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ORIGINAL ARTICLE

Cigarette smoke condensate-induced level of adenomatous polyposis coli blocks long-patch base excision repair in breast epithelial cells

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Our previous studies have shown that treatment with cigarette smoke condensate (CSC) transforms normal breast epithelial cell line, MCF-10A. In the present study, the mechanism of CSC-induced transformation of breast epithelial cells was examined. We first determined whether benzo[*a*]pyrene (B[*a*]P)- and CSC-induced levels of APC are capable of inhibiting long-patch base excision repair (LP-BER) since our earlier studies had shown that an interaction of APC with DNA polymerase β (pol-β) blocks strand-displacement synthesis. With the use of a novel *in vivo* LP-BER assay, it was demonstrated that increased and decreased APC levels in different breast cancer cell lines were associated with a decrease or increase in LP-BER activity, respectively. The effect of APC on LP-BER in malignant and pre-malignant breast epithelial cell lines was produced by either overexpression or knockdown of APC. Furthermore, it was shown that the decreased LP-BER in B[*a*]P- or CSC-treated pre-malignant breast epithelial cells is associated with an increased level of APC and decreased cell growth. Our results suggest that the decreased growth allows cells to repair the damaged DNA before mitosis, and failure to repair damaged DNA has the potential to transform pre-malignant breast epithelial cells.
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Keywords: adenomatous polyposis coli; long-patch base excision repair; benzo[*a*]pyrene; cigarette smoke condensate; breast carcinogenesis

Introduction

Several epidemiological studies have examined the relationship between smoking and breast cancer risk. Review of these studies show negative, positive or null association between smoking and breast malignancies (Palmer and Rosenberg, 1993; Hamajima *et al*., 2002; Fink and Lash, 2003). More recent studies suggest that smoking does not decrease but may enhance breast cancer risk depending upon the duration and frequency of smoking, age at the commencement of smoking and smoking before a first full-term pregnancy (Terry and Rohan, 2002; Gram *et al*., 2005; Terry and Goodman, 2006). Role of passive smoking in breast cancer has also been investigated and while some studies have found no association (Egan *et al*., 2002), others report a positive association between passive smoking and breast cancer risk (Morabia *et al*., 1996; Johnson *et al*., 2000). There are also reports suggesting that cigarette smoking has little or no independent effect on breast cancer risk (Baron, 1984; London *et al*., 1989; Baron *et al*., 1996). Furthermore, a meta analysis of 53 epidemiological studies suggested a little or no independent effect of smoking on the risk of developing breast cancer (Hamajima *et al*., 2002), but the recent California Teachers Study has provided evidence that active, but not passive smoking increases breast cancer risk (Reynolds *et al*., 2004).

Studies have also examined if polymorphism of detoxifying, activating, or DNA damage-repair genes influence the breast cancer risk in smokers (Terry and Goodman, 2006). The results show either a positive or null association between long-term smoking and *N-acetyltransferase (NAT-2), CYP1A1, glutathione S-transferase, sulfotransferase 1A1* genes (Terry and Goodman, 2006). Studies of the BER enzyme, *XRCC1*, have shown a positive association of long-term smoking with breast cancer risk (Duell *et al*., 2001; Terry and Rohan, 2002; Patel *et al*., 2005; Pachkowski *et al*., 2006). Thus, mutations in the *XRCC1* gene may influence the BER activity and reduce the repair of nonbulky DNA adducts generated by cigarette smoke constituents.

Significant experimental evidence exists to support a role of cigarette smoke in breast carcinogenesis. Cigarette smoke is a complex mixture containing over 4000 different compounds, and approximately 122 of them have been classified into six biological categories based upon their effective average concentrations (Smith and Hansch, 2000). Among them, 81 compounds, which have been classified as carcinogens by the International Agency for Research on Cancer (IARC), are pharmacologically active, toxic, mutagenic and carcinogenic in nature (Hecht, 2002). Cigarette smoke condensate

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(CSC), which has been used as a surrogate for cigarette smoke in many experimental studies, is a highly genotoxic substance capable of causing various types of DNA damage in different biological systems (DeMarini, 2004). Recently, we have shown that the spontaneously immortalized normal breast epithelial cell line MCF10A is transformed in culture by treatment with CSC (Narayan *et al*., 2004). However, the mechanism by which CSC induces breast carcinogenesis is not clear.

Cigarette smoke carcinogens require metabolic activation to form intermediates that are often more reactive than the parent compound and can form DNA adducts (Chakravarti *et al*., 2000; Hecht, 2002). Individual cigarette smoke carcinogens such as polycyclic hydrocarbons benzo[*a*]pyrene (B[*a*]P), 7,12-dimethylbenz(*a*)anthracine (DMBA) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) have been found to transform cultured cells and/or induce mammary tumors in mammals (Soule *et al*., 1990; el-Bayoumay *et al*., 1995; Mei *et al*., 2003; Currier *et al*., 2005). On the other hand, when rats were exposed to tobacco smoke via inhalation for 4 h/day for 100 days, differences in the development of mammary tumors between exposed and unexposed animals were not observed. However, when these 100 day smoke-exposed rats were treated with *N*-methyl-*N*-nitrosourea (MNU), rapid development of mammary tumors occured (Steinetz *et al*., 2006) indicating that exposure to tobacco smoke sensitizes rats to MNU-induced mammary tumorigenesis.

Most B[*a*]P-DNA adducts result from an interaction of reactive metabolites with the nucleophilic groups on two purine bases, adenine and guanine. Those adducts that are repaired by the BER pathway, can be either stable or depurinating. Through comprehensive studies of the DNA adducts of B[*a*]P, Cavalieri and Rogan (1998) and Chakravarti *et al*. (1995) have demonstrated a strong association between depurinating adducts and oncogenic mutations in the *H-ras* gene, suggesting that these adducts are important in tumor initiation and progression. Furthermore, it has been found that in lung cancer cells, approximately 30% of GC to TA transversions occur at the hot spot codon 248 and 273 of *TP53* gene and at codon 12, 13 or 61 of *K-ras* gene (Slebos *et al*., 1991; Husgafvel-Pursiainen *et al*., 1993; Westra *et al*., 1993; Greenblatt *et al*., 1994). A similar mutation spectrum has been detected in the *TP53* gene of bronchial epithelial cells treated with B[*a*]P-7,8-diol-9,10-epoxide (Pfeifer *et al*., 2002). GC to TA transversions have also been found in *Salmonella typhimurium* strains by CSC treatment, suggesting a role of PAHs in inducing this class of mutations (DeMarini *et al*., 1995).

