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Letter

The correlations between BRCA1 defect and environmental factors in the risk of breast cancer

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ABSTRACT — The risk factors for breast cancer, the most common female malignant cancer, include environmental factors such as radiation, tobacco, a high-fat diet, and xenoestrogens as well as hormones. In addition, BRCA1 and BRCA2 are the most well-known genetic factors that increase risk for breast cancer. Coincidence of those environmental and genetic factors might augment the risk of tumorigenesis of breast. To verify this hypothesis, we briefly evaluated the carcinogenic potency of various environmental factors in the absence or presence of BRCA1 as a genetic factor in a normal mammary epithelial cell line, MCF10A. Many environmental factors tested increased cellular ROS level in the absence of other insult. In addition, TCDD, DMBA, 3MC, and BPA enhanced the BaP-induced ROS production. BRCA1 knockdown (BRCA1-KD) cells by siRNA significantly induced cellular accumulation of ROS compared to control cells. In this setting, the addition of paraquat, TCDD, DMBA, 2OHE2 or 4OHE2 significantly augmented ROS generation in BRCA1-KD MCF10A cells. Measurements of BaP-DNA adduct formation as a marker of DNA damage also revealed that BRCA1 deficiency leads increased DNA damage. In addition, TCDD and DMBA significantly increased BaP-DNA adduct formation in the absence of BRCA1. These results imply that elevated level of ROS is correlated with increase of DNA damage in BRCA1 defective cells. Taken together, our study suggests that several environmental factors might increase the risk of tumorigenesis in BRCA1 defective breast epithelial cells.

Key words: BRCA1, Genetic factor, Environmental factors, Tumorigenesis, Breast cancer

INTRODUCTION

Primary risk factors of breast cancer include exposure to environmental factors such as radiation, tobacco and xenoestrogen (Ibarluzea *et al*., 2004; Wolff *et al*., 1996; Lichtenstein *et al*., 2000; Nathanson *et al*., 2001). Main molecular pathogenesis of these environmental factors is attributed to oxidative stresses. There are accumulating data that residual oxidative stresses from these xenobiotics promote tumorigenesis (Dunnick *et al*., 1995). For complete detoxification and excretion of xenobiotics, the cooperative processes of phase I and phase II enzymes are required (Xu *et al*., 2005). In phase I, enzymes such as cytochrome P450 oxidases (CYPs) introduce reactive or polar groups into xenobiotics. These modified compounds are then conjugated to polar compounds in phase II, and excreted out by phase III enzymes (Denison and Nagy, 2003).

Mutations of BRCA1 have been identified as to be responsible for about half of inherited cases of breast

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cancer (Easton *et al*., 1993). Although major function of BRCA1 is known as a classical tumor suppressor gene, we have demonstrated that BRCA1 regulates transcription of phase I and II enzymes upon exposure to various exogenous stresses (Kang *et al*., 2006, 2008a and 2008b). BRCA1 can stimulate antioxidant gene expression and modulate intracellular reactive oxygen species (ROS) levels through enhancing the activity of the antioxidant response transcription factor, NRF2 (Bae *et al*., 2004; Kang *et al*., 2012). Furthermore, BRCA1 is also engaged in the cells' responses to xenobiotic stresses by up-regulating AhR/ARNT (aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator)-driven transcription (Kang *et al*., 2006, 2008a). BRCA1 stabilizes ARNT and modulates transcriptional regulation of CYP1A1 and CYP1B1 following xenobiotic stress exposure (Kang *et al*., 2006). Therefore, BRCA1 can preserve the integrity of cellular macromolecules, especially genomic DNA by reducing protein nitration and hydrogen peroxide levels (Saha *et al*., 2009).

In this context, defects in both phase I and II systems resulting from a BRCA1 deficiency may hamper sufficient cytoprotection against environmental insults, which could result in increased ROS production, DNA damage and tumorigenesis in the mammary gland. Here, we evaluated the role of environmental risk factors in the absence of BRCA1 on oxidative stress and DNA damage.

