Journal of Cellular Biochemistry 103:1607–1620 (2008)

Signaling of DNA Damage is Not Sufficient to Induce p53 Response: (Re)Activation of wt p53 Protein Strongly Depends on Cellular Context

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Abstract It is generally accepted that exposure of cells to a variety of DNA-damaging agents leads to up-regulation and activation of wild-type (wt) p53 protein. We investigated the (re)-activation of p53 protein in two human cancer cell lines in which the gene for this tumor suppressor is not mutated: $HeLaS_3$ cervix carcinoma and MCF-7 breast cancer cells, by induction via different genotoxic and cytotoxic stimuli. Treatment of human cells with the alkylating agent N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) or different anti-cancer drugs resulted in a strong DNA damage as evidenced by Comet assay and a marked increase in site-specific phosphorylation of H2AX. Unlike in MCF-7 cells, in $HeLaS_3$ cells the expression of p53 protein did not increase after MNNG treatment despite a strong DNA damage. However, other agents for example doxorubicin markedly induced p53 response in $HeLaS_3$ cells. After exposure of these cells to MNNG, the ATM-dependent effector proteins Chk2 and NBS1 were phosphorylated, thereby evidencing that MNNG-induced DNA breakage was recognized and properly signaled. In $HeLaS_3$ cells wt p53 protein is not functional due to E6-mediated targeting for accelerated ubiquitylation and degradation. Therefore, the activation of a p53 response to genotoxic stress in $HeLaS_3$ cells seems to depend on the status of E6 oncoprotein. Indeed, the induction of p53 protein in $HeLaS_3$ cells in response to distinct agents inversely correlates with the cellular level of E6 oncoprotein. This implicates that the capability of different agents to activate p53 in $HeLaS_3$ cells primarily depends on their inhibitory effect on expression of E6 oncoprotein. J. Cell. Biochem. 103: 1607–1620, 2008. © 2007 Wiley-Liss, Inc.

Key words: cell cycle arrest; DNA breaks; checkpoints; ATM kinase; single cell gel electrophoresis

The p53 tumor suppressor protein is a central player in the decision-making process after cellular stress induced by various stimuli, for example, DNA-lesions, nutritional deficiencies, growth factor deprivation, and imbalance in the nucleotide pool. p53 integrates the incoming positive and negative signals and induces the appropriate cellular response, depending on the severity of the damage. In case the cell is able to repair the lesions or to overcome the nutritional shortage, a reversible cell cycle arrest is induced. When the problems are solved, for example repair of the DNA, the cells are able to re-enter cell cycle and continue to grow and divide. On the other hand, if the damage is too severe, up-regulated p53 induces apoptosis, thereby

Abbreviations used: ATM, ataxia telangiectasia mutated; ATR, ATM-Rad3-related; BER, base excision repair; Chk1, checkpoint kinase 1; Chk2, checkpoint kinase 2; CP, cisplatin; DOX, doxorubicin; E, etoposide (VP-16); HDM2, human double minute 2; HIPK2, homeodomain-interacting protein kinase 2; HPV, human papilloma virus; HRR, homologous recombinational repair; LMP, low melting point; MCM7, minichromosome maintenance 7; MDM2, mouse double minute 2; MMR, mismatch repair; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; NBS1, Nijmegen breakage syndrome 1; NER, nucleotide excision repair; NHEJ, non-homologous end joining; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PD, Petri dish; PVDF, polyvinylidene difluoride membrane; RING, really interesting new gene; WCL, whole cell lysate; wt, wild-type.

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Received 10 July 2007; Accepted 23 July 2007

DOI 10.1002/jcb.21548

removing the cell from the multicellular organism via a strictly regulated process, making sure that the damaged or mutated cell cannot harm the organism for example by developing a tumor. The central role of p53 in DNA damage repair and signaling is also reflected by the fact that it is involved in all five major repair pathways: nucleotide excision repair (NER) [Kastan et al., 1991] and base excision repair (BER) [Offer et al., 1999; Seo and Jung, 2004], as well as in non-homologous end joining (NHEJ), homologous recombinational repair (HRR), and mismatch repair (MMR) [Bernstein et al., 2002; Viktorsson et al., 2005].

