The effect of 17β-estradiol-DNA adducts on the replication of exon # 5 of the human suppressor gene p53

Fu-Li Yu[a,*], Wei-Yun Zheng[a], Mian-Ying Wang[a], Wanda Bender[a], Alexandra Cheerva[b], James Miller[c]

[a] *Department of Biomedical Sciences, University of Illinois College of Medicine at Rockford, Rockford, IL 61107, USA*
[b] *Department of Pediatrics, University of Illinois College of Medicine at Rockford, Rockford, IL 61107, USA*
[c] *Department of Obstetrics and Gynecology, University of Illinois College of Medicine at Rockford, Rockford, IL 61107, USA*

Received 26 April 1999

Abstract Using a PCR technique, exon # 5 of the human tumor suppressor gene p53 was amplified and ligated into the pCRII vector and transformed into *Escherichia coli* INVαF' competent cells. The cloned exon # 5 was 184 bp long. Evidence is presented to show that after dimethyldioxirane epoxidation, 17β-estradiol was able to form 17β-estradiol-DNA adducts and to strongly inhibit the replication of the cloned exon # 5 producing smaller sizes of DNA fragments and introducing errors of incorporation at the 3′-end of the terminating DNAs. The errors occurred mainly at the clusters of the complementary 'G' and 'A' bases on the template strand DNA, presumably, the major sites where the 17β-estradiol-DNA adducts were formed.

Key words: Exon # 5 of p53; 17β-estradiol; Dimethyldioxirane; DNA adduct; DNA replication

1. Introduction

Estrone (E_1) and 17β-estradiol (E_2) are endogenous female hormones required for the growth and development in the target tissues, including the mammary glands. However, animal and epidemiological studies have clearly indicated that they are also carcinogenic causing uterine [1,2] and breast [3–5] cancers. The mechanism of their carcinogenic action is still largely unknown. Recently, we found that E_1 and E_2 could be activated by the epoxide-forming oxidant dimethyldioxirane (DMDO), resulting in the inhibition of rat liver nuclear and nucleolar RNA synthesis in vitro [6]. Since epoxidation is required for the activation of many well-known chemical carcinogens, we proposed that epoxidation is the underlying mechanism for the initiation of estrogen carcinogenesis [6]. Chemical carcinogenesis is a multistage process including initiation, promotion and progression [7–9]. Initiation requires the covalent binding of a carcinogen to DNA [7–9]. Therefore, one of the important tests of our hypothesis is to determine whether E_1 and E_2 after DMDO activation are able to bind to DNA. In support to our hypothesis, we have found that ^{3}H-labelled E_1 and E_2 are able to bind to both G-C and A-T containing DNAs but only after DMDO activation [10,11]. Additionally, the covalent binding nature of E_1 and E_2 to DNA after DMDO activation was further confirmed by ^{32}P-post-labelling analysis using calf thymus as well as several synthetic DNAs of a known base content and sequence [10,11]. However, these are in vitro experiments and it is critical that estrogen-DNA adducts are detected in vivo. A recent report has shown that when a continuous treatment of E_2 was delivered through Silastic tubing implants, female ACI rats developed 100% mammary tumors [12]. We found that when the female ACI rats were given intramammallary injection of E_2 or DMDO-activated E_2, identical DNA adducts were formed in vivo and that the DMDO-activated E_2 was at least 25 000-fold more active than E_2 in the forming of DNA adducts in the mammary glands [13,14].

P53 is a human tumor suppressor gene [15,16]. Mutation of p53 is known to be involved in many types of cancers including breast cancer [15,17,18]. Exon # 5 of p53 has been identified as one of the mutational hotspots in breast cancer patients [15,17,18]. This work is to report the cloning of exon # 5 of the human tumor suppressor gene p53 and the study of the effect of E_2-DNA adducts on the replication of exon # 5 DNA in vitro.

