Journal of Cellular Biochemistry 100:1191–1199 (2007)

Gadd45α Does Not Modulate the Carboplatin or 5-Fluorouracil-Induced Apoptosis in Human Papillomavirus-Positive Cells

Sandeep Singh, Ankur Kumar Upadhyay, Amrendra Kumar Ajay, and Manoj Kumar Bhat*

National Centre for Cell Science, Ganeshkhind, Pune 411 007, India

Abstract Gadd45α is shown to be induced by a wide spectrum of DNA-damaging agents and implicated in negative regulation of cell growth by causing G2-M arrest or induction of apoptosis. In the present study, we explored the involvement of p53 in the promoter activation of Gadd45α as well as the role of Gadd45α in carboplatin (Carb) or 5-fluorouracil (5-FU)-induced apoptosis in human papillomavirus virus (HPV)-positive HEp-2 and HeLa cells. We report that Carb or 5-FU upregulate Gadd45α and p53 in both these cells. Transient transfection of chloramphenicol acetyl transferase (CAT)-reporter construct driven by Gadd45α promoter clearly indicated that Gadd45α upregulation was mediated through activation of its promoter. Inhibition of p53 function by dominant-negative-p53 expression partially suppressed the activation of Gadd45α promoter. Further, the induction of apoptosis was assessed by detection of poly (ADP-ribose) polymerase (PARP) cleavage by Western blot analysis. Inhibition of upregulated Gadd45α expression by antisense expression vector did not modulate the Carb or 5-FU-induced apoptosis. Overall, we conclude that Gadd45α promoter activation partially depends on p53 function in HPV-positive cells. Moreover, Gadd45α protein does not modulate Carb or 5-FU-induced apoptosis in these cells. J. Cell. Biochem. 100: 1191–1199, 2007.

Key words: HPV-positive carcinoma; p53; Gadd45α; apoptosis

In response to DNA damage, various molecular and cellular processes are activated as a part of the cellular stress response that result in cell cycle arrest and induction of the DNA-repair machinery to restore the damaged DNA or to activate cell death program. The tumor suppressor p53 has been demonstrated to play an important role in these biological events, in part through its downstream target genes [Slee et al., 2004]. Among various downstream genes regulated by p53, the growth arrest and DNA damage-inducible gene alpha (Gadd45α) acts as an important player in the control of G2-M checkpoint [Wang et al., 1999; Zhan et al., 1999; Jin et al., 2000a, 2002], DNA repair [Smith et al., 1994, Hollander et al., 2001], and apoptosis [Takekawa and Saito, 1998; Harkin et al., 1999; Hildesheim et al., 2002; Zhan et al., 2002; Tong et al., 2005]. In addition, the potential roles of Gadd45α in malignant transformation and tumor progression have also been described [Hollander et al., 2001; Hildesheim et al., 2002].

Gadd45α is generally found in very low abundance under normal circumstances and it is reported to be induced by a wide range of genotoxic stress agents and growth arrest treatments such as methylmethane sulfonate (MMS), nitrogen mustard, melphalan, hydrogen peroxide, hypoxia, many cancer chemotherapeutic drugs, ionizing radiation (IR), growth factor withdrawal, and medium depletion [Zhan, 2005]. The signaling pathways that regulate Gadd45α expression after genotoxic stress are complex and involve different mechanism(s) depending on the types of DNA-damaging agents as well as on different cellular genetic statuses. Gadd45α is the only member of the *Gadd* gene family which is regulated by p53, but p53 independent induction is also described depending on the type of stress and cell type. IR-induced Gadd45α induction is strictly dependent on normal cellular p53 function [Kastan

*Correspondence to: Dr. Manoj Kumar Bhat, Scientist E, National Centre for Cell Science, NCCS Complex, Ganeshkhind, Pune 411 007, India.
E-mail: manojkbhat@nccs.res.in; manojkbhat@yahoo.com
Received 1 May 2006; Accepted 21 July 2006
DOI 10.1002/jcb.21111

et al., 1992; Zhan et al., 1994a] whereas its induction by MMS, UV, medium starvation, and other non-IR agents does not require p53 function [Zhan, 2005]. Further, transcription factors Oct-1 and NF-YA along with BRCA-1 have been implicated in upregulation of *Gadd45α* gene in response to non-IR stresses [Jin et al., 2000b, 2002; Zhao et al., 2000; Takahashi et al., 2001; Fan et al., 2002; Hirose et al., 2003]. Although these reports do not support the role of p53, a number of investigations have demonstrated that the Gadd45α response to non-IR treatments is stronger in cells having functional p53 than that observed in cells harboring negative p53 status [Zhan et al., 1996, 1998]. The effect of p53 on Gadd45α promoter to non-IR treatments is likely mediated through p53 interaction with WT1 protein [Zhan et al., 1998].

Several studies have demonstrated that overexpression of Gadd45α substantially inhibits cell growth by blocking the cells at G2-M transition [Zhan et al., 1994b; Wang et al., 1999]. This function of Gadd45α is found to be p53 dependent, since Gadd45α failed to arrest p53-null Li-Fraumeni fibroblasts and human cells with abnormal p53 status [Wang et al., 1999]. In addition to cell cycle arrest, ectopic expression of Gadd45α or its induction after DNA damage has been linked with the induction of apoptosis [Takekawa and Saito, 1998; Harkin et al., 1999; Hildesheim et al., 2002; Zhan et al., 2002; Tong et al., 2005]. Overall, these findings suggest that Gadd45α negatively regulates cell proliferation and exert antioncogenic effect by regulating G2-M checkpoint or the induction of apoptosis, depending on the nature of stress and the cell type. Although Gadd45α regulation is correlated with induction of apoptosis, it remains unclear whether Gadd45α activates apoptosis or whether Gadd45a upregulation occurs as a consequence of apoptotic response to genotoxic stress [Zhan, 2005].

