the mouse liver (Martin et al., 1997), our results indicated that the repair of TAM-DNA adducts in mice was not rapid, as observed for rats. Although the level of hepatic TAM-DNA adducts in the Xpc knockout mice would tend to be higher than that observed with the wild type at 5 h and 7 days after the termination of TAM treatment, the removal of TAM-DNA adducts from the Xpc knockout mice in 1 week was similar to that observed with the wild type. NER may not efficiently remove TAM-DNA adducts during the period of TAM treatment and after the termination of treatment.

Like \( \text{trans-dG-N}^2\text{-TAM adducts, } 3\text{-}(\text{deoxyguanosin-}N^2\text{-yl})-2\text{-}

acetylamino fluorescence (dG-N\textsuperscript{2}-AAF) persists in the liver of rats treated with AAF, whereas N-(deoxyguanosin-8-yl)-2-acetylaminofluorenone (dG-C\textsuperscript{8}-AAF) and N-(deoxyguanosin-8-yl)-2-aminofluorenone (dG-C\textsuperscript{8}-AF) are rapidly excised by NER (Westra et al., 1976). dG-N\textsuperscript{2}-AAF is accommodated within a minor groove without disruption of the Watson-Crick pair in DNA (Grad et al., 1997), as is also observed with cis-dG-N\textsuperscript{2}-TAM (Shimotakahara et al., 2000). Therefore, non-disruptive dG-N\textsuperscript{2}-adducts including dG-N\textsuperscript{2}-TAM may not be efficiently recognized by NER enzymes, resulting in the persistence in the rat liver.

There are several pieces of evidence showing mutagenic potential of TAM-DNA adducts. Site-specific mutagenesis studies revealed that dG-N\textsuperscript{2}-TAM, a major TAM-DNA adduct detected in liver of rodents treated with TAM and in endometrial tissue of patients treated with TAM, promoted primarily G\textsuperscript{C}→T A mutations in the endometrium of breast cancer patients treated with TAM; the presence of K-ras mutation in endometrium was significantly influenced by the duration of TAM treatment and menstrual status of the patient (Hachisuga et al., 2005). The mutational spectrum was consistent with that observed in our mutagenesis study (Terashima et al., 1999) and rodent studies (Davies et al., 1999), suggesting that the mutations that occurred at the K-ras gene are due to the genotoxic damage induced by TAM.

When DNA-adducted plasmid induced by α-acetylo TAM was transferred into nucleotide excision-proficient or -deficient (XPA) human fibroblast, mutation frequency in NER-deficient cells was 1.3 to 3.6 times higher than that observed with NER-proficient cells (McLuckie et al., 2005). Unlike the 10- to 15-fold higher multiple mutations observed in NER-proficient cells by damaged UV, the portion of multiple mutations induced by α-acetylo TAM was not significantly different between cell lines. This result may indicate that TAM-DNA adducts are repaired inefficiently. Therefore, if the mutagenic TAM-DNA adducts are not rapidly repaired, they could accumulate over extended periods of time in the specific genes like K-ras, leading to the development of cancers.


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