2. Materials and methods

2.1. Materials

E_2 was purchased from Sigma Chemical Company (St. Louis, MO, USA). A Sequenase Version 2.0 DNA sequencing kit and 5′-[α-^{35}S]dATP were purchased from Amersham Life Science (Arlington Heights, IL, USA). The PHP53B plasmid (ATCC 57254) in *Escherichia coli* containing the human tumor suppressor gene p53 was bought from American Type Culture Collection (Rockville, MD, USA). The QIAprep Plasmid kit was from QIAGEN (Chadsworth, CA, USA). The p53 exon # 5 sense (5′-TACTCCCCTGCCCTCAACAA-3′) and antisense (5′-CATCGCTATCTGAGCAGCGC-3′) primers were made by The Great American Gene Company (Ramona, CA, USA). GeneAmp PCR Reagent kit including Ampli Taq DNA polymerase was from Perkin Elmer (Branchburg, NJ, USA). TA Cloning kit Dual Promoter was purchased from Invitrogen (Carlsbad, CA, USA). The universal SP6 and T7 promoter primers were purchased from Promega (Madison, WI, USA).

2.2. Cloning and sequencing of exon # 5 of the human tumor suppressor gene p53

The recombinant plasmid PHP53B (ATCC 57254) containing the pBR322 vector (size 4363 kb) with a 2 kb DNA insert that included the 135 bp upstream of the first ATG codon and the entire encoding sequence of the 393 amino acids of the human tumor suppressor gene p53 (DNA sequence accession number X02469) in *E. coli* was incubated in 10 ml L-broth medium containing ampicillin (50 μg/ml final concentration) at 37°C for 3 h. Then, 200 μl of these cells was inoculated into a 5 ml fresh L-broth medium containing ampicillin for an overnight culture at 37°C with shaking. The cells were then collected by centrifugation at 3000 rpm for 10 min and lysed. The plasmid DNA was isolated and purified using the QIAprep Spin Plasmid kit.

For PCR reaction, 0.5–1.0 μg of the purified plasmid DNA was mixed in a total of 100 μl of the PCR reaction mixture that contained 10 μl 10×PCR buffer, 200 μM of each dNTPS (final concentration), 1 pM (final concentration) each of the exon # 5 sense and antisense

*Corresponding author. Fax: (1) (815) 395 5666.
E-mail: fuliyu@uic.edu

PII: S0014-5793(99)00769-3

primers and 2.5 U Ampli Taq DNA polymerase. The PCR reaction was carried out at 94°C for 60 s, 55°C for 45 s and 72°C for 90 s, for a total of 29 cycles. Then, 10 μl of the PCR product was taken for agarose (2%) gel electrophoresis to check the size of the amplified p53 exon # 5 DNA after ethidium bromide staining and it was visualized under UV light. For cloning, 1 μl of the PCR-amplified p53 exon # 5 DNA was ligated to the pCRII vector (3.9 kb) using T4 DNA ligase at 14°C for 7 h according to the TA Cloning kit protocol. The recombinant pCRII vector was transformed into the INVαF' *E. coli* competent cells and the white-colored clones were selected and incubated in 5 ml L-broth medium containing ampicillin at 37°C, overnight with shaking. The recombinant plasmids were purified with the QIAprep Plasmid kit. The p53 exon # 5 DNA insertions were confirmed by both PCR and *Eco*RI double digestion methods. The sequence of the encoding (sense) strand of the p53 exon # 5 DNA cloned in pCRII vector replicated from the T7 promoter primer is shown below:

5′-TACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGC-
CAAGACCTGCCCTGTGCAGCTGTGGGTTGATTCCA-
CACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTA-
CAAGCAGTCACAGCACATGACGGAGGTTGT-
GAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGCGATG-
3′ (184 bp).