Based on studies of B[*a*]P-induced mouse skin papilloma it has been suggested that the H-*ras* mutations are generated by misrepair, rather than misreplication, of the apurininc sites (Chakravarti *et al*., 1998). The B[*a*]P-induced apurinic sites in cellular DNA are 15- to 120-fold higher than those formed spontaneously, suggesting that this large increase in apurinic sites has the potential of overwhelming the cell repair capacity before replication occurs (Chakravarti *et al*., 1995; Cavlieri and Rogan, 1998). Thus, it is likely that nonrepair of apurinic sites in the DNA induced by carcinogenic constituents of CSC may generate critical mutations which in turn play a role in initiating transformation of normal breast epithelial cells; however, the mechanisms remain obscure.

Biochemical and genetic studies indicate that the repair of apurinic/apyrimidinic (AP)-sites in DNA occurs through two subpathways of BER that differ on the basis of repair gap size and the enzymes involved in these repair pathways (Wilson, 1998). These subpathways are designated as 'single-nucleotide BER' also referred to as 'short patch (SP)-BER' and 'multinucleotide BER' also referred to as 'long patch (LP)-BER'. In both subpathways, repair is initiated by excision of a damaged base by a DNA glycosylase leaving an abasic or AP-site in DNA. The resulting AP-sites, which are also generated spontaneously or by radiation and chemicals, are subsequently acted upon by an AP endonuclease (APE) to generate a 3′ hydroxyl group and a 5′-deoxyribosephosphate (dRP) terminus. In SP-BER, DNA polymerase β (pol-β) extends the 3′ terminus by a single nucleotide and removes the dRP moiety with its dRP lyase activity. Finally, the nick is sealed by DNA ligase I or DNA ligase III/XRCC1 (Srivastava *et al*., 1998). When AP-sites are oxidized or reduced, they become resistant to β-elimination and cannot be excised by the dRP lyase activity of pol β. In these cases, the modified AP-site is repaired *via* the long-patch base excision repair (LP-BER) pathway in which pol-β, δ or ε incorporates 2–15 nucleotides displacing the strand containing the modified AP-site. The DNA flap structure is cleaved by Fen-1, and the nick is sealed by a DNA ligase (Bambara *et al*., 1997; Klungland and Lindahl 1997).

We have previously shown that *adenomatous polyposis coli* (*APC*) gene expression is induced in colon cancer and breast epithelial cell lines upon exposure to the DNA-damaging agents *N*-methyl-*N*′-nitro-*N*-nitrosoguanine (MNNG), methylmethane sulfonate (MMS), dimethylhyrdazine (DMH), as well as cigarette smoke carcinogen, DMBA (Narayan and Jaiswal, 1997; Jaiswal and Narayan, 2001a; Narayan *et al*., 2005; Jaiswal *et al*., 2006), suggesting a possibility of interaction between APC and DNA repair machinery. Recently, we have shown that APC interacts with pol-β and blocks LP-BER by blocking strand-displacement synthesis and then DNA repair (Narayan *et al*., 2005). As B[*a*]P can generate abasic lesions in DNA that can be repaired by the BER pathway, a compromised BER can lead to accumulation of mutations and possibly transformation of normal breast epithelial cells. Based on these observations, we hypothesized that CSC-induced DNA damage increases APC protein level, which interacts with pol-β to block pol-β-mediated strand-displacement synthesis of LP-BER and lead to cell transformation. B[*a*]P studies were run parallel to establish the similarities between CSC and B[*a*]P action.

Results

Increased levels of APC are associated with decreased LP-BER in breast cancer cell lines

To test the hypothesis that APC blocks LP-BER, we examined LP-BER activity in several breast cancer cell lines (MCF7, BT20, MDA-MB-231 (MB-231) and MDA-MB-468 (MB-468)) with different levels of APC. A novel *in vivo* plasmid-based LP-BER assay was established which is based on the principle that the reduced R-p21P plasmid when transfected into cells would show poor promoter activity as compared to the unmodified p21P plasmid (see Materials and methods). However, the promoter activity can be restored if the modified DNA is allowed to go through DNA repair process(es) within the cell. This quick, sensitive and quantitative assay provides valuable information but suffers from the limitation that it does not indicate if the damage and repair is more efficient on a transcribed or nontranscribed strand of DNA and the number of C-residues modified and repaired within the cell in a given time period. Nonetheless, it provides valuable information about the DNA repair capacity of the cell.

To establish the association of APC levels with LP-BER activity, MCF7, BT20 and MB-231 cell lines with very low APC protein and MB-468 cells with very high APC protein levels were used (Figure 1a). Cells were harvested at 0 and 30 h time points to measure the p21P or R-p21P promoter activity. The p21P promoter activity increased with time in all of the four cell lines; however, the activity was much higher in the MCF7 and MB-231 cell lines (Figure 1b). The R-p21P promoter activity at the 30 h time point compared to the 0 h time point was increased in the MCF7, BT20 and MB-231 cell lines, which have a low level of APC expression (Figure 1b, compare lane 3 with 4, 7 with 8 and 11 with 12, respectively). However, in the MB-468 cell line, which expresses a high level of APC, the R-p21P promoter activity was 50% lower at the 30 h time point (Figure 1b, compare lane 15 with 16). These experiments suggested that the unmodified p21P promoter is active in all of the cell lines; however, the modified R-p21P promoter activity is linked with the APC level. The breast cancer cell lines with lower APC levels showed a higher R-p21P promoter activity, and the cell line with higher APC activity showed a lower R-p21P promoter activity. As the modified R-p21P can be repaired primarily by the LP-BER pathway, the change in R-p21P promoter activity is indicative of the repair of R-p21P DNA by LP-BER.