Studies so-far suggests that induction as well as function of Gadd45α is directly or indirectly regulated by p53. Since, in human papillomavirus (HPV)-positive cells p53 function is abrogated by endogenous E6 expression, the involvement of p53 in Gadd45α expression as well as the function of Gadd45α in response to DNA damage is not very clear. Our present findings for the first time demonstrate that Carb or 5-fluorouracil (5-FU) treatment resulted in activation of Gadd45α promoter in HPV-positive HEp-2 and HeLa cells which was partially dependent on upregulated p53. Further, data showed that Gadd45α induction does not modulate the drug-induced apoptosis in HPV-positive carcinoma cells.

MATERIALS AND METHODS

Cell Culture and Reagents

HEp-2 and HeLa cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and maintained in our in-house National Cell Repository. Both these cell lines are reported to contain human papillomavirus type 18 (HPV-18) sequences [Yee et al., 1985; Min et al., 1994]. Cells were routinely cultured in minimum essential medium (MEM) (Sigma, St. Louis, MO) supplemented with heat-inactivated fetal bovine serum (FBS) (HyClone, Utah) (10%), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen, MD), at 37°C with 5% CO_2. Carb and 5-FU were purchased from Sigma and dissolved in sterile water to prepare a stock of 50 mM. HEp-2 and HeLa cells were treated with 150 μM of Carb or 750 μM of 5-FU for 24 h at 37°C.

Plasmid Constructs, Transfection, and CAT Assay

pHG45-CAT2 plasmid construct containing the *Hind*III-*Sma*I fragment of the Gadd45α promoter spanning from −909 to +144 relative to transcription start site inserted in chloramphenicol acetyl transferase (CAT) reporter gene and Gadd45α expression vector pCMV45 were kind gift of Dr. Albert J. Fornace Jr., NIH, Bethesda, MD. PG_{13}-CAT which contains 13 repeats of p53-binding site inserted in the 5′ to polyomavirus basal promoter linked to CAT reporter gene and p53 dominant-negative expression vector, pC53-SCX3, which expresses a mutant p53 protein containing a substitution of Ala for Val-143 was kindly gifted by Dr. Bert Vogelstein, John Hopkins, Baltimore, MD. pTRE45-AS was constructed to express Gadd45α in antisense orientation under the control of tetracycline activator in presence of doxycycline. First Gadd45α cDNA (obtained from pCMV45) was inserted into *Xba*I-*Hind*III sites of pBIISK(+) (Stratagene, CA). From here Gadd45α cDNA was excised and cloned into *Hind*III-*Not*I site of pTRE2 vector (Clontech, CA) to obtain pTRE45-AS. pTRE2 vector is a response plasmid that can be used to express any gene of interest when it is present with

doxycycline and pTet-On (Clontech) gene expression system [Gossen and Bujard, 1992]. To express Gadd45α in antisense orientation, cells were transiently transfected with pTRE45-AS and pTet-On plasmids and subsequently treated with 0.1 or 1 μg/ml of doxycycline, as indicated in the experiments. Semi-confluent cells were transfected by Lipofectamine 2000 reagent as suggested by the manufacturer (Invitrogen). Each transfection mixture contained 7 μg of DNA including 1 μg of EGFP expression vector pEGFPN1 (Clontech) as an internal control for normalization of transfection efficiencies. In each transient transfection experiments more than 50% transfection efficiency was achieved as determined by the expression of green fluorescent protein. After 18 h of transfection, cells were washed and fresh medium was added. Cells were harvested after 24 h following drugs treatment, collected and resuspended in 0.25 M Tris (pH 7.5). Cells were disrupted by five cycles of freeze-thaw. Equal amount of protein was used for the CAT assay. The CAT reaction mixture containing 1 μCi ^{14}C-chloramphenicol (NEN, Boston, MA) and 100 μg of acetyl CoA (Amersham, Aylesbury, UK) was incubated at 37°C for 6 h. CAT activity was determined by measuring the acetylation of ^{14}C-chloramphenicol by thin layer chromatography. TLC plates were analyzed by autoradiography and by scanning on a phosphorimager (Bio-Rad, CA). The specific CAT activity was calculated by determining the fraction of chloramphenicol that had been acetylated during the reaction. CAT values were normalized for EGFP expression. EGFP expression was quantitated by the method as described earlier [Dandekar et al., 2005]. All transfections were performed in duplicate and all were repeated for at least three times.

Reverse Transcription-PCR

HEp-2 and HeLa cells were treated with Carb or 5-FU. Following treatment, total cellular RNA from treated and untreated cells was extracted using TRIzol™ reagent (Invitrogen), according to the manufacturer's instructions and analyzed by RT-PCR as described earlier [Chhipa et al., 2005]. The primer pairs used were as follows: Gadd45α 5′-AGA GCA GAA GAC CGA AAG GAT G-3′ (F) 5′-ACG CGC AGG ATG TTG ATG TCG-3′ (R), β-actin 5′-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3′ (F) 5′-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3′ (R).