The carcinogenic compound N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) is an alkylating agent which causes DNA lesions [Singer, 1975] that can lead to programmed cell death [Eadie et al., 1984; Loveless, 1969]. Importantly, in the majority of cells the DNA damage response is p53-dependent. It has also been shown that the activation of p53 as a consequence of DNA damage induced by MNNG, is mediated via the ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) kinases, and subsequently by the checkpoint kinase 1 (Chk1), checkpoint kinase 2 (Chk2), and Nijmegen breakage syndrome 1 (NBS1) effector kinases [Vidal et al., 2005]. The information about DNA damage is thereby transferred to p53 by the mentioned kinases via phosphorylation of a specific serine amino acid moiety of the tumor suppressor protein at position 15 in the human protein [Vidal et al., 2005; Parra et al., 2001] which corresponds to serine 18 in the mouse protein [Oren, 1999]. It has been shown, that ATM is responsible for the early phosphorylation of p53 whereas ATR induces the late modification at the serine residue [Vidal et al., 2005].

A specific phosphorylation of p53 at serine 15 abolishes the MDM2-mediated degradation of the protein via the ubiquitin-dependent pathway leading to a significant elevation of the half-life and of the level of p53 protein. In normal cells MDM2 is the partner of p53 in an autoregulatory, negative feedback loop, and keeps the amount of p53 under tight control. This is highly important because an elevated level of p53 in healthy cells would inhibit cell cycle progression or induce apoptosis, leading to a strong selection against these cells. However, after DNA injury the level and activity of p53 have to rise immediately and significantly and this is provided by the specific phosphorylation of p53 that makes it inaccessible to MDM2-mediated polyubiquitylation.

In human $HeLaS_3$ cervix carcinoma cells p53 is usually not detectable even after severe DNA damage induced by MNNG [Wesierska-Gadek et al., 2003] although the tumor suppressor protein is not mutated in these cells. This phenomenon is attributable to the action of the human papilloma virus (HPV)-encoded E6 oncoprotein [Wesierska-Gadek et al., 2002] that is present in $HeLaS_3$ cells. In contrast, in human MCF-7 breast cancer cells, the treatment with MNNG leads to a strong up-regulation and activation of p53. Importantly, MCF-7 cells do not harbor the E6 oncoprotein. To clarify the mechanisms by which different agents and drugs act in tumor cells, we compared the effect of MNNG with the chemotherapeutic drugs cisplatin (CP) (cis-diammine-dichloro-platinum; CDDP), doxorubicin (DOX), and etoposide (VP-16) (E) on MCF-7 and $HeLaS_3$ cells.

A careful analysis of the cellular response in reaction to various treatments of the cells showed that MNNG treatment resulted in an accumulation of phosphorylated Chk2 and NBS1, the effector proteins of ATM, indicating that the DNA damage-dependent signaling upstream of p53 was not compromised in both human tumor cell lines. The lack of an up-regulation of p53 protein in $HeLaS_3$ cells that is in contrast to the situation in MCF-7 cells, indicates that the presence of the E6 oncoprotein is responsible for the missing activation and elevation of the tumor suppressor protein. Indeed, expression of viral E6 protein in MCF-7 cells abolished their p53 response to MNNG-generated DNA injury. Most importantly, inhibition of E6 protein with distinct chemotherapeutics facilitated the up-regulation of p53 protein also in the HPV positive cervix carcinoma cell line.

This finding provides a rationale for the activation of p53 in tumor cell lines that possess a wild-type (wt) *p53* gene where the protein is only down-regulated or rendered inactive through the action of proteins that have an adverse impact on p53. A fine-tuned chemotherapeutic intervention based on these findings might provide a strong benefit for patients who bear a tumor in which wt p53 protein is apparently solely inhibited by the cellular background.

MATERIALS AND METHODS

Drugs and Chemicals

CP (PLIVA-Lachema, Czech Republic), DOX, and E (Calbiochem-Novabiochem, La Jolla, CA) as well as MNNG and caffeine (Sigma Co., St. Louis, MO) were used in this study.