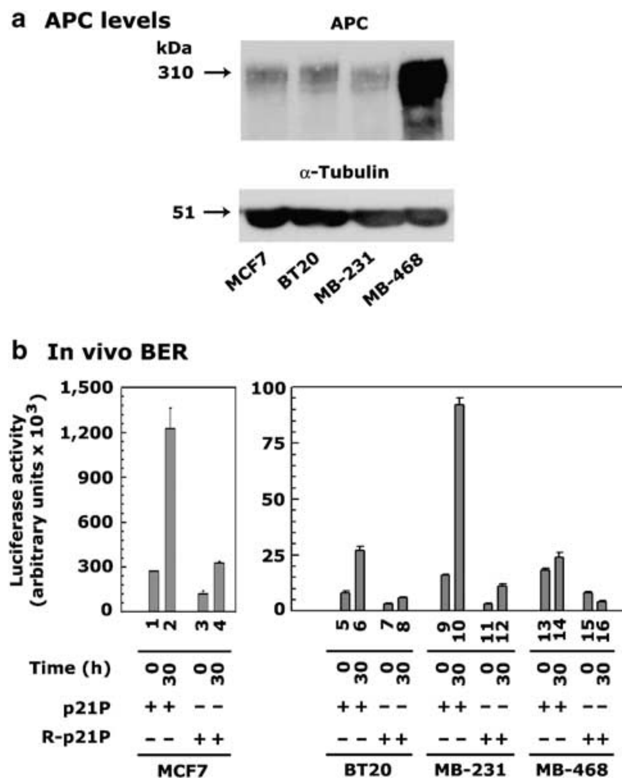

Figure 1 Association of APC levels with *in vivo* LP-BER in different breast cancer cell lines. (**a**) Western blot analysis. Whole cell lysate was prepared from MCF7, BT20, MB-231 and MB-468 breast cancer cell lines and processed for the determination of APC protein levels by Western blot analysis. The lower panel shows the α-tubulin level in these cell lines, which serves as a control to assess the loading of the proteins in each lane. (**b**) An *in vivo* LP-BER assay. The luciferase gene-reporter assay for *in vivo* LP-BER was performed after co-transfection of p21P or R-p21P and β-gal plasmids in different breast cancer cell lines. Data are the mean ± s.e. of three different experiments.

Overexpression of APC blocks in vivo *LP-BER in BT20 breast cancer cell line*

As the BT20 cell line expresses a low level of APC and shows an improved level of LP-BER, then it is likely that overexpression of APC would decrease LP-BER. To test this possibility, we coexpressed pCMV-APC with either p21P or R-p21P plasmids in BT20 cells and determined the luciferase-reporter activity to assess the LP-BER. We observed a time-dependent increase in the APC protein level after overexpression with pCMV-APC in BT20 cells (Figure 2a). The p21P promoter activity was more at the 12 h time point and then slightly decreased at the 24 and 48 h time points but still maintained a twofold higher activity. The R-p21P promoter activity was lower than the p21P promoter activity at early time points, but it reached close to the activity at the 48 h time point (Figure 2b, upper panel). This suggested that in the presence of low levels of APC there is an increased level of LP-BER in BT20 cells. On the other hand, once the APC levels were increased in BT20 cells with pCMV-APC, the p21P promoter activity was increased in a dose-dependent manner; however, the R-p21P activity decreased (Figure 2b, lower panel). These results suggest that the increased levels of APC cause a decreased level of LP-BER in BT20 cells.

Silencing of APC improves LP-BER in MB-468 breast cancer cell line

If the increased level of APC decreases LP-BER, then it is likely that the decreased level of APC would improve

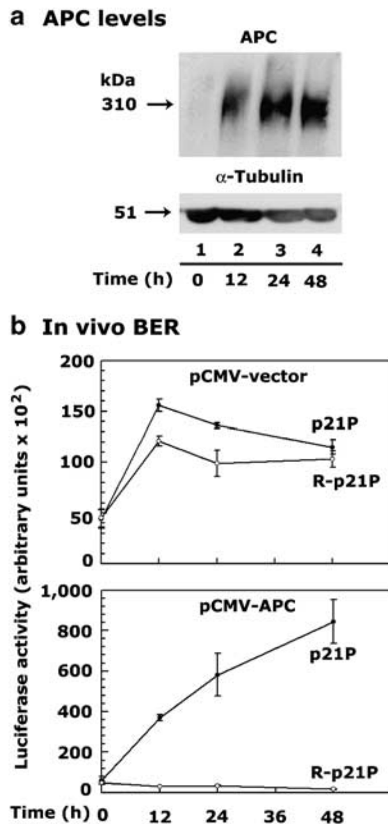

Figure 2 Effect of overexpression of APC on *in vivo* LP-BER in BT20 breast cancer cell line. (**a**) Western blot analysis. The APC was overexpressed in BT20 cell lines by using pCMV-APC plasmid. Cells were harvested at different time periods and APC protein determined by Western blot analysis. The α-tubulin level in BT20 cells served as a control to assess the loading of the proteins in each lane. (**b**) *In vivo* LP-BER. The BT20 cells were co-transfected with pCMV-APC and either p21P or R-p21P and β-gal plasmids. Cells were harvested at different time points and luciferase activity was determined to assess the effect of overexpression of APC on LP-BER. Data are the mean ± s.e. of three different experiments.