2.3. Activation of E_2 by DMDO

The procedure to activate E_2 using the specific epoxide-forming oxidant DMDO was the same as described previously [6,10,11]. Briefly, aliquots of 1 ml of the freshly prepared DMDO were mixed with 1 mg E_2 at room temperature for 60 min with gentle shaking. After activation, the reaction mixture was vacuumed to dryness and was redissolved in dimethylsulfoxide (DMSO) before use (the E_2 epoxide group). In parallel, tubes containing 1 mg E_2 were mixed with 1 ml acetone and reacted the same way as DMDO at room temperature for 60 min, then, they were dried. Again, it was dissolved in DMSO before use (the E_2 control group).

2.4. Detection of E_2-DNA adducts by ^{32}P-post-labelling

For this study (Fig. 1), three groups of samples were used. (a) The solvent control group, 5 μg of the plasmid DNA containing exon # 5 was incubated alone in the presence of 33% DMSO. (b) The E_2 control group, 5 μg of the plasmid DNA was incubated with 200 μg E_2 in the presence of 33% DMSO. (c) The E_2 epoxide group, 5 μg of the plasmid DNA was incubated with 200 μg of the DMDO-activated E_2 in the presence of 33% DMSO. The binding reaction for each group was carried out at room temperature for 1 h. The nuclease P1-enhanced version of the ^{32}P-post-labelling technique was used for DNA adduct analysis [10,11]. Kodak XAR-5 or Du Pont Cronex 4 X-ray films and Du Pond Lightning Plus intensifying screens were used for the detection of DNA adducts after the films were exposed for 16 h at −80°C [10,11].

2.5. Assay of DMDO-activated E_2 on the replication of exon # 5 DNA of p53

For the study on the inhibitory effect of E_2-DNA adducts on the replication of exon # 5 DNA of p53 (Table 1), 5 μg of the pCRII vector DNA containing exon # 5 of the human tumor suppressor gene p53 was reacted with 200 μg of the DMDO-activated E_2 for 30 min (30 min group) or for 2 h (2 h group) at room temperature in a 33% final concentration of DMSO. For the control, 5 μg of the plasmid DNA containing exon # 5 was incubated alone in the presence of 33% of DMSO for 2 h. The DNA was then denatured in 0.2 M NaOH (10 min, room temperature) and precipitated with alcohol in the presence of 3% sodium acetate overnight at −20°C. The denatured DNA was annealed with the T7 promoter primer and the replication was carried out at room temperature for 5 min using the Sequenase Version 2.0 DNA Sequencing kit according to the conditions specified by the protocol (Amersham Life Science, Arlington Heights, IL, USA), but in the absence of 2′,3′- dideoxynucleoside 5′-triphosphate (ddNTP) terminators. 5′-[α-^{35}S]dATP was used to monitor the synthesis of DNA. After incubation, 1 μl aliquots of the reaction mixture in triplicates were taken from each group for quantitative radioactivity incorporation determination using TCA washing and GF/C filter

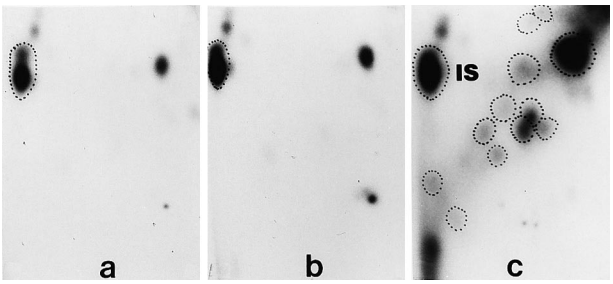

Fig. 1. ^{32}P-post-labelling evidence for the formation of E_2-DNA adducts after DMDO activation with the cloned exon # 5 of the tumor suppressor gene p53 in vitro. (a) Solvent control group. (b) E_2 control group. (c) DMDO-activated E_2 group. The conditions for E_2 activation by DMDO and the binding of the activated E_2 to DNA were all the same as described in Table 1, except in the case when the binding reaction for each group was carried out in the presence of 33% DMSO for 1 h. 2 μg of the DNA samples were used for the ^{32}P-post-labelling experiments. 'is' is the internal standard DNA marker that was included in each sample to monitor the efficiency of the ^{32}P-post-labelling procedure.