Cell Culture

The human $HeLaS_3$ cervical carcinoma and the MCF-7 breast cancer cell line (American Type Culture Collection) were used. Some experiments were performed using MCF-7 cells transfected with CMV empty vector or with HPV-encoded E6 oncoprotein. $HeLaS_3$ cells were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS) at 37°C in an atmosphere of 5% CO_2 and MCF-7 cells were cultured in Dulbecco's medium without phenol red supplemented with 10% FCS at 37°C in an atmosphere of 8% CO_2. MCF-7/CMV-E6 cells were cultured in conventional Dulbecco's medium supplemented with 10% FCS at 37°C in a an atmosphere of 8% CO_2. Cells were grown to 60–70% confluence and then treated with 5 and 50 μM MNNG or with anti-cancer drugs (2 μg/ml DOX, 10 μg/ml E, or 40 μM CP) for indicated periods of time. In some cases cells were exposed to UV radiation (50 J/m^2) or pretreated with caffeine at a final concentration of 10 mM.

Antibodies

We used the following antibodies: monoclonal anti-p53 antibody DO-1, a kind gift from Dr. B. Vojtesek (Masaryk Memorial Cancer Institute, Brno), monoclonal anti-PARP-1 antibody (C-2-10), monoclonal anti-MCM-7 antibody (DCS-141), anti-Chk1 and anti-HPV16/18 E6 oncoprotein were from Santa Cruz Biotechnology (Santa Cruz, CA), polyclonal anti-caspase-3 antibody was from DAKO (Glostrup, Denmark). Monoclonal anti-proliferating cell nuclear antigen (PCNA) antibodies (clone PC-10) and anti-HPV 16/18 E6 oncoprotein were purchased from Oncogene Research Products (Cambridge, MA) and anti-Ran antibodies were from Transduction Laboratories (Lexington, KY). Antibodies directed against site-specific phosphorylated and total proteins Chk1, Chk2, NBS1/p95, and histone H2AX were from Cell Signalling Technology, Inc., (Beverly, MA). Monoclonal anti-actin (clone C4) antibodies were from ICN (ICN Biomedicals, Aurora, OH).

Appropriate secondary antibodies linked to horseradish peroxidase (HRP) were from Amersham International (Little Chalfont, Buckinghamshire, England).

Comet Assay

The generation of DNA-strand breaks was assessed by the single cell gel electrophoresis assay performed under alkaline conditions [Singh et al., 1988; Tice et al., 2000]. The experiments were carried out according to the guidelines published by Tice [Tice et al., 2000]. Untreated $HeLaS_3$ and MCF-7 control cells and cells exposed for 1 h to 5 or 50 μM MNNG were harvested in phosphate-buffered saline (PBS) and the viability of cells was determined using the Trypan blue exclusion test. Only samples exhibiting a viability of at least 80% were used for DNA-migration analysis. Cell suspensions (1×10^5 cells) were mixed with low melting point (LMP) agarose and spread on agarose-precoated slides. After a short incubation at +4°C cells were lysed for 1 h and submitted to horizontal electrophoresis at 25 V for 30 min under alkaline conditions. After electrophoresis slides were neutralized and stained with propidium iodide (20 μg/ml). For each experimental point at least three cultures were analyzed. From each culture 50 cells were evaluated. Comet tail length (μm) and tail moment were measured under a fluorescence microscope (Nikon Model 027012) using an automated image analysis system based on a public domain NIH image program [Helma and Uhl, 2000].

Measurement of DNA Concentration of Single Cells by Flow Cytometry

The measurement of DNA content was performed by flow cytometric analysis based on a slightly modified method of Vindelov [Vindelov et al., 1983] as described previously in detail [Wesierska-Gadek and Schmid, 2000]. Propidium iodide-stained cells were measured using the Becton Dickinson FACScan. DNA histograms were prepared using the CellQuest evaluation program. The distribution of cells in distinct cell cycle phases was determined using ModFIT cell cycle analysis software.