Immunoblotting

HEp-2 and HeLa cells were treated with Carb or 5-FU. Following treatment, cells were washed thrice with ice cold phosphate buffered saline (PBS) and lysed in ice cold lysis buffer (50 mM Tris-HCl, pH 7.5, with 120 mM NaCl, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40 and protease inhibitor cocktail). Cellular lysates were clarified by centrifugation at 15,000 rpm for 30 min and used for Western blot analysis. Gadd45α, p53, PARP, and β-actin were detected by Western blot analysis using 50 μg of cellular lysates. The Western blot analysis of cleaved PARP from a p116 polypeptide to p85 fragment was assessed to demonstrate the induction of apoptosis in Carb or 5-FU-treated cells. Samples were resolved on denaturing SDS-polyacrylamide gel and transferred to membrane. The immunoblots were detected by enhanced chemiluminescence (ECL) reagent (New England Biolabs, MA).

Immunofluorescence Confocal Microscopy

HEp-2 and HeLa cells were grown on collagen-coated cover slips and treated with Carb or 5-FU. Cells were washed with PBS and fixed with 3.7% paraformaldehyde for 10 min at room temperature. Cells were permeabilized in PBS containing 0.1% Triton X-100 on ice and subsequently blocked with 10% FBS for 1 h at 4°C. Primary antibodies against Gadd45α (1:100) in the blocking solution was added and incubated for 2 h at room temperature. Following incubation, five 5 min washes were given. Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:100) was added in blocking solution and incubated for further 1 h at room temperature. After five additional 5 min washes, samples were examined on a confocal microscope (LSM510, Carl Zeiss, Germany). Images were subsequently processed by Adobe Photoshop software. More than 100 cells were inspected per experiment, and cells with typical morphology are presented.

Statistical Analysis

Data are expressed as the mean of three independent results. Statistical comparisons

are made using student's *t*-test and *P*-value <0.05 was considered as significant.

RESULTS

p53 Is Upregulated After Carb or 5-FU Treatment

p53 is induced in various cell types including HPV-positive cells, after treatment with DNA-damaging agents like Carb or 5-FU [Koivusalo et al., 2002; Yim et al., 2004]. We investigated the status of p53 by Western blot analysis, in HEp-2 and HeLa cells, treated for 24 h with Carb or 5-FU. As shown in Figure 1, treatment with Carb or 5-FU resulted in almost twofold increase in p53 levels in HEp-2 cells, whereas 1.4- to 1.5-fold increase in HeLa cells.

Gadd45α Is Upregulated by Carb or 5-FU Treatment

In many reports, it has been demonstrated that DNA-damaging chemotherapeutic drugs including platinum adducts like cisplatin induces Gadd45α mRNA and protein in various cell types [Butz et al., 1999; Zhan, 2005]. To investigate its response in HPV-positive cells, HEp-2 and HeLa cells were treated with Carb or 5-FU and the expression of Gadd45α mRNA was analyzed by RT-PCR. As shown in Figure 2A, cells exposed for 24 h to Carb or 5-FU, the level of Gadd45α mRNA was induced markedly in both HEp-2 and HeLa cells. Since, induction of Gadd45α in response to non-IR agents is mediated through the activation of its promoter element [Jin et al., 2000b, 2002; Zhao et al., 2000b; Takahashi et al., 2001; Fan et al., 2002; Hirose et al., 2003], we next investigated, whether increased Gadd45α mRNA in response to Carb or 5-FU treatment is mediated through activation of its promoter. CAT reporter construct pHG45-CAT2 which contains Gadd45α promoter elements upstream of *CAT* gene [Jin et al., 2000, 2001; Fan et al., 2002] was transiently transfected in HEp-2 and HeLa cells. Subsequently, cells were treated with Carb or 5-FU and CAT activity was measured in the lysates of untreated as well as treated cells. As shown in Figure 2B, a marked induction of Gadd45α promoter was observed in drugs-treated cells. Compared to untreated cells, Carb treatment resulted in approximately 3.5- and 1.7-fold increase in Gadd45α promoter activity in HEp-2 and HeLa cells, respectively. Similarly, 5-FU treatment led to almost 2.8- and 1.5-fold increase in Gadd45α promoter activity in HEp-2 and HeLa cells, respectively. Further, to verify whether upregulated mRNA levels results in increased expression of Gadd45α protein, we performed immunofluorescence staining for Gadd45α protein in untreated as well as Carb or 5-FU-treated HEp-2 and HeLa cells. As expected, in Carb and 5-FU-treated cells, increase in Gadd45α protein expression was detected (Fig. 1C). These data clearly demonstrate that Carb or 5-FU treatment results in increased expression of Gadd45α in HPV-positive HEp-2 and HeLa cells. Moreover, the upregulation of Gadd45α in response to Carb or 5-FU treatment is through the activation of its promoter element.

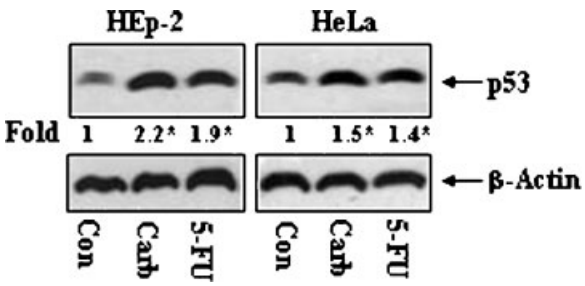

Fig. 1. p53 is upregulated by Carb or 5-FU treatment. HEp-2 and HeLa cells were treated with Carb and 5-FU for 24 h. Cells were harvested and processed for Western blot analysis as described in Materials and Methods. Expression levels of p53 protein were determined by quantitation of Western blotting signals using densitometry scanner analysis (Phosphorimager, Bio-Rad). These results are representative of three independent experiments. Fold expression was calculated with reference to untreated cells. The * indicates the mean value is significantly different ($P < 0.05$) than the control.