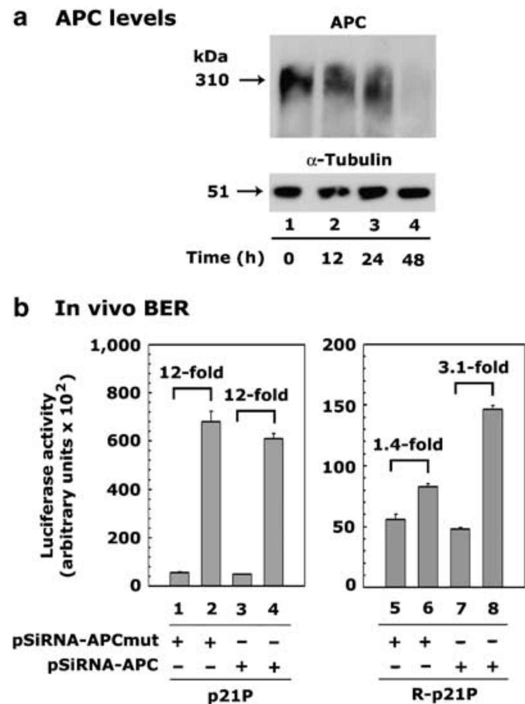

Figure 3 Effect of knockdown levels of APC on *in vivo* LP-BER in MB-468 breast cancer cell line. (**a**) Western blot analysis. The APC was knocked-down by pSiRNA-APC in MB-468 cells. Cells were harvested at different periods and APC protein determined by Western blot analysis. The α-tubulin level in MB-468 cells served as a control to assess the loading of the proteins in each lane. (**b**) *In vivo* LP-BER. The MB-468 cells were co-transfected with pSiRNA-APC or pSiRNA-APCmut and with p21P or R-p21P and β-gal plasmids as described in Materials and methods. Cells were harvested at different time points, and luciferase activity was determined to assess the effect of downregulation of APC on LP-BER. Data are the mean ± s.e. of three different experiments.

LP-BER. To test this hypothesis, we used the MB-468 breast cancer cell line which expresses a high level of APC (Figure 1a). We downregulated APC expression in MB-468 cells by SiRNA technique and then determined the R-p21P promoter activity. The APC protein levels were decreased more than 90% at 48 h in MB-468 cells transfected with pSiRNA-APC plasmid (Figure 3a, compare lane 1 with 4). Then we used the 48 h time point and determined the p21P or R-p21P promoter activity in these cells to assess the effect of APC on LP-BER. The p21P promoter activity was increased 12-fold in MB-468 cells either transfected with pSiRNA-APCmut or pSiRNA-APC plasmids (Figure 3b, compare lane 1 and 2, with 3 and 4, respectively). On the other hand, the R-p21P promoter activity increased only 1.4-fold in pSiRNA-APCmut transfected than 3.1-fold in pSiRNA-APC transfected MB-468 cells (Figure 3b, compare lane 5 with 6 and 7 with 8). The p21P promoter activity was much higher in both pSiRNA-APCmut and pSiRNA-APC transfected conditions; however, the R-p21P promoter activity was much lower under the similar treatment conditions. This difference may have been due to a poor LP-BER capacity of MB-468 cells. Nonetheless, the LP-BER was higher in pSiRNA-APC transfected cells than in pSiRNA-APCmut transfected cells. Thus, these results further suggest that the decreased levels of APC increase LP-BER.

Silencing of APC increases LP-BER in the pre-malignant breast epithelial cell line MCF10AT

The above experiments were performed with breast cancer cells. To further examine whether similar effects of APC are observed with normal breast epithelial cells, we used MCF10AT cells. The MCF10AT cell line has been derived from the spontaneously immortalized human normal breast epithelial cell line MCF10A cells by stable transfection with mutant T24-H-*ras* (Russo *et al*., 1991). These are pre-malignant but nontumorigenic cells (Basolo *et al*., 1991; Miller *et al*., 1993). In our preliminary experiments, we found that the endogenous APC protein level in MCF10A cells was much lower than in MCF10AT cells (data not shown). Since our aim was to understand the role of APC in BER after CSC

treatment, we used MCF10AT cells in our experiments to manipulate APC levels by SiRNA technique. These cells were co-transfected either with R-p21P plasmid along with pSiRNA-APC or pSiRNA-APCmut and β-galactosidase plasmids. The level of APC was knocked-down by pSiRNA-APC as shown in Figure 4a (compare lane 1 with 2). The R-p21P promoter activity was 8.6-fold higher at 48 h in pSiRNA-APC transfected (Figure 4b, compare lane 3 with 4) versus 2.9-fold increase in pSiRNA-APCmut transfected MCT10AT cells (Figure 4b, compare lane 1 with 2). These results are similar to the MCF7, BT20 and MB-231 breast cancer cell lines suggesting that MCF10AT cells have higher LP-BER capacity with low levels of APC than with high levels of APC such as with MB-468 cells (Figure 1).

B[a]P and CSC induce APC levels and decrease DNA repair in MCF10AT cells

As B[*a*]P and CSC generate abasic lesions in DNA that can be repaired by the BER pathway, we determined whether MCF10AT cells with different levels of APC will show an effect on DNA repair after treatment with B[*a*]P and CSC. The DNA damage and repair was measured by single-cell gel-electrophoresis or comet assay. It was hypothesized that cells with a wild-type *APC* gene expression will be more sensitive to B[*a*]P and CSC treatment. If the DNA damage-induced by B[*a*]P and CSC are repaired by the LP-BER pathway, then the increased levels of APC should be expected to block DNA repair and exhibit increased comet formation. We observed an increased level of APC in MCF10AT cells treated with 25 μM of B[*a*]P or 25 μg/ml of CSC for 20 and 30 h (Figure 5), which was parallel to the increased level of comet formation (Figure 6,

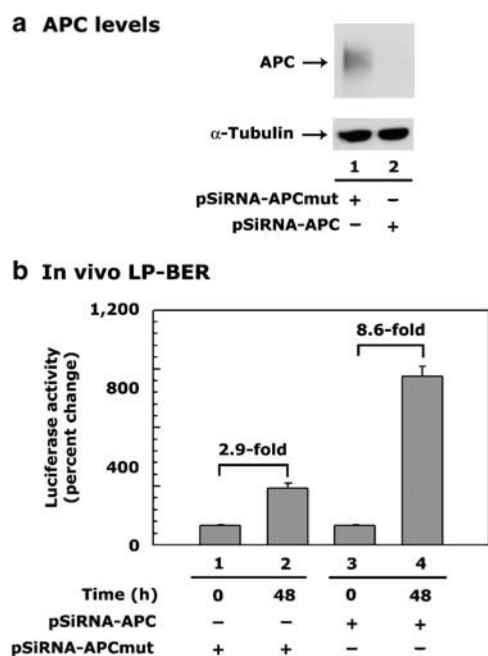

Figure 4 APC-knocked-down in pre-malignant breast epithelial cell line MCF10AT improves LP-BER capacity. (**a**) APC protein levels in MCF10AT cells after transfection with pSiRNA-APCmut (lane 1) and pSiRNA-APC (lane 2) plasmids. The α-tubulin level serves as a control to assess protein loading in each lane. (**b**) Luciferase-reporter assay. The luciferase gene-reporter activity of the R-p21P promoter was determined after co-transfection with pSiRNA-APC or pSiRNA-APCmut plasmids in MCF10AT cells. Data are the mean ± s.e. of three different experiments.