MATERIALS AND METHODS

Cell culture and reagents

MCF10A and 293 cells from American Type Culture Collection (Manassas, VA, USA) were cultured as described previously (Kang *et al*., 2008a, 2012). Benzo[a]pyrene (BaP), 7,12-Dimethylbenz[a]anthracene (DMBA), 3-Methylcholanthrene (3MC), 2-hydroxyestradiol (2OHE2), 4-hydroxyestradiol (4OHE2), sodium selenite, styrene oxide, cadmium chloride, and bisphenol A (BPA) were purchased from Sigma (St. Louis, MO, USA). PCB (3,3',4,4',5-Pentachlorobiphenyl) was obtained from AccuStandard, Inc. (New Haven, CT, USA) and TCDD (2,3,7,8-Tetrachlorodibenzodioxin) was purchased from Ultra Scientific, Inc. (North Kingstown, RI, USA). Radio-labeled [^{3}H]BaP was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Irradiations of UVA and UVC were performed using CL-1000L UV crosslinker (UVP, Inc., Upland, CA, USA) and Spectrolinker XL-1000 UV crosslinker (Spectronics, Westbury, NY, USA), respectively.

Transfection of siRNA

Control (non-targeting scrambled) and BRCA1-siRNAs were purchased from Dharmacon, Inc. (Lafayette, CO, USA). Their sequences and transfection method were described previously (Kang *et al*., 2011a, 2012).

Measurement of ROS production

Measurements of ROS were performed by using CM-H_2DCFDA (2′,7′-dichlorofluorescin diacetate) as described previously (Kang *et al*., 2011b). After incubation of environmental factors with or without 5 μM BaP for 24 hr, cells were treated with 5 μM of CM-H_2DCF-DA. Fluorescence was measured using Ultra 384 Fluorometer (Tecan, Männedorf, Switzerland) at 495/535 nm at the Genomics and Epigenomics Shared Resource at Georgetown University Medical Center.

Measurement of [^{3}H]BaP-induced DNA adducts

To determine BaP-induced DNA adducts, we used [^{3}H]BaP-DNA binding assay (Kang and Lee, 2005; Kang *et al*., 2011a, 2011b). After 24 hr incubation of cells with environmental factors in the absence or presence of 5 nM of [^{3}H]BaP, genomic DNAs were isolated using Wizard SV Genomic DNA purification system (Promega, Madison, WI, USA). The radioactivity of [^{3}H]BaP-DNA adducts in equal amount of DNA was counted using Beckman Coulter liquid scintillation counter LS6500 (Fullerton, CA, USA).

Reporter gene assay

Cells seeded in 24-well-plates and transfected with a reporter gene (GAL4-DBD-Luc) and expression vectors for GAL4-BRCA1 AD1 and AhR (Kang *et al*., 2008a) using Lipofectamine Plus (Invitrogen). Then cells were treated with various agents for 24 hr when they were harvested, lysed and used for luciferase assays were performed as previously (Kang *et al*., 2008a). The luminescence signal was measured by the Wallac Victor^2 microplate reader (Perkin-Elmer Life Sciences, Boston, MA, USA) at the Genomics and Epigenomics Shared Resource at Georgetown University Medical Center.

Statistical analysis

All experiments were performed more than three times. We used ANOVA analysis and Tukey's multiple comparison procedure to adjust for p values. The test is performed at 5% significance level. * or $^{\#}$ means the difference is significant after adjusting for multiple comparison.

RESULTS AND DISCUSSION

For evaluation of carcinogenesis in cellular model system, we have employed an *in vitro* assay system using benzo[a]pyrene (BaP) as a carcinogenic insult in BRCA1-knockdown (BRCA1-KD) cells to demonstrate oxidative stress induction and genomic DNA damage in BRCA1 defective cells (Kang *et al.,* 2011b). To address the tumorigenic potency of the various environmental factors when genetic factors are involved, we assessed the risk changes using our monitoring system.