Gadd45α Promoter Activation Is Partially Regulated by p53

Even though, p53-binding motif is not present in Gadd45α promoter, p53 can participate in transcriptional activation of Gadd45α promoter without direct DNA binding [Zhan et al., 1998]. Thus, it was of interest to study the involvement of p53 in the induction of Gadd45α with or without genotoxic stress in HEp-2 and HeLa cells. Cells were cotransfected with Gadd45α promoter reporter construct, pHG45-CAT2 and dominant-negative p53 expression vector; pC53-SCX3. Interestingly, expression of mutant p53 resulted in inhibition of Gadd45α promoter activity by 20% in both HEp-2 and HeLa cells (Fig. 3A). Further, to study the role of p53 in

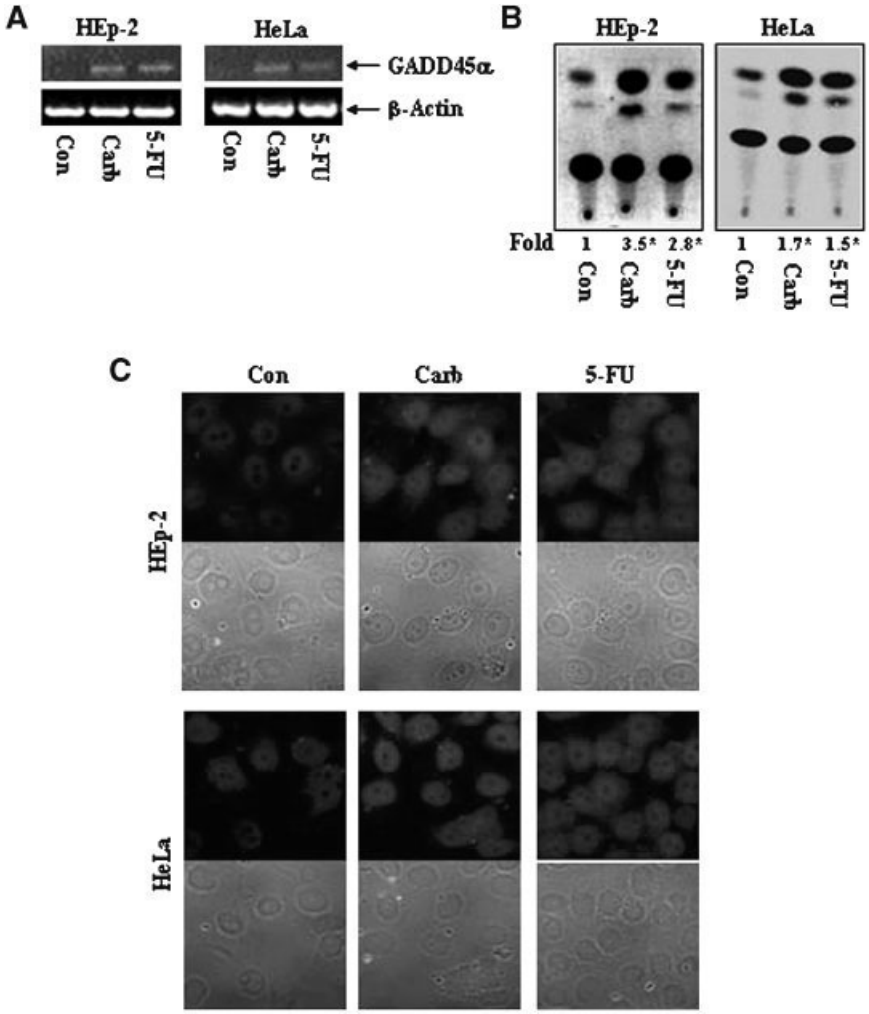

Fig. 2. Gadd45α is induced by Carb or 5-FU treatment. **A**: mRNA expression of Gadd45α in 24 h treated (Carb and 5-FU) and untreated (Con) HEp-2 and HeLa cells. Expression of β-actin mRNA was used as control for RNA integrity and equal loading. Expression levels of Gadd45α mRNA were determined by quantitation of bands using densitometry scanner analysis (Phosphorimager, Bio-Rad). Fold expression was calculated with reference to untreated cells. **B**: HEp-2 and HeLa cells were transiently transfected with Gadd45α promoter construct pHG45-CAT2 and treated with Carb or 5-FU for 24 h. Equal amount of protein was used to estimate CAT activity. CAT activity (normalized to EGFP expression) is reported as fold of the %-CAT activity, calculated as given in Materials and Methods. **C**: Gadd45α protein levels in drugs-treated cells. HEp-2 and HeLa cells were treated with Carb or 5-FU for 24 h. Gadd45α protein was detected by immunofluorescence staining. Pictures were taken in confocal microscope (LSM510, Carl Zeiss) and images were subsequently processed by Adobe Photoshop software. These results are representative of three independent experiments. The * indicates the mean value is significantly different ($P<0.05$) than the control.