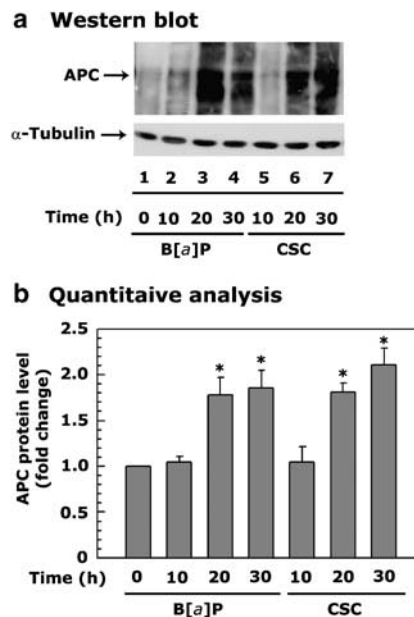

Figure 5 APC protein levels are increased in MCF10AT cells treated with B[*a*]P and CSC. (**a**) Western blot analysis. MCF10AT cells were treated with 25 μg/ml CSC or 25 μM B[*a*]P for different time intervals. Cells were harvested and lysate prepared for Western blot analysis of APC and α-tubulin levels. (**b**) Quantitative analysis of APC protein level. The APC protein levels of autoradiograms were quantified and presented as a graph. Data represent the mean ± s.e. of three different experiments.

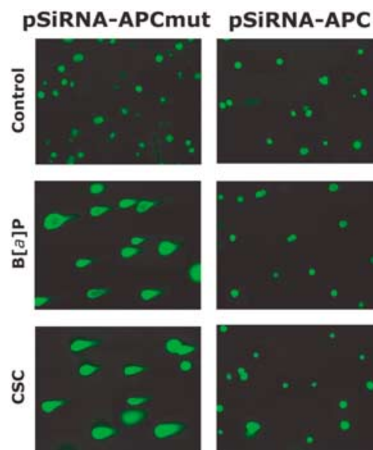

Figure 6 B[*a*]P- and CSC-induced levels of APC block DNA repair in MCF10AT cells. A comet assay was performed for these studies. MCF10AT cells were transfected with either pSiRNA-APCmut or pSiRNA-APC plasmids and treated with 25 μM of B[*a*]P or 25 μg/ml of CSC for 30 h. After treatment, cells were processed for comet assay as described under 'Materials and methods'. Data are representative of three different experiments.

left-hand side panel). From these results we conclude that B[*a*]P and CSC treatment increased APC protein levels which decreased DNA repair in MCF10AT cells. We further recapitulated the role of B[*a*]P- and CSC-induced APC levels in DNA damage repair by silencing APC expression in MCF10AT cells by the transfection of the pSiRNA-APC plasmid. The results show that comet formation was drastically reduced in both B[*a*]P- and CSC-treated cells which were transfected with pSiRNA-APC (Figure 6, right-hand side panel). As APC blocks the LP-BER pathway (Narayan *et al*., 2005) and since the comet was abolished in the absence of APC, it is concluded that the major damage introduced by B[*a*]P and CSC is repaired by LP-BER.

We also examined whether the B[*a*]P- and CSC-induced levels of APC were at the transcriptional level. For these experiments, we treated MCF10AT with different concentrations of B[*a*]P or CSC for 24 h, and the *APC* mRNA levels were determined by Northern blot analysis. We found an increased level of *APC* mRNA in these cells treated with both B[*a*]P and CSC (Figure 7a). The maximum increase in the *APC* mRNA level was found up to 25–50 μM of B[*a*]P and 10–25 μg/ml of CSC treatment. Then, to further examine whether the increased *APC* mRNA levels were due to increased transcriptional activity, we determined the promoter activity of the *APC* gene in MCF10AT cells. The pAPCP promoter (Jaiswal and Narayan, 2001b) was transfected into MCF10AT cells and then treated with 25 μM of B[*a*]P or 25 μg/ml of CSC for various time intervals. Results showed a significantly increased pAPCP promoter activity in a time-dependent manner (Figure 7b). Further, we optimized B[*a*]P and CSC concentrations for pAPCP activity. The MCF10AT cells were transfected with the pAPCP plasmid and then treated with different concentrations of B[*a*]P and CSC for 24 h. We found a dose-dependent increase in the pAPCP activity in which the concentration of 25 μM of B[*a*]P and 25 μg/ml of CSC treatment was found to be optimum for these studies (Figure 7c). From these results it is concluded that the increased protein levels in MCF10AT cells after treatment with CSC and B[*a*]P are due to increased transcriptional regulation of *APC* gene expression.