Electrophoretic Separation of Proteins and Immunblotting

Total cellular proteins dissolved in SDS sample buffer were separated on 10 or 15%

SDS slab gels and transferred electrophoretically onto polyvinylidene difluoride membrane (PVDF) (Amersham International). Equal protein loading was confirmed by Ponceau S staining. Membranes were saturated in 5% non-fat milk in PBS-Tween-20 as previously described. Blots were incubated with specific primary antibodies and the immune complexes were detected autoradiographically using appropriate peroxidase-conjugated secondary antibodies and chemiluminescent detection reagent ECL+ (Amersham International) [Wesierska-Gadek et al., 2002; Wesierska-Gadek and Schmid, 2000]. In some cases, blots were used for sequential incubations. For detection of phosphorylated proteins, blots were saturated in 3% BSA in Tris-saline-Tween-20 and then incubated and washed in Tris-saline-Tween-20 according to the manufacturer's recommendations [Wesierska-Gadek et al., 2004].

RESULTS

Strong DNA Damage After Exposure of Human Cancer Cells to N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)

To assess the effect of the alkylating agent MNNG on human cancer cells, we examined the direct cytotoxicity of the drug on cells using a dye exclusion assay. Treatment with 5 μM MNNG for 1 h resulted in an increased accumulation of Trypan blue (approximately 10–15%) and 7-actinomycin D (7-AAD) in $HeLaS_3$ and MCF-7 cells as compared to the untreated control. At 50 μM MNNG the ratio of Trypan-blue positive cells increased approximately two-fold. After 3 h at 50 μM MNNG almost all $HeLaS_3$ cells accumulated the dye, thereby evidencing increased cytotoxicity of MNNG at high doses and especially after longer treatment. The determination of cell viability was a prerequisite for the Comet assay. Usually, only samples possessing approximately 80% viability are used for the single cell gel electrophoresis (Comet assay).

The cells were exposed to MNNG for 1 h at a final concentration of 5 or 50 μM. As depicted in Figure 1A cells exposed to MNNG generated Comets during cell electrophoresis. MNNG at higher concentration resulted in massive DNA strand breaks. Upon single cell electrophoresis, damaged DNA migrated almost completely out of the nuclei (Fig. 1A) and there remained only residual DNA anchored to the nuclear envelope or in the nucleoli. Therefore, the Comet assay was not evaluable by the image analysis program of cells exposed to 50 μM MNNG.

The damage of DNA was quantified by determination of tail length (μM) and tail moment according to the international recommendations [Tice et al., 2000; Helma and Uhl, 2000]. As shown in Figure 1B values of tail length as well as of tail moment after 1 h at 5 μM MNNG increased about five-fold or eight-fold, respectively, as compared to untreated $HeLaS_3$ controls. MNNG also heavily damaged DNA in human MCF-7 cells (Fig. 1B). Statistical analysis revealed the high statistical significance of the increase. After post-incubation of MNNG-treated $HeLaS_3$ cells for 3 h in a drug-free medium a slight increase in tail length was observed (Fig. 1B). The severe DNA injury after action of 50 μM MNNG coincided with the increased loss of cell viability. These results unequivocally show that MNNG generates massive DNA damage in the examined human cancer cells even at low concentrations.

N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) Does Not Affect the Distribution of Cells in the Cell Cycle Phases

The flow cytometric measurement of DNA concentration in single cells revealed that exposure to MNNG for increasing periods of time neither affected the distribution of HeLa cells in distinct cell cycle phases nor induced apoptosis (Fig. 2). Unlike MNNG, CP induced apoptosis in $HeLaS_3$ cells after 15 h of treatment as evidenced by the accumulation of sub-G_1 cells (Fig. 2). These results are consistent with our previous observations [Wesierska-Gadek et al., 2002; Wesierska-Gadek et al., 2003] of accumulation of $HeLaS_3$ cells in the sub-G_1 phase after CP treatment. On the other hand, treatment of MCF-7 cells with CP did not induce apoptosis and only weakly affected their cell cycle progression. After exposure of MCF-7 cells to MNNG, changes in the distribution of cells in the cell cycle phases occurred. An accumulation of G_1-arrested cells was accompanied by a slight decrease in the G_2 population of cells after 15 h (Fig. 2).