First, we profiled the potency of ROS production by various environmental factors in MCF10A normal mammary epithelial cell (Soule *et al.,* 1990). The concentrations of various environmental factors were comparable to US Drinking Water Standards and Health Advisories (Supplementary Table 1). As expected, exposure to several xenobiotics increased cellular ROS level (Table 1). Most of the environmental factors tested (except for PCB, cadmium, BPA, UVA, and UVC) significantly induced cellular ROS level. We also measured the changes of ROS level by these environmental factors in the presence of BaP as a carcinogenic insult. Incubation of 5 μM BaP alone significantly increased ROS level (2.25 fold higher than control). In this setting, co-incubation with TCDD, DMBA and 3MC significantly augmented BaP-induced

Table 1. Effects of various environmental factors on the level of cellular ROS production in the absence or presence of BaP.

Environmental Factor	Conc.	ROS	
		(-)	w/ BaP
Control	-	1.00 ± 0.04	2.25 ± 0.24
Paraquat	10 μM	1.47 ± 0.05*	3.62 ± 0.34
	25 μM	2.02 ± 0.20*	4.12 ± 0.68
TCDD	1 nM	1.16 ± 0.13	2.83 ± 0.15
	10 nM	1.31 ± 0.14*	3.38 ± 0.21#
DMBA	1 μM	1.74 ± 0.24*	7.24 ± 0.87#
	10 μM	1.93 ± 0.20*	8.18 ± 0.32#
3MC	1 μM	1.29 ± 0.23*	4.49 ± 0.05#
	5 μM	1.39 ± 0.04*	5.02 ± 0.23#
PCB	1 μM	0.97 ± 0.07	2.42 ± 0.12
	10 μM	0.82 ± 0.08	1.94 ± 0.06
2OHE2	1 μM	1.43 ± 0.04*	2.85 ± 0.00
	5 μM	2.54 ± 0.35*	4.20 ± 0.22
4OHE2	1 μM	1.50 ± 0.18*	3.00 ± 0.05
	5 μM	1.99 ± 0.21*	4.31 ± 0.13
Sodium Selenite	1 μM	1.40 ± 0.04*	2.93 ± 0.26
	5 μM	1.63 ± 0.23*	3.66 ± 0.10
Styrene Oxide	50 μM	1.77 ± 0.32*	2.55 ± 0.29
	100 μM	1.82 ± 0.00*	2.75 ± 0.11
Cadmium Chloride	100 μM	0.93 ± 0.26	2.03 ± 0.03
	200 μM	0.58 ± 0.03	1.43 ± 0.09
BPA	1 nM	0.94 ± 0.16	2.44 ± 0.10
	10 nM	1.05 ± 0.04	2.76 ± 0.38#
Ethanol	1 %	1.19 ± 0.11	2.84 ± 0.10
	5 %	1.31 ± 0.16*	2.26 ± 0.15
UVA	0.2 J/cm^2	1.21 ± 0.12	2.56 ± 0.30
UVC	0.2 J/cm^2	1.22 ± 0.12	2.93 ± 0.48

*and #; significant increase compared to without or with BaP treated control, respectively. $P <$. 05.