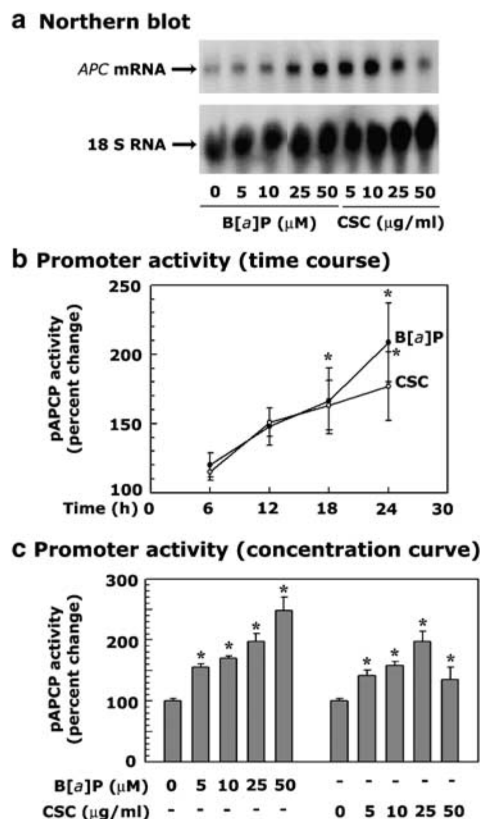

Figure 7 *APC* mRNA levels and pAPCP promoter activity in MCF10AT cells treated with CSC and B[*a*]P. (**a**) *APC* mRNA levels. Cells were treated with different concentrations of CSC and B[*a*]P for 24 h and then processed for Northern blot analysis. A representative autoradiogram of *APC* mRNA and 18S RNA is shown here. (**b**) Time course of the pAPCP promoter activity in MCF10AT cells treated with 25 μg/ml of CSC and 25 μM of B[*a*]P for different time intervals. (**c**) Concentration curve of the pAPCP promoter activity in MCF10AT cells treated with different concentrations of CSC and B[*a*]P for 24 h. Results were normalized with β-gal activity. Data are the mean$\pm$s.e. of three different experiments. *Significantly different than untreated control.

Increased levels of APC decrease the growth of MCF10AT cells after B[a]P and CSC treatment

If the BER capacity of MCF10AT cells is compromised after B[*a*]P and CSC treatment, then the affected cells would arrest in cell cycle for repair and grow slowly as compared to the cells with efficient BER. As B[*a*]P and CSC treatment induce APC levels and since increased levels of APC block LP-BER in MCF10AT cells, it is hypothesized that the blocked LP-BER will cause growth retardation of these cells. To test this hypothesis, we silenced *APC* gene expression by the SiRNA technique (Figure 4). The pSiRNA-APCmut plasmid transfected cells showed growth retardation after B[*a*]P and CSC treatment (Figure 8, left-hand panel). However, the growth of MCF10AT cells transfected with pSiRNA-APC, in which the APC protein levels were knocked-down, was better than pSiRNA-APCmut transfected cells after CSC and B[*a*]P treatment (Figure 8, right-hand panel). As the difference in these two sets of experiments was the expression of APC, these results suggest that the decreased levels of APC resulted in increased growth of MCF10AT cells due to improved LP-BER after treatment with CSC and B[*a*]P (Figure 8, right-hand panel). From these results we conclude that APC blocks LP-BER in MCF10AT cells and puts them at higher risk of carcinogenesis after treatment with B[*a*]P and CSC. We also observed that the presence of APC in pSiRNA-APCmut transfected MCF10AT cells showed slower cell growth than the absence of APC in pSiRNA-APC transfected MCF10AT cells without treatment with B[*a*]P or CSC, suggesting a role of APC on cell cycle control.

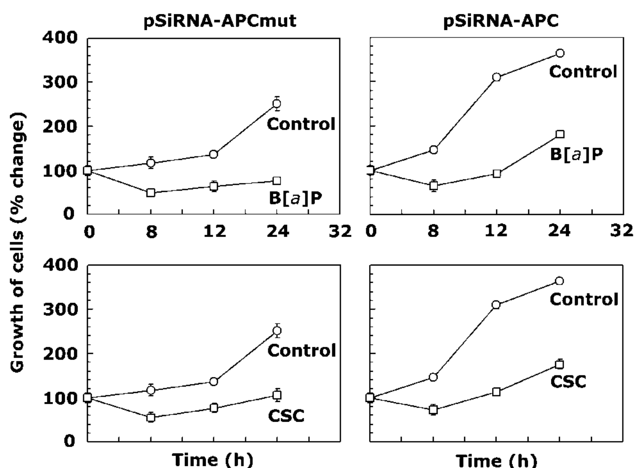

Figure 8 MCF10AT cells with wild-type APC are resistant to growth after B[*a*]P and CSC treatment. MCF10AT cells were transfected with pSiRNA-APC or pSiRNA-APCmut plasmids as described in Figure 4. Cells were treated with 25 μM B[*a*]P or 25 μg/ml CSC for 24 h. The growth of these cells was determined by MTT assay at different time intervals (control = ○, treated = □). Data are the mean ± s.e. of three different experiments.

Discussion

In the past several years evidence has been provided that various molecular mechanisms resulting in genomic instability play an important role in human carcinogenesis (Raptis and Bapat, 2006). Once a normal cell is challenged with a DNA-damaging agent, two possibilities arise immediately within the cell. First, the DNA repair activity of the cell increases and fixes the damaged DNA. Second, if the DNA repair mechanism of the cell is compromised, then the accumulation of multiple genetic mutations may occur. The consequence of the accumulation of mutations in a susceptible cell may lead to the perturbation of regulatory network(s) that controls proliferation, survival, cellular function and ultimately results in cancer (Coates *et al*., 2005). Many of the cigarette smoke carcinogens are well-known initiators and promoters of carcinogenesis in various organs such as the lung, oropharynx, stomach, pancreas, liver and colon (Nishikawa *et al*., 2004). However, whether cigarette smoking is related to breast carcinogenesis is not well established.