N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) Strongly Up-Regulates p53 Protein in MCF-7 but Not in $HeLaS_3$ Cells

Since DNA damage is known to induce a p53 response, we proved the action of MNNG on p53

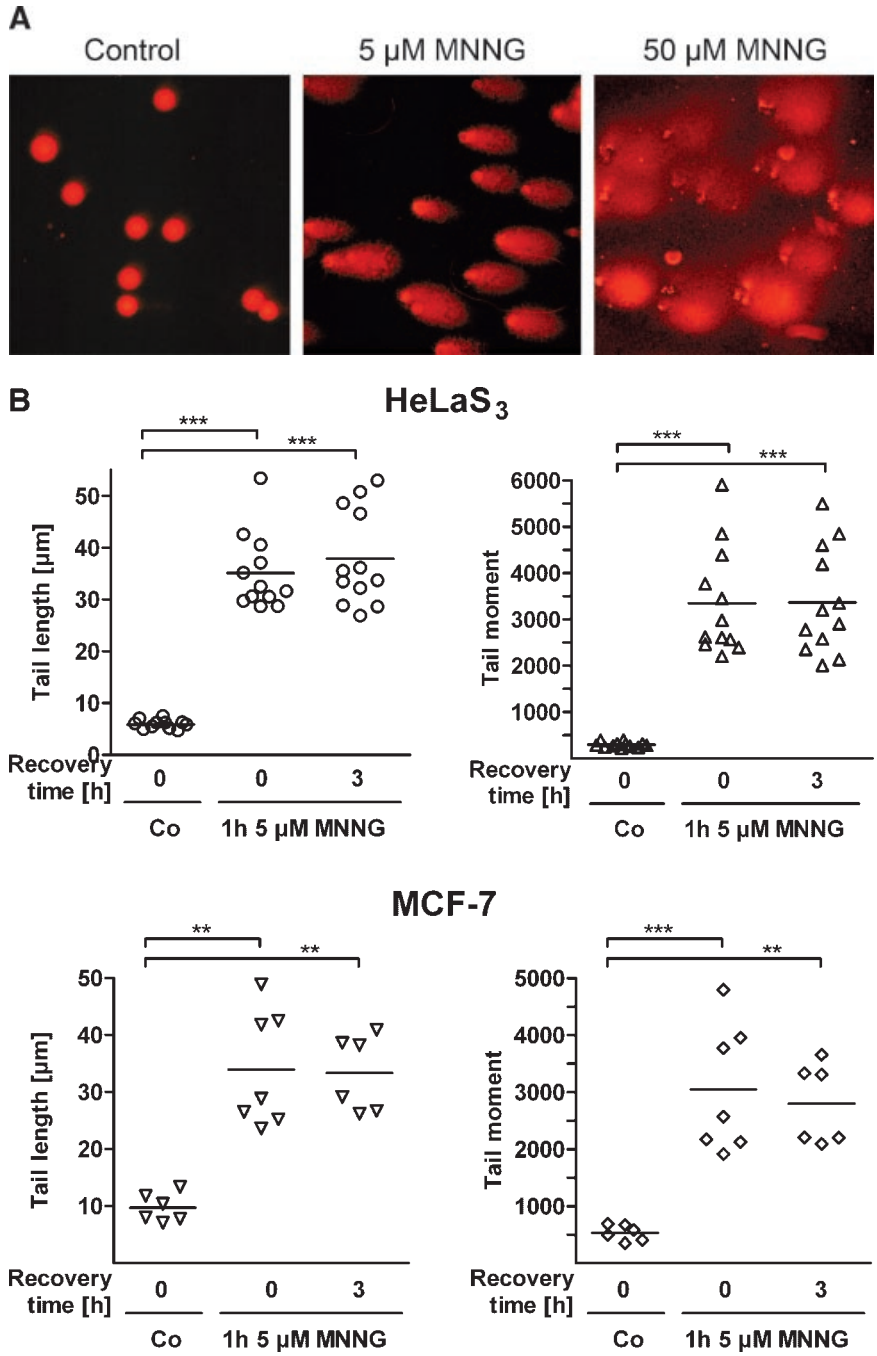

Fig. 1. Short exposure of human cancer cells to MNNG results in strong DNA damage. Human HeLaS$_3$ and MCF-7 cells were treated with 5 or with 50 μM MNNG for 1 h. Thereafter, in some samples medium was changed and cells were post-incubated in a drug-free medium for 3 h. Untreated cells and cells exposed to MNNG were subjected to the single cell gel electrophoresis assay. The DNA was stained