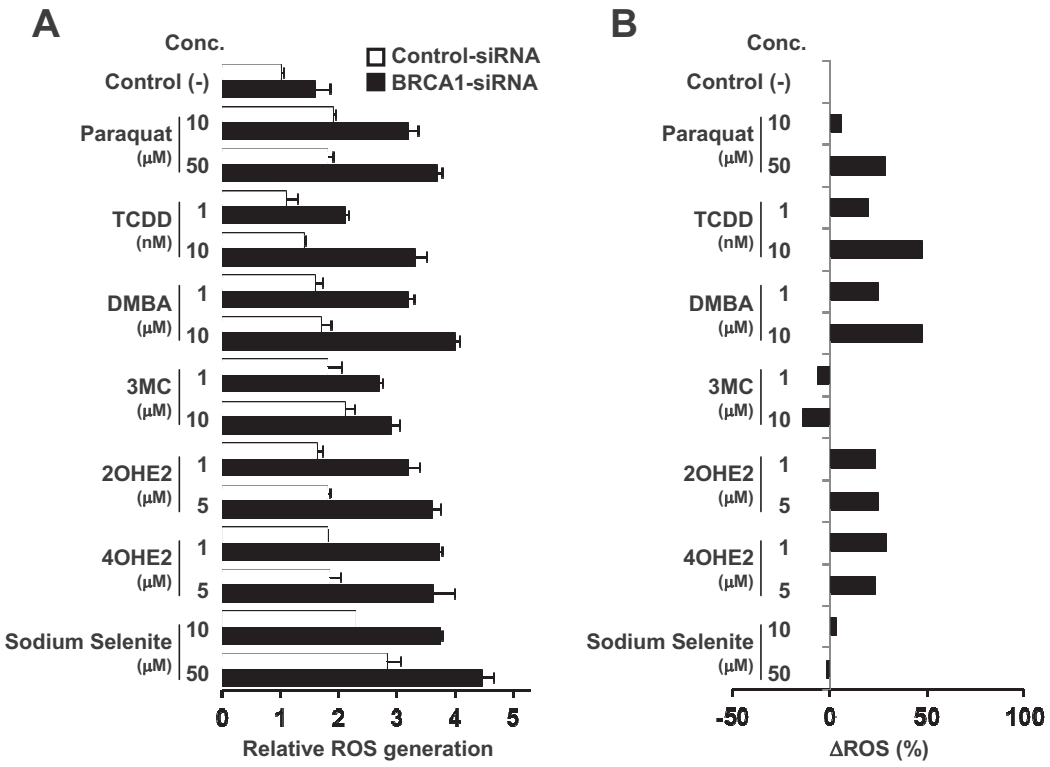

Fig. 1. Effect of various environmental factors on the ROS production in BRCA1 deficient MCF10A cells. (A) The ROS levels were measured after incubation with indicated environmental factors in the presence of BaP (5 μM) for 24 hr in control- or BRCA1-siRNA treated MCF10A cells. (B) The change of ROS (ΔROS) calculated by $[(R_{BF}/R_{CF})-(R_B/R_C)]/(R_B/R_C)*100$ from values in (A). Where R_C is ROS level in control-siRNA-transfected cells, R_B is ROS level in BRCA1-siRNA-transfected cells, R_{CF} is ROS level in control-siRNA-transfected cells treated with environmental factor, and R_{BF} is ROS level in BRCA1-siRNA-transfected cells treated with environmental factor.

ROS level in MCA10A cells (Table 1). Intriguingly, BPA (10 nM) incubation synergistically increased BaP induced ROS level, while BPA itself did not induce ROS generation.

Next, we investigated whether a genetic factor modulates ROS production induced by environmental factors. After knockdown of BRCA1 in MCF10A cells, the changes of ROS generation was measured. As reported earlier (Saha *et al*., 2009; Kang *et al*., 2011a; Martinez-Outschoorn *et al*., 2012a, 2012b; Kang *et al*., 2012), abrogation of BRCA1 significantly increase ROS level in the control-siRNA treated cells (Fig. 1A). Although ROS levels were changed by all environmental factors in some degrees in control-siRNA transfected MCA10A cells, significant enhancement of ROS production was only observed in paraquat, TCDD, DMBA, 2OHE2, 4OHE2 treated BRCA1-KD MCF10A cells (Fig. 1B). Interestingly, paraquat, TCDD, and DMBA commonly enhanced ROS production both in the presence of BaP and in the absence of BRCA1. These results implicate that several factors might potentiate the risk factors (i.e., ROS production) in BRCA1 deficiency related breast cancer.

Since oxidative stresses lead to genotoxicity, we examined whether there are positive correlations between ROS levels and DNA damage. We determined the [^{3}H] BaP-DNA binding as a measure of DNA damage (Kang and Lee, 2005; Kang *et al*., 2011a, 2011b). Previously we reported that the results from [^{3}H]BaP-DNA binding assay are well correlated to the results from *in vitro* [^{32}P] postlabeling assay using TLC plates (Kang *et al*., 2011a, 2011b). In control MCF10A cells, the level of [^{3}H]BaP-DNA adduct was significantly elevated in BRCA1-KD cells. Interestingly, TCDD itself markedly increased the [^{3}H]BaP-DNA adduct formation in control-siRNA transfected MCF10A cells. Under this condition, only TCDD and DMBA significantly raised DNA damage in BRCA1 deficient cells (Fig. 2). Unexpectedly,