counting as described previously [19,20]. For sequencing gel analysis, the assay was conducted in the presence of ddNTPs as specified by the Sequenase Version 2.0 DNA Sequencing kit. However, the samples were analyzed in two ways. One was that the samples from each group containing the same amount of DNA template were applied to a gel (Fig. 2) and the other was that the samples containing the same amount of radioactivity were applied to the gel (Fig. 3). DNA sequencing analysis using an 8% polyacrylamide/7 M urea gel in TBE buffer was performed on a Model S2 Sequencing Gel Electrophoresis Apparatus (Life Technologies, Gibco BRL Apparatus, Gaithersburg, MD, USA). The gel was pre-run at 1500 V for 45–60 min. Then, the samples were applied onto the gel after heat denaturation and electrophoresed at 1500 V for an additional 1.5–2 h. After the run, the gel was vacuum-dried and exposed to the 35×43 Kodak XAR-5 autoradiographic films at room temperature for several days before they were developed.

3. Results and discussion

Fig. 1 shows the results of the detection of E_2-DNA adducts by ^{32}P-post-labelling analysis using the cloned p53 exon # 5 DNA. Fig. 1a indicates that the solvent DMSO was not able to form adduct with the p53 exon # 5 DNA. Similarly, no DNA adducts were found using E_2 before activation (Fig. 1b). On the other hand, E_2-DNA adducts were formed after E_2 activation by DMDO (Fig. 1c). These results are in good agreement with our earlier observations that E_2 requires activation by epoxidation before it is able to inhibit the DNA-dependent RNA synthesis [6] and to bind DNA-forming DNA adducts [13,14]. The important question here is what is the effect, if any, of these E_2-DNA adducts on the replication of the p53 exon # 5 suppressor gene? For this study, 5 μg of the pCRII recombinant DNA containing exon # 5 of the suppressor gene p53 was reacted with 200 μg of the DMDO-activated E_2 at room temperature in 33% DMSO (final concentration) for either 30 min (30 min group) or 2 h (2 h group). As a control, 5 μg of the exon # 5 recombinant DNA was incubated in 33% DMSO alone for 2 h. DNA replication was carried out with T7 promoter primer, but in the absence of ddNTP terminators and 5′-[α-^{35}S]dATP was used to monitor the synthesis of DNA. After incubation, 1 μl aliquots in triplicates of the reaction mixture from each group were taken for quantitative radioactivity determination using

the TCA washing and GF/C filter counting procedure as described previously [19,20]. The results from this study, as shown in Table 1, indicate that when compared with the control, the DMDO-activated E_2 was able to strongly inhibit the replication of p53 exon # 5 DNA. The inhibition was 50 and 64% when the plasmid DNA was reacted with DMDO-activated E_2 for 30 min or for 2 h, respectively.

In order to understand the mechanism of this inhibition, an equal amount (i.e. 0.1 μg) DNA template in the final reaction mixture from each group was used for sequencing gel electrophoresis. Fig. 2 shows the results from this study. There are two points that can be made. (1) The intensity of the radioactivity labelling bands on the sequencing gel from the DMDO-activated E_2 groups was greatly reduced as compared with the control group. This confirms the fact that less radioactivity was incorporated into the activated E_2 groups as shown in Table 1. (2) It appears that larger DNA fragments which are located on the top of the gel preferentially disappeared. Instead, many additional faint bands appeared in the middle or lower portion of the gel. However, because of the low levels of radioactivity incorporation in the DMDO-activated E_2 groups, the additional faint bands are difficult to see. For this reason, we decided to run the gel using an equal amount of radioactivity from each group. The result from this study is shown in Fig. 3. Again, there are two points that can be made. (1) Indeed, there are many additional, although weaker, radioactive bands that can be seen from both of the 30 min and 2 h groups. These radioactive bands appeared mostly toward the middle and the lower portion of the gel and represent smaller pieces of the DNA chain fragments (the arrow on the left of the gel indicates the start of exon # 5 DNA replication from the 5′-end). These bands are not from the normal p53 exon # 5 sequencing bands because the corresponding control lanes do not show these bands. (3) The larger DNA chain fragments on top of the gel disappeared from both 30 min and 2 h groups as a result of the activated E_2 treatment. These results confirmed the data obtained in Fig. 2, suggesting that the activated E_2 was able to interfere with the p53 exon # 5 DNA replication producing smaller sizes of DNA fragments.