In the present study, CSC was used as a surrogate for cigarette smoke carcinogens instead of individual components. It contains most of the particulate chemicals identified in the cigarette smoke (Smith and Hansch, 2000; Hecht, 2002; Stabbert *et al*., 2003). The transformation of the normal breast epithelial cells by pure cigarette smoke carcinogens such as B[*a*]P, DMBA and NNK have been well documented (Soule *et al*, 1990; el-Bayoumay *et al*., 1995; Mei *et al*., 2003; Currier *et al*., 2005). However, the effect of chemically complex cigarette smoke, which contains both carcinogenic substances as well as their inhibitors, still remains an unresolved question. Thus, we treated pre-malignant MCF10AT cells with B[*a*]P and CSC in parallel experiments and compared our results. The referenced cigarettes, 1R4F, have a tar or CSC content of about 10 mg/cigarette, which contains about 6.94 ng of B[*a*]P (Stabbert *et al*., 2003; Roemer *et al*., 2004). Clearly, the concentrations of CSC (0–50 μg/ml) employed in our studies were very low in comparison to the amount present in a cigarette. However, how much of the CSC was taken up by the cells is not known. Based on urinary metabolites, it has been estimated that the uptake of nicotine and B[*a*]P amounts to 1.1 mg and 0.01 μg/cigarette, respectively (Roemer *et al*., 2004). In our experiments based upon IC_{50} concentration, 0–12.5 μg/ml of B[*a*]P was used, which was much higher than the amount taken up by the cells as estimated by Roemer *et al*. (2004). However, it is important to consider that only a small portion of the B[*a*]P present in the culture medium comes in contact with cells, and their uptake is further influenced by cellular factors. From these estimates it is clear that the amount of B[*a*]P present in CSC is much lower than used in the experiments as a reference compound. In our experiments, the APC levels and DNA damage in MCF10AT cells induced by 25 μg/ml of CSC and 12.5 μg/ml of B[*a*]P were comparable. Thus, it is reasonable to conclude that the CSC effects observed represent not only the B[*a*]P effects but the total effect of all similar CSC constituents.

Our earlier study had already established that CSC is capable of inducing the transformation of normal breast epithelial cells (Narayan *et al*., 2004). In previous studies, we have also observed an increased level of APC in cancer cell lines treated with DNA-damaging agents (Narayan and Jaiswal, 1997; Jaiswal and Narayan, 2001a; Narayan *et al*., 2005; Jaiswal *et al*., 2006). We have recently demonstrated a role of APC in DNA repair suggesting a block of DNA pol-β-mediated strand-displacement synthesis of long-patch base-excision repair (LP-BER) by increased APC (Narayan *et al*., 2005). As compromised DNA repair capacity of breast epithelial cells can play a role in the carcinogenic process, we examined whether CSC treatment also increases APC levels which in turn compromises with LP-BER capacity of the breast epithelial cells.

To examine the effect of CSC and B[*a*]P on breast carcinogenesis, we studied three different parameters. First, we established an *in vivo* LP-BER assay system to examine the role of APC in LP-BER. This assay system utilizes a known type of DNA damage introduced in a p21P promoter whose activity can be monitored to assess the efficiency of the repair in the cell. As described previously, we randomly modified C residues of the p21P promoter to a reduced abasic site (R-p21P) plasmid by chemical and enzymatic modification (Jaiswal *et al*., 2002). The reduced abasic site of the R-p21P plasmid can be repaired by the LP-BER pathway involving pol-β. The compromised LP-BER is indicated by the reduced R-p21P activity. Indeed, we found that the R-p21P activity was higher in breast cancer cell lines with lower levels of APC and vice versa. These findings were further confirmed either by overexpression of APC in cells with low levels of endogenous APC or down-regulation of APC in cells with high levels of endogenous APC. In the case of overexpression of APC,

we found a decreased level of LP-BER in breast cancer cells. While in the case of the downregulation of APC, we found an increased level of LP-BER in breast cancer cells. These observations were extended to pre-malignant breast epithelial cells and the similar results were observed. Thus, our *in vivo* plasmid-based LP-BER assay was a useful tool to describe the role of APC in DNA repair. These results were consistent with the comet assay results in which an increased DNA repair was seen with the silenced APC than with the wild-type APC in MCF10AT cells treated with B[*a*]P and CSC.

Second, we examined whether the expression of APC is increased in MCF10AT cells after treatment with B[*a*]P and CSC. Our results described that the APC expression is increased at both mRNA and protein levels in B[*a*]P- and CSC-treated MCF10AT cells. Previously, we have also reported an increase in the APC level in MCF10A cells after the treatment with DMBA (Jaiswal *et al*., 2006). These results suggest that the increase in the APC level by CSC is as similar as with its pure components; however, the mechanism of *APC* gene expression by different compounds may differ from each other.

Third, we looked at whether APC affects the growth of MCF10AT cells after treatment with B[*a*]P and CSC. CSC, B[*a*]P, and other constituents of CSC have been shown to generate abasic DNA damage in cells (Chakravarti *et al*., 1995; DeMarini *et al*., 1995; Cavlieri and Rogan, 1998). However, the mechanism by which they are repaired is not yet well described. B[*a*]P metabolite, benzo[*a*]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide-(±)-*anti* (BPDE) also produces bulky DNA adducts, which are repaired by nucleotide excision repair (NER) as well as BER pathways (Braithwaite *et al*., 1998; Wani *et al*., 2002; Porter *et al*., 2005). Our results demonstrate that the abasic lesions which are introduced by CSC and B[*a*]P are primarily repaired by the LP-BER pathway. If B[*a*]P and CSC induced other types of DNA damage than the one produced by reduced or oxidized abasic sites, then we may have seen no increased DNA repair and thus no reduction in the comet in the absence of APC. Thus, our results suggest that B[*a*]P and CSC induce abasic lesions in DNA and induce *APC* gene expression in breast epithelial cells. The impaired LP-BER can cause growth retardation in breast epithelial cells with wild-type APC after B[*a*]P- and CSC-induced DNA damage. This provides an opportunity for cells to repair the damage before mitosis. If the damage reaches beyond the repair capacity of the cell, then the cell either goes through apoptosis or transformation. Thus, the increased levels of APC block the LP-BER pathway and may contribute to the accumulation of mutations and the carcinogenic transformation of normal breast epithelial cells. In previous studies, we have shown that the treatment of B[*a*]P and CSC caused growth retardation and transformation of immortalized normal breast epithelial cell line, MCF10A cells (Narayan *et al*., 2004). On the other hand, an efficient LP-BER in the APC-knocked-down breast epithelial cells may result in normal growth and avoid accumulation of mutation and thus transformation of normal breast epithelial cells after B[*a*]P and CSC treatment.