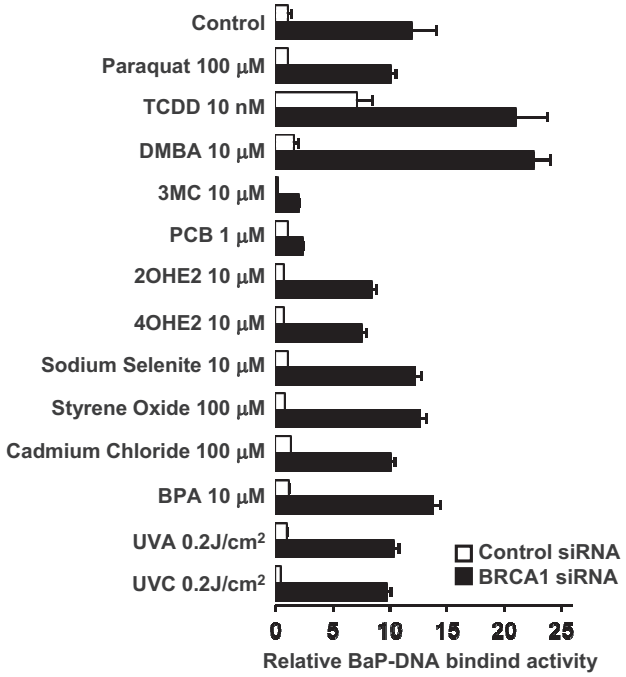

Fig. 2. Effect of various environmental factors on BaP-DNA adduct formation. To facilitate DNA damage, MCF10A cells, transfected with either control- or BRCA1-siRNA, were further incubated 5 nM of [^{3}H]BaP for 24 hr then the amounts of BaP-DNA adduct were measured as described in materials and methods.

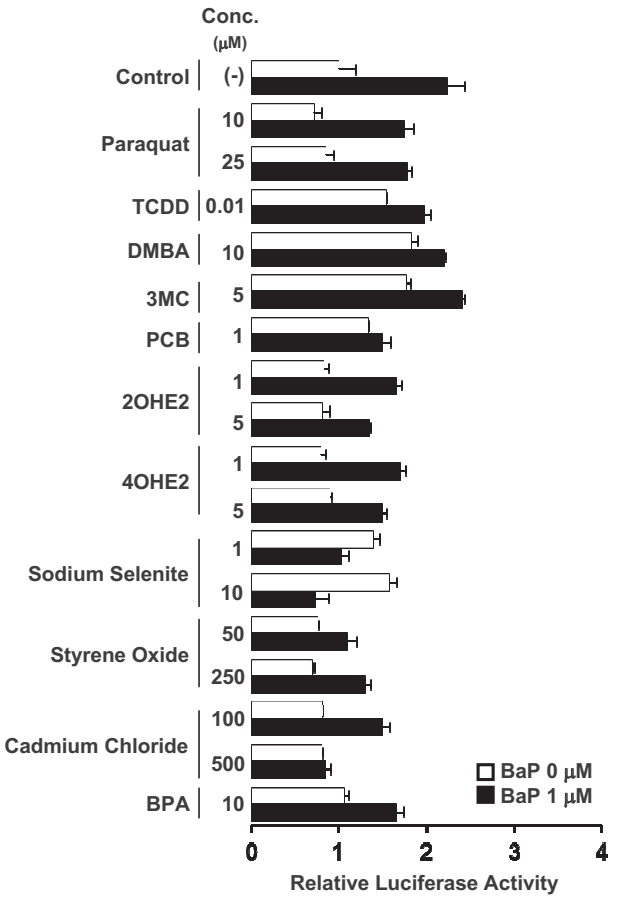

Fig. 3. Effect of various environmental factors on the transcriptional activation by BRCA1 AD1-AhR. 293 cells were transfected with a reporter plasmid (GAL4-DBD-Luc) and expression vectors for GAL4-BRCA1 AD1 and AhR. The environmental factors were treated as indicated for 24 hr and luciferase activity was measured as described in materials and methods.