Additionally, we believe that the appearance of additional radioactive bands on the sequencing gel from the activated E_2 groups represents errors of incorporation of wrong bases at the 3′-end of the terminated DNAs. The fact that a DNA sequencing gel separates DNA fragments is based on the chain length of the DNA and the sequencing ladder is able to distinguish two pieces of DNA differing only one base in length. When one compares the cluster of 'C' ladders in the 'C' lanes of the control group with the 30 min and 2 h of the activated E_2 groups just above the 'arrow' sign from the sequencing gel in Fig. 3, it is evident that there have no other radioactive bands appeared next to the 'C' clusters in the corresponding 'T', 'G' and 'A' lanes of the control group. On the other hand, one finds that many radioactive bands, although weaker, appeared in the 'T', 'G' and 'A' lanes from the 30 min and 2 h activated E_2 groups. This result is interpreted to mean that the DNA fragments that were terminated normally with a 'C' at the 3′-end of the DNA chain were replaced with a 'T', 'A' or 'G' base in the activated E_2 groups. Since the DNA sequencing ladders on the sequencing gel represent the coding strand copied from the template strand of

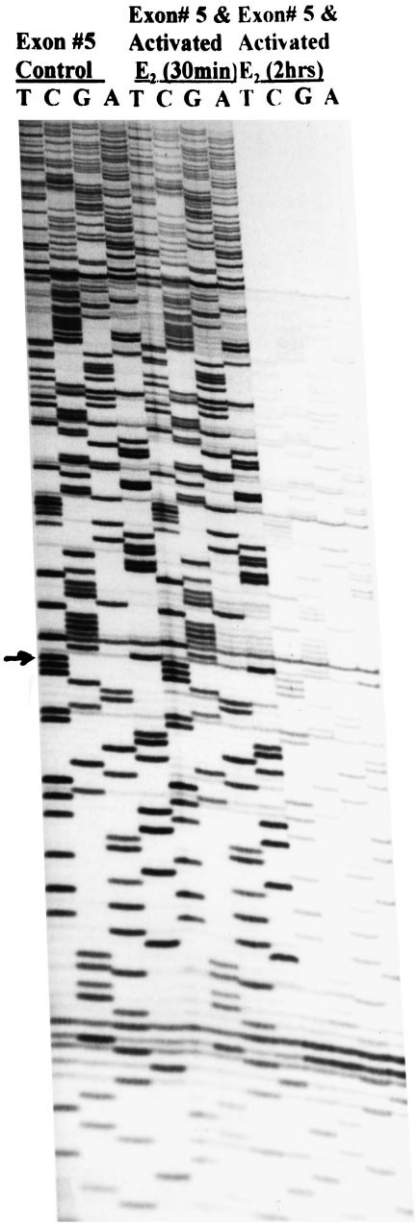

Fig. 2. Effect of DMDO-activated E_2 on the replication of exon # 5 of the human tumor suppressor gene p53 by DNA sequencing gel analysis using equal amounts of DNA template. DMDO-activated E_2 (200 μg) was reacted with 5 μg of the pCRII vector containing exon # 5 DNA as described in Table 1. 0.1 μg DNA of the final reaction mixture from each group was used and analyzed on 8% denatured polyacrylamide sequencing gel. After the run, the gel was vacuum-dried and exposed to the 35×43 Kodak XAR-5 autoradiographic film, at room temperature for several days before the film was developed. The arrow on the left of the gel indicates the start of exon # 5 DNA replication from the 5′-end.