Carb or 5-FU-induced activation of Gadd45α promoter; cells were transfected with pHG45CAT2 with or without pC53-SCX3 and then treated with Carb or 5-FU. As expected, drugs treatment significantly activated Gadd45α promoter (Fig. 3B; lanes 2 and 4 as compared to lane 1), which was reduced in presence of mutant p53 overexpression (Fig. 3B; lane 3 as compared to lane 2 for Carb and lane 5 as compared to lane 4 for 5-FU). HEp-2 cells, overexpressing dominant-negative p53 and subsequently treated with Carb or 5-FU, Gadd45α promoter activity reduced by 30 and 40%, respectively. Similarly, HeLa cells overexpressing dominant-negative p53 and subsequently treated with Carb or 5-FU, Gadd45α promoter activity reduced by 25 and 30%, respectively. Relative folds of Gadd45α promoter activation is represented as bar graph. These results clearly demonstrate that abrogation of p53 function results in

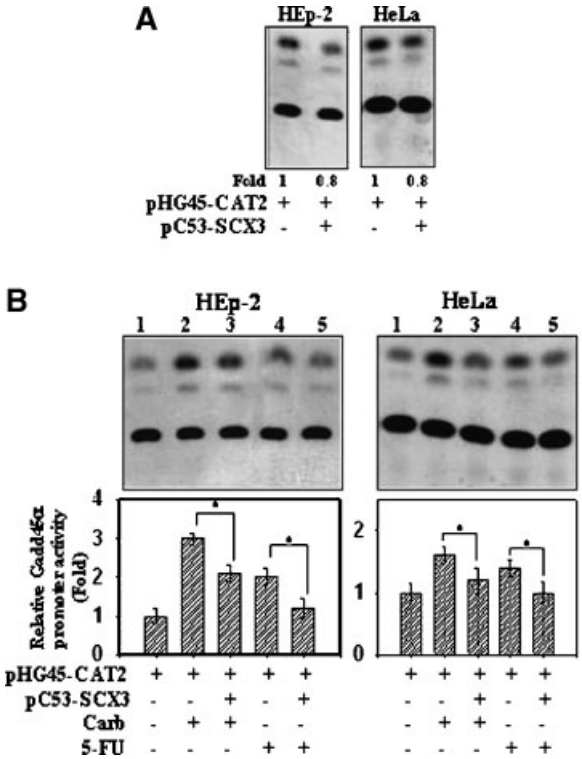

Fig. 3. Gadd45α promoter activation is partially regulated by p53. **A**: HEp-2 and HeLa cells were transfected with pHG45-CAT2 along with pC53-SCX3 or vector backbone. Cells were harvested and equal amount of protein was used for estimation of CAT activity. CAT activity (normalized to EGFP expression) is reported as fold of the %-CAT activity, calculated as described in Materials and Methods. **B**: HEp-2 and HeLa cells were transiently transfected with pHG45-CAT2 along with pC53-SCX3 or vector backbone. Transfected cells were treated as indicated. Cells were harvested and equal amount of protein was used to estimate CAT activity. The fold of Gadd45α promoter driven CAT activity (normalized to EGFP expression) is calculated as given in Materials and Methods. Data are also represented as bar graph. These results are representative of three independent experiments. The * indicates the mean value is significantly different ($P < 0.05$) within the groups.

diminished activation of Gadd45α promoter in response to Carb or 5-FU.

Upregulation of Gadd45α Does Not Modulate the Carb or 5-FU-Induced Apoptosis

Expression or induction of Gadd45α has been linked to inhibition of cell growth by inducing cell cycle arrest at G2-M phase as well as apoptosis [Takekawa and Saito, 1998; Wang et al., 1999]. In our previous study, we have demonstrated that Carb or 5-FU exerts its cytotoxic effect by the induction of apoptosis in HPV-positive cells. Carb or 5-FU treatment resulted in decreased Bcl-2 protein level accompanied by caspase-9 activation and PARP cleavage [Singh et al., 2006]. In the present study, we next evaluated, whether upregulated Gadd45α is also involved in the process of apoptosis induced by Carb or 5-FU in HPV-positive cells. To inhibit the induction of Gadd45α, we expressed antisense Gadd45α in the cells. The functionality of the Gadd45α antisense expression vector (pTRE45-AS) was initially ascertained by transfecting pTRE45-AS along with pCMV45 and pTet-On in HeLa cells. Cells, transfected with pTRE45-AS and subsequently treated with doxycycline exhibited a dose-dependent inhibition of Gadd45α expression (Fig. 4A). Next, as shown in Figure 4B, HeLa cells were transiently transfected with pTRE45-AS together with pTet-On plasmid and the expression of Gadd45α antisense was initiated by addition of doxycycline. Subsequently, these cells were treated with drugs as indicated in Figure 4B. Drug treatment resulted in significant PARP cleavage as compared to untreated cells (Fig. 4B, lanes 3 and 5 as compared to lane 1). Doxycycline

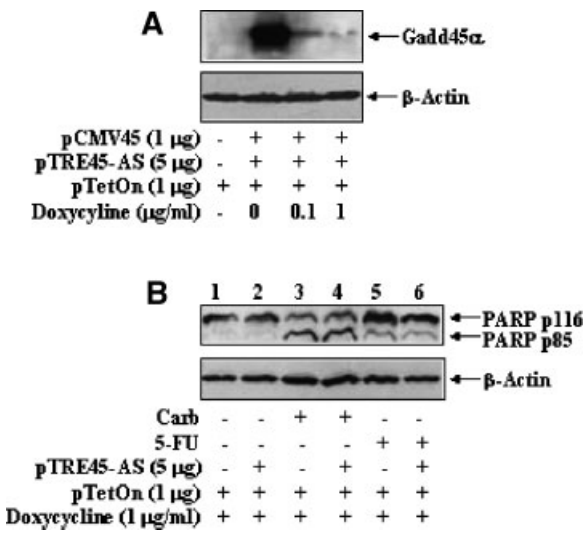

Fig. 4. Gadd45α does not modulate the drugs-induced apoptosis. **A**: HeLa cells were transiently transfected with Gadd45α expression vector pCMV45 along with pTet-On and pTRE45-AS as indicated. Eighteen hours after transfection, cells were treated with increasing concentrations of doxycycline for 24 h. Cells were harvested and equal amount of protein (50 μg) was processed for Western blot analysis to determine the Gadd45α expression. **B**: HeLa cells were transiently transfected with pTRE45-AS or vector backbone along with pTet-On. Cells were treated with doxycycline to express Gadd45α in antisense orientation. Transfected cells were treated with Carb or 5-FU, as indicated. Cells were harvested and equal amount of protein (50 μg) was processed for Western blot analysis for PARP cleavage as described in Materials and Methods. These results are representative of three independent experiments.

treatment and inhibition of Gadd45α expression did not result in PARP cleavage (Fig. 4B, lanes 1 and 2). Moreover, drug-induced PARP cleavage was not altered by inhibition of Gadd45α expression in Carb (Fig. 4B; lane 4 as compared to lane 3) as well as 5-FU (Fig. 4B; lane 6 as compared to lane 5) treated cells. Similar results were obtained in HEp-2 cells (data not shown).