Materials and methods

Maintenance and treatment of cells

The MCF10AT cells were grown in DMEM/F-12 (50:50, v/v) medium supplemented with 5% (v/v) horse serum (Sigma Chemical Co., St Louis, MO, USA), 100 U/ml of penicillin, 100 μg/ml of streptomycin, 0.5 μg/ml of hydrocortisone, 100 ng/ml of cholera toxin, 10 μg/ml of insulin, 10 ng/ml of epidermal growth factor and 1% (w/v) of L-glutamine at 37°C under a humidified atmosphere of 5% CO_2. BT20, MB-231 and MB-468 cells were grown in MEM and MCF7 cells were grown in Rosewell's Park Memorial Institute media medium. Both medium were supplemented with 10% fetal bovine serum (FBS). After cells reached 60% confluence, they were treated with B[*a*]P (Sigma Chemical Co., St Louis, MO, USA) or CSC for various time intervals as shown in figure legends.

Preparation of CSC

The CSC was prepared from the University of Kentucky Reference Cigarette 1R4F (9 mg tar and 0.8 mg. nicotine/cigarette) adapting the previously described procedure (Hsu *et al*., 1991). Briefly, the 'tar' or particulate phase of smoke was collected on a Cambridge filter pad from cigarettes smoked under standard Federal Trade Commission conditions (35 ml puff volume of a 2 s duration) and dissolved in dimethylsulfoxide (Pillsbury and Bright, 1972).

Luciferase gene-reporter assay for in vivo *BER*

The C-residues of a plasmid DNA of p21(Waf-1/Cip1) promoter (pGL3-p21) was deaminated by 3 M sodium bisulfite in the presence of 50 mM hydroquinone (Shortle and Botstein, 1983). The deamination of C-produces U-residues (U-p21P), which become a substrate for SP-BER. The resulting U-p21P DNA was further treated with uracil-DNA glycosylase (UDG) and then reduced with 0.1 M sodium borohydride to generate reduced AP-sites (R-p21P), which become a substrate for LP-BER. Cells were grown to 60–70% confluence in 60 mm tissue culture dishes and transfected with 2.0 μg/ml of either p21P or R-p21P plasmid and 0.5 μg/ml of pCMV-β-galactoside (β-gal) plasmid using 5 μl/ml of FuGENE6 reagent. The pCMV-β-gal served as an internal control to correct the differences in the transfection efficiency. With 5 h of acclimatization after transfection, one set of cells was harvested, and the promoter activity determined at this time point was considered as zero time point. The medium of the remaining dishes was aspirated and replaced with complete medium supplemented with 5% horse serum and growth factors. Cells were harvested at different time intervals as shown in figure legends. The luciferase gene-reporter activity of the cellular lysate was determined by using a Moonlight 3010 Illuminometer (Promega, San Diego, CA, USA). The reporter activity was interpreted as the extent of DNA repair in these cells.

Overexpression of APC in BT20 cell line

The wild-type APC protein was overexpressed in BT20 cells (expressing very low level of endogenous APC) by transient transfection of pCMV-APC plasmid. Cells were grown in 60 mm tissue culture dishes to 60% confluence and then transfected with 6 μg pCMV-APC plasmid with 9 μl FuGENE-6 transfection reagent at room temperature for 45 min. After 5 h of transfection, the medium was replaced with the complete

medium supplemented with 10% FBS. Cells were harvested at different time intervals, and lysates were prepared to analyse the APC protein by Western blot analysis.

Silencing of APC in MB-468 cell line
The wild-type *APC* gene expression was silenced in MB-468 cells by the procedure described earlier (Narayan *et al*., 2005). In brief, cells were grown in 60 mm tissue culture dishes to 60% confluence and then transfected with 4 μg of pSiRNA-APC or pSiRNA-APCmut plasmids with 9 μl of FuGENE-6 transfection reagent. Cells were harvested at different time intervals, and the APC protein level was determined by Western blot analysis as described by Narayan and Jaiswal (1997).

Northern blot analysis
For northern blot analysis, the total RNA from untreated and treated cells was isolated by TRIzol TM reagent as described by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA, USA). Fifty micrograms of total RNA were separated on 1% formaldehyde-agarose gel and transferred onto a Hybond-N^+ membrane (GE Healthcare, Piscataway, NJ, USA). The membrane was incubated with ^{32}P-labeled *APC* probe (*Eco*RI fragment of APC-HFBCI43; ATCC, Manassas, VA, USA). Later, the same membrane was reprobed with ^{32}P-labeled *Eco*RI fragment of 18S RNA probe for normalization of RNA loading and transfer efficiency (Jaiswal *et al*., 2006). The membrane was exposed to X-ray films for detection of specific mRNA signal. Data are the representative of two different experiments.

Comet assay
The MCF10AT cells were transfected with pSiRNA-APC or pSiRNA-APCmut plasmids and treated with CSC or B[*a*]P. For evaluating DNA damage and repair in MCF10AT cells after treatment, a single-cell gel electrophoresis (comet assay) was performed as described by the manufacturer (Trivigen, Gaithersburg, MD, USA). DNA from cells was visualized by staining with SYBR green dye. Images were captured using a fluorescence microscope (Zeiss Axioplan-2 Imaging, Thornwood, NY, USA) at 20-times magnification.

Growth assay
MCF10AT cells were grown in 96-well tissue culture plates and transfected with pSiRNA-APC plasmid (Narayan *et al*., 2005) using FuGENE-6 reagent as described by the manufacturer (Roche Diagnostics Corporation, Indianapolis, IN, USA). Briefly, 4 μg pSiRNA-APC plasmid was mixed with 9 μl of the FuGENE-6, and DNA/lipid mixture was assembled for 45 min at room temperature. DNA/lipid mixture was slowly added and gently mixed with medium. After 24 h post-transfection, cells were treated with CSC or B[*a*]P for various time intervals as shown in figure legends. The survival of the cells was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (ATCC, Manassas, VA, USA).

Abbreviations

APC, adenomatous polyposis coli; B[*a*]P, benzo[*a*]pyrene, CSC, cigarette smoke condensate; LP-BER, long-patch base excision repair; pol-β, DNA polymerase β.

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