Table 1
Inhibition of DMDO-activated E_2 on the replication of exon # 5 DNA of the human tumor suppressor gene p53

Group	Cpm/μg DNA	%
Control	169 038 ± 40 888	100
30 min	84 588 ± 11 508	50
2 h	60 807 ± 6 691	36

Values given are means ± S.E.M. of two separate experiments with six independent determinations.

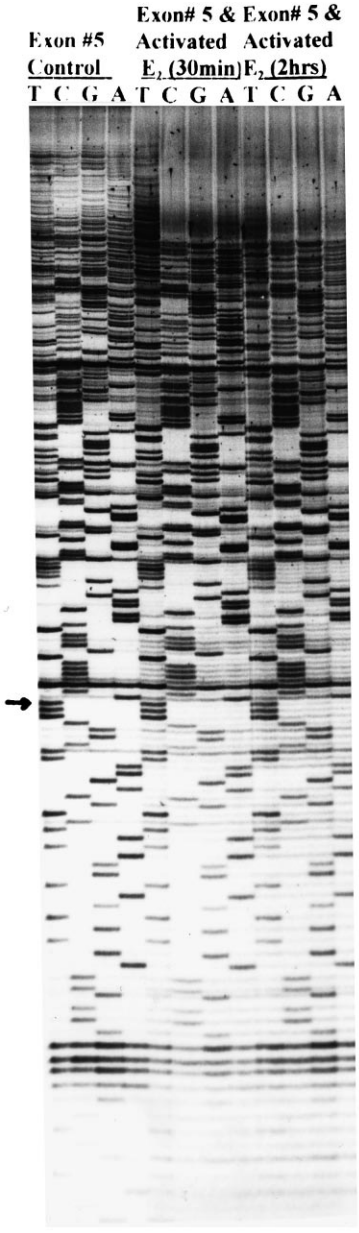

Fig. 3. Effect of DMDO-activated E_2 on the replication of exon # 5 of the human tumor suppressor gene p53 by DNA sequencing gel analysis using equal amounts of radioactivity. DMDO-activated E_2 (200 μg) was reacted with 5 μg of the pCRII vector containing exon # 5 DNA as described in Table 1. An equal amount of radioactivity estimated by the filter counting results from each group was applied onto an 8% denatured polyacrylamide sequencing gel. After the run, the gel was vacuum-dried and exposed to the 35×43 Kodak XAR-5 autoradiographic film, at room temperature for several days before the film was developed. The arrow on the left of the gel indicates the start of exon # 5 DNA replication from the 5′-end.

the double-stranded DNA, the errors of incorporation of wrong bases by DNA polymerase are believed to be a result of the formation of E_2-DNA adducts along the clusters of the complementary 'G' bases on the template strand DNA. Following the same reasoning, one finds a similar situation with the cluster of four 'T's, 10 bases above the 'C' clusters from the sequencing gel. In this case, the source of error was believed to be a result of the presence of E_2-DNA adducts from the complementary four 'A' base cluster on the template strand of DNA. In contrast, the errors of incorporation reflected from the 'G' and 'A' lanes on the sequencing gel were hardly detectable, suggesting that either the interaction of the activated E_2 with C and T bases on the template-strand of DNA was less favorable or the interaction did not result in frequent errors of incorporation.