Overall, the results suggest that increase in Gadd45α levels does not significantly contribute to the Carb or 5-FU-induced apoptosis in HPV-positive HEp-2 and HeLa cells.

DISCUSSION

It is well established that upon DNA damage, p53 protein is stabilized and in turn, regulates many target genes that may play roles in different aspects of cellular response [Slee et al., 2004]. In HPV-positive cells, E6 oncoprotein inactivates p53 by inducing its degradation [zur Hausen, 2002]. Several reports have demonstrated that even in HPV-positive cells, the DNA-damaging drugs like Carb or 5-FU are able to induce physiologically enough functional p53 to execute its functions [Koivusalo et al., 2002; Yim et al., 2004]. In agreement with these reports, we also demonstrate the induction of p53 in HEp-2 and HeLa cells after Carb or 5-FU treatment (Fig. 1). As a well-defined p53 downstream gene, Gadd45α is the only member of the *Gadd* gene family that is p53 regulated [Zhan, 2005]. The signaling that regulate Gadd45α induction is complex and is based on differences in genetic status of the cell as well as the type of DNA damage stress [Zhan, 2005]. Moreover, induced Gadd45α is implicated in several important biological events such as maintenance of genomic fidelity [Hollander et al., 1999] possibly due to its involvement in the control of cell cycle G2-M checkpoint [Wang et al., 1999; Zhan et al., 1999; Jin et al., 2000a, 2002], induction of cell death [Takekawa and Saito, 1998; Harkin et al., 1999; Hildesheim et al., 2002; Zhan et al., 2002; Tong et al., 2005], and DNA repair process [Smith et al., 1994; Hollander et al., 2001]. In the present study, we clearly demonstrate that Carb or 5-FU treatment leads to Gadd45α upregulation in HPV-positive, HEp-2, and HeLa cells. Carb or 5-FU treatment resulted in a significant increase in Gadd45α promoter activity, leading to its increased expression in HEp-2 as well as HeLa cells (Fig. 2). Induction of *Gadd45α* gene in HPV-positive cells by various DNA-damaging drugs and UV radiation has been reported earlier [Butz et al., 1999]. However in the present study, we for the first time report that Carb and 5-FU-mediated Gadd45α induction is due to the activation of its promoter. Since, p53 is reported as direct or indirect activator of Gadd45α, we explored the involvement of basal as well as induced p53 in the activation of Gadd45α promoter. Interestingly, cells in which p53 activity was abrogated by the expression of dominant-negative p53, diminished Gadd45α promoter activity was observed, both at basal as well as in drugs-induced states (Fig. 3). Similar results were obtained when cells were pretreated with pifithrinα, a physiological inhibitor of p53 functions (data not shown). Even though, Gadd45α promoter does not contain p53 responsive motif in its promoter region, the results suggest that functional p53 may contribute to the basal as well as Carb or 5-FU-mediated activation of Gadd45α promoter in HPV-positive cells. Several evidences indicate that p53 exists in the WT1-associated complex that has a synergistic effect with WT1 in induction of the Gadd45α promoter in response to the genotoxic stresses [Zhan et al., 1998]. This may be one of the mechanisms by which p53 might contribute to the stress responsiveness of Gadd45α promoter in these cells.

Although, the role of Gadd45α is yet to be clearly understood, it is well known that Gadd 45α protein binds to the regulators of DNA replication and cell cycle, such as PCNA (proliferating cell nuclear antigen) and p21 protein [Smith et al., 1994; Kearsey et al., 1995a]. Moreover, it has been proposed that Gadd45α is directly responsible for the DNA excision repair process, although this hypothesis is still controversial [Kearsey et al., 1995b; Smith et al., 1996; Hollander et al., 2001]. Nonetheless, Gadd45α is strongly proposed to be involved in the induction of apoptosis [Takekawa and Saito, 1998; Harkin et al., 1999; Hildesheim et al., 2002; Zhan et al., 2002; Tong et al., 2005]. Thus, overall Gadd45α acts as negative regulator of cell growth either by inducing cell cycle arrest or by inducing apoptosis [Zhan, 2005]. Therefore, we investigated its involvement in induction of apoptosis in HEp-2 and HeLa cells. In our earlier reports, we have demonstrated that apoptosis induced by Carb or 5-FU in HPV-positive cells is mediated through downregulation of Bcl-2 via NF-κB

inactivation [Singh and Bhat, 2004; Singh et al., 2006]. Here, we investigated the role of upregulated Gadd45α in Carb or 5-FU-induced apoptosis in HPV-positive HEp-2 and HeLa cells. Proteolytic cleavage of PARP from a p116 polypeptide to p85 and p29 fragments is one of the hallmarks of apoptosis [Kaufmann et al., 1993], therefore the cleavage of PARP was assessed to demonstrate the induction of apoptosis in Carb or 5-FU-treated cells. As shown in Figure 4B, in Carb or 5-FU-treated cells, inhibition of Gadd45α expression did not show any significant difference in PARP cleavage as compared to vector alone transfected cells. This implies that Carb or 5-FU-induced apoptosis is independent of the induced Gadd45α expression in HEp-2 and HeLa cells. These findings are in accordance with the report in which it was demonstrated that Gadd45α induction did not affect viability of airway and type II epithelial cells during hypoxia exposure [Roper et al., 2005].