[^{3}H]BaP-DNA levels in 3MC and PCB treated BRCA1-KD MCF10A cells were significantly reduced compared to control. It is still unclear how 3MC and PCB inhibits the formation of BaP-DNA adduct formation. Previously it has been reported that BaP-DNA adduct formation was reduced when [^{3}H]BaP and calf thymus DNA were incubated with microsomal protein from 3MC-induced rats in the presence of unoxidized beta carotene (Salgo *et al.*, 1999). It was also reported that pretreatment of CB 126 (3,3',4,4',5-pentachlorobiphenyl; a dioxin-like PCB) reduced the hepatic BaP-DNA adduct formation in marine flatfish dab (van Schanke *et al*., 2000). We could postulate that these results might come from the activation of AhR by 3MC and PCB. As 3MC and PCB are well-known to activate AhR (Abdelrahim *et al*., 2006), activated AhR system might clear metabolites of [^{3}H]BaP before incorporation into DNA. Alternatively, these environmental factors might differentially affect enzyme activities of phase I system. As an example, CB 126 was reported as a strong inhibitor of CYP1B1 (Pang *et al.*, 1999). Although TCDD is also known as an AhR ligand, mutagenic analyses of AhR suggested that TCDD has a different AhR binding mode (Denison *et al.*, 2011). Indeed, TCDD has been reported to increase the BaP-DNA adducts in several experimental settings (Carvan *et al.*, 1995; Harrigan *et al.*, 2006; de Waard *et al*., 2008). Similar to BaP, DMBA itself can form high level of DNA adduct even in the absence of enzymatic or chemical activation (Bryla and Weyand, 1992). Under our experimental setting, DMBA enhanced the BaP-DNA adduct formation in a BRCA1-dependent manner. These data implicate that there might be positive correlation between elevated level of ROS and DNA damage or tumorigenesis by these environmental factors.

Since BRCA1 plays crucial role in detoxification of xenobiotics through interaction with AhR (Kang *et*

al., 2008b), we measured effects various environmental factors on the transcriptional activity of BRCA1. 293 cells were transfected with a reporter plasmid containing GAL4-DNA binding domain (DBD) in the upstream of luciferase reporter gene and expression plasmids for BRCA1 AD1 fused to GAL4 DBD (GAL4-BRCA1 AD1) and AhR (Kang *et al.*, 2008a). Then, the transfected cells were further treated with various environmental factors and reporter gene activity was monitored to determine the effect of these factors on BRCA1 AD1-AhR-mediated transcriptional activity. Under these conditions, BaP enhanced BRCA1 AD1-AhR-mediated transcriptional activation. Most of environmental factors themselves showed little or no effects on the transcriptional activation by BRCA1 AD1-AhR except for TCDD, DMBA, 3MC, PCB, and sodium selenite (Fig. 3). Interestingly, sodium selenite markedly induced the BRCA1 AD1-AhR-mediated transcription in the absence of BaP. Co-treatment of BaP with these environmental factors exhibited no significant effects (paraquat, TCDD, DMBA, and 3MC) or rather antagonistic effects (PCB, 2OHE2, 4OHE2, odium selenite, sodium oxide, cadmium, and BPA) on the BaP-induced transcriptional activation of BRCA1 AD1-AhR. Thus combination of some environmental factors might augment the impairment of defense mechanism of BRCA1 against xenobiotic stress.

In this study, we assessed the risks of various environmental factors for increase of ROS production or ROS-induced DNA damage. We included the environmental factors such as 1) the polycyclic aromatic hydrocarbon (PAH) family (BaP and DMBA), 2) pesticides (PCB and paraquat), 3) chemicals causing mammary gland tumors in mice (styrene oxide), 4) heavy metal (cadmium), 5) radiation (UVA and UVC), 6) catechol estrogen (2OHE2 and 4OHE2), 7) a herbicide (TCDD), and 8) xenoestrogen BPA. Interestingly, TCDD and DMBA commonly increased the BaP-induced ROS production in both control and BRCA1 defective normal breast epithelial MCF10A cells. In addition, TCDD and DMBA drastically enhanced the BaP-DNA adduct formation in BRCA1 deficient cells. These results suggest that concurrent exposure to environmental factors increases the risk of breast cancer carrying genetic factors such as BRCA1 defect.

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