Our earlier studies have concluded that E_2, after activation by epoxidation, is able to bind to DNA-forming DNA adducts both in vitro [10,11] and in vivo [13,14]. However, no functional tests have been conducted for these E_2-DNA adducts on any of these substrate DNAs. It is known that each chemical carcinogen has its own DNA base [7–9] and sequence [19,20] binding specificities and that the same carcinogen-DNA adduct may not have the same biological effect depending on the base, sequence as well as the conformation of the DNA where the adducts are formed [20]. Since exon # 5 of p53 has been identified as one of the mutational hotspots in breast cancer patients, it is basically important to study the effect of E_2-DNA adducts on the functional role of this important biological DNA molecule. We believe that the results from this study are significant and informative. It is our hope that this study will lead to further investigations into the molecular mechanism of E_2 as an initiator in carcinogenesis.

Acknowledgements: This investigation was supported in part by PHS Grant # CA-70466 awarded by the National Cancer Institute DHHS (F.L.Y.) and by an Excel Academic Award from the Swedish American Hospital (F.L.Y. and J.M.).

References

[1] Mcgonigle, K.F., Karlan, B.Y., Barbuto, D.A., Leuchter, R.S., Lagasse, L.D. and Judd, H.L. (1994) Gynecol. Oncol. 55, 126–132.
[2] Grady, D., Gebretsakid, T., Kerlikowske, K., Ernster, V. and Petitti, D. (1995) Obstet. Gynecol. 85, 304–313.
[3] Toniolo, P.G., Levitz, M., Zeleniuch-Jacquotte, A., Banerjee, S., Koenig, K.L., Shore, R.E., Strax, P. and Pasternack, B.S. (1995) J. Natl. Cancer Res. 87, 190–197.
[4] Colditz, G.A. (1998) J. Natl. Cancer Inst. 90, 814–823.
[5] Hankinson, S.E., Willett, W.C., Manson, J.E., Colditz, G.A., Hunter, D.J., Speigelman, D., Barbieri, R.L. and Speizer, F.E. (1998) Cancer Inst. 90, 1292–1299.
[6] Yu, F.L. and Bender, W. (1996) Carcinogenesis 17, 1957–1961.
[7] Miller, E.C. and Miller, J.A. (1981) Cancer 47, 2327–2345.
[8] Weisburger, E.K. (1980) Biochemistry 32, 95–104.
[9] Farber, E. (1984) Cancer Res. 44, 5463–5474.
[10] Yu, F.L., Wang, M.Y., Li, D.H., Bender, W. and Zheng, W.Y. (1998) Chem. Biol. Interact. 110, 173–187.
[11] Yu, F.L., Bender, W., Zheng, W.Y. and Wang, M.Y. (1998) Carcinogenesis 19, 1127–1131.
[12] Shull, J.D., Spady, T.J., Snyder, M.C., Johansson, S.L. and Pennington, K.L. (1997) Carcinogenesis 18, 1595–1601.
[13] Yu, F.L., Wang, M.Y. and Bender, W. (1999) in: International Symposium on Hormonal Carcinogenesis (Li, J. and Li, S., Eds.), Vol. III, Springer-Verlag Press, New York (in press).
[14] Yu, F.L., Wang, M.Y. and Bender, W. (1999) 90th Annual Proc. Am. Assoc. Cancer Res. 40, p. 45, April 10–14, Philadelphia, PA.
[15] Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C.C. (1991) Science 253, 49–53.
[16] Levine, A.J., Momand, J. and Finlay, C.A. (1991) Nature 351, 453–456.
[17] Harris, C.C. (1996) J. Natl. Cancer Inst. 88, 1442–1455.
[18] Sato, T., Yuyama, Y., Watabe, K., Okazaki, A., Toda, K., Okazaki, M. and Hirata, K. (1997) Cancer Lett. 115, 47–55.
[19] Yu, F.L., Bender, W. and Geronimo, I.H. (1990) Carcinogenesis 11, 475–478.
[20] Yu, F.L., Bender, W. and Hutchcroft, A. (1994) Carcinogenesis 15, 1737–1741.