Overall, we demonstrate that Gadd45α promoter is partially regulated by p53-functions in HPV-positive cells. Importantly, the functions of induced Gadd45α in response to DNA-damage stress may vary with the cell type, because in the present study, apoptosis induced by Carb or 5-FU was found to be independent of Gadd45α functions in HPV-positive HEp-2 and HeLa cells. Therefore, we propose that even though Gadd45α upregulation occurs as a consequence of apoptotic response to genotoxic stress, its significance in the induction of apoptosis is solely dictated by the nature of stress and probably, by the cell type differences.

ACKNOWLEDGMENTS

We thank Dr. G.C. Mishra, Director, NCCS for being very supportive and giving all the encouragement to carry out this work. We also thank Department of Biotechnology (DBT), Government of India for providing financial support and research fellowship to SS. AKU thanks University Grants Commission (UGC), New Delhi and AKA thanks Indian Council for Medical Research (ICMR), New Delhi for providing research fellowships.

REFERENCES

Butz K, Whitaker N, Denk C, Ullmann A, Geisen C, Hoppe-Seyler F. 1999. Induction of the p53-target gene GADD45 in HPV-positive cancer cells. Oncogene 18:2381–2386.

Chhipa RR, Singh S, Surve SV, Vijayakumar MV, Bhat MK. 2005. Doxycycline potentiates antitumor effect of cyclophosphamide in mice. Toxicol Appl Pharm 202:268–277.

Dandekar DH, Kumar M, Ladha JS, Ganesh KN, Mitra D. 2005. A quantitative method for normalization of transfection efficiency using enhanced green fluorescent protein. Anal Biochem 342:341–344.

Fan W, Jin S, Tong T, Zhao H, Fan F, Antinore MJ, Rajasekaran B, Wu M, Zhan Q. 2002. BRCA1 regulates GADD45 through its interactions with the OCT-1 and CAAT motifs. J Biol Chem 277:8061–8067.

Gossen M, Bujard H. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 89:5547–5551.

Harkin DP, Bean JM, Miklos D, Song YH, Truong VB, Englert C, Christians FC, Ellisen LW, Maheswaran S, Oliner JD, Haber DA. 1999. Induction of GADD45 and JNK/SAPKdependent apoptosis following inducible expression of BRCA1. Cell 97:575–586.

Hildesheim J, Bulavin DV, Anver MR, Alvord WG, Hollander MC, Vardanian L, Fornace AJ, Jr. 2002. Gadd45a protects against UV irradiation-induced skin tumors, and promotes apoptosis and stress signaling via MAPK and p53. Cancer Res 62:7305–7315.

Hirose T, Sowa Y, Takahashi S, Saito S, Yasuda C, Shindo N, Furuichi K, Sakai T. 2003. p53-independent induction of Gadd45 by histone deacetylase inhibitor: Coordinate regulation by transcription factors Oct-1 and NF-Y. Oncogene 22:7762–7773.

Hollander MC, Sheikh MS, Bulavin DV, Lundgren K, Augeri-Henmueller L, Shehee R, Molinaro TA, Kim KE, Tolosa E, Ashwell JD, Rosenberg MP, Zhan Q, Fernandez-Salguero PM, Morgan WF, Deng CX, Fornace AJ, Jr. 1999. Genomic instability in Gadd45a-deficient mice. Nat Genet 23:176–184.

Hollander MC, Kovalsky O, Salvador JM, Kim KE, Patterson AD, Haines DC, Fornace AJ, Jr. 2001. Dimethylbenzanthracene carcinogenesis in Gadd45a-null mice is associated with decreased DNA repair and increased mutation frequency. Cancer Res 61:2487–2491.

Jin S, Antinore MJ, Lung FD, Dong X, Zhao H, Fan F, Colchagie AB, Blanck P, Roller PP, Fornace AJ, Jr., Zhan Q. 2000a. The GADD45 inhibition of Cdc2 kinase correlates with GADD45-mediated growth suppression. J Biol Chem 275:16602–16608.

Jin S, Zhao H, Fan F, Blanck P, Fan W, Colchagie AB, Fornace AJ, Jr., Zhan Q. 2000b. BRCA1 activation of the GADD45 promoter. Oncogene 19:4050–4057.

Jin S, Fan F, Fan W, Zhao H, Tong T, Blanck P, Alomo I, Rajasekaran B, Zhan Q. 2001. Transcription factors Oct-1 and NFYA regulate the p53-independent induction of the GADD45 following DNA damage. Oncogene 20:2683–2690.

Jin S, Tong T, Fan W, Fan F, Antinore MJ, Zhu X, Mazzacurati L, Li X, Petrik KL, Rajasekaran B, Wu M, Zhan Q. 2002. GADD45-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity. Oncogene 21:8696–8704.

Kastan MB, Zhan Q, el-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, Fornace AJ, Jr. 1992. A mammalian cell cycle checkpoint pathway

utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 71:587–597.
Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. 1993. Specific proteolytic cleavage of poly (ADP-ribose) polymerase: An early marker of chemotherapy-induced apoptosis. Cancer Res 53:3976–3985.
Kearsey JM, Coates PJ, Prescott AR, Warbrick E, Hall PA. 1995a. Gadd45 is a nuclear cell cycle regulated protein which interacts with p21Cip1. Oncogene 11:1675–1683.
Kearsey JM, Shivji MK, Hall PA, Wood RD. 1995b. Does the p53 up-regulated Gadd45 protein have a role in excision repair? Science 270:1004–1005.
Koivusalo R, Krausz E, Ruotsalainen P, Helenius H, Hietanen S. 2002. Chemoradiation of cervical cancer cells: Targeting human papillomavirus E6 and p53 leads to either augmented or attenuated apoptosis depending on the platinum carrier ligand. Cancer Res 62:7364–7371.
Min BM, Baek JH, Shin KH, Gujuluva CN, Cherrick HM, Park NH. 1994. Inactivation of the p53 gene by either mutation or HPV infection is extremely frequent in human oral squamous cell carcinoma cell lines. Eur J Cancer B Oral Oncol 30B:338–345.
Roper JM, Gehen SC, Staversky RJ, Hollander MC, Fornace AJ, Jr., O'Reilly MA. 2005. Loss of Gadd45a does not modify the pulmonary response to oxidative stress. Am J Physiol Lung Cell Mol Physiol 288:L663–L671.
Singh S, Bhat MK. 2004. Carboplatin induces apoptotic cell death through downregulation of constitutively active nuclear factor-kB in human HPV-18 E6 positive HEp-2 cells. Biochem Biophys Res Commun 318:346–353.
Singh S, Chhipa RR, Vijayakumar MV, Bhat MK. 2006. DNA damaging drugs-induced down-regulation of Bcl-2 is essential for induction of apoptosis in high-risk HPV-positive HEp-2 and KB cells. Cancer Lett 236:213–221.
Slee EA, O'Connor DJ, Lu X. 2004. To die or not to die: How does p53 decide? Oncogene 23:2809–2818.
Smith ML, Chen IT, Zhan Q, Bae I, Chen CY, Gilmer TM, Kastan MB, O'Connor PM, Fornace AJ, Jr. 1994. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen [see comments]. Science 266:1376–1380.
Smith ML, Kontny HU, Zhan Q, Sreenath A, O'Connor PM, Fornace AJ, Jr. 1996. Antisense GADD45 expression results in decreased DNA repair and sensitizes cells to u.v.-irradiation or cisplatin. Oncogene 13:2255–2263.
Takahashi S, Saito S, Ohtani N, Sakai T. 2001. Involvement of the Oct-1 regulatory element of the gadd45 promoter in the p53-independent response to ultraviolet irradiation. Cancer Res 61:1187–1195.
Takekawa M, Saito H. 1998. A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. Cell 95:521–530.
Tong T, Ji J, Jin S, Li X, Fan W, Song Y, Wang M, Liu Z, Wu M, Zhan Q. 2005. Gadd45a expression induces Bim dissociation from the cytoskeleton and translocation to mitochondria. Mol Cell Biol 25:4488–4500.
Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L. Hollander MC, O'Connor PM, Fornace AJ, Jr., Harris CC. 1999. GADD45 induction of a G2/M cell cycle checkpoint. Proc Natl Acad Sci USA 96:3706–3711.
Yee C, Krishnan-Hewlett I, Baker CC, Schlegel R, Howley PM. 1985. Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. Am J Pathol 119:361–366.
Yim EK, Lee KH, Bae JS, Namkoong SE, Um SJ, Park JS. 2004. Proteomic analysis of antiproliferative effects by treatment of 5-fluorouracil in cervical cancer cells. DNA Cell Biol 23:769–776.
Zhan Q. 2005. Gadd45a-a p53- and BRCA1-regulated stress protein, in cellular response to DNA damage. Mutation Res 569:133–143.
Zhan Q, Bae I, Kastan MB, Fornace AJ, Jr. 1994a. The p53-dependent gamma-ray response of GADD45. Cancer Res 54:2755–2760.
Zhan Q, Lord KA, Alamo I, Jr., Hollander MC, Carrier F, Ron D, Kohn KW, Hoffman B, Liebermann DA, Fornace AJ, Jr. 1994b. The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. Mol Cell Biol 14: 2361–2371.
Zhan Q, Fan S, Smith ML, Bae I, Yu K, Alamo I, Jr., O'Connor PM, Fornace AJ, Jr. 1996. Abrogation of p53 function affects gadd gene responses to DNA base-damaging agents and starvation. DNA Cell Biol 15: 805–815.
Zhan Q, Chen IT, Antinore MJ, Fornace AJ, Jr. 1998. Tumor suppressor p53 can participate in transcriptional induction of the GADD45 promoter in the absence of direct DNA binding [published erratum appears in Mol. Cell. Biol. 18 (9) (1998) 5620]. Mol Cell Biol 18:2768–2778.
Zhan Q, Antinore MJ, Wang XW, Carrier F, Smith ML, Harris CC, Fornace AJ, Jr. 1999. Association with Cdc2 and inhibition of Cdc2/cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene 18:2892–2900.
Zhan Q, Jin S, Ng B, Plisket J, Shangary S, Rathi A, Brown KD, Baskaran R. 2002. Caspase-3 mediated cleavage of BRCA1 duringUV-induced apoptosis. Oncogene 21: 5335–5345.
Zhao H, Jin S, Fan F, Fan W, Tong T, Zhan Q. 2000. Activation of the transcription factor Oct-1 in response to DNA damage. Cancer Res 60:6276–6280.
zur Hausen H. 2002. Papillomaviruses and cancer: From basic studies to clinical application. Nat Rev Cancer 2: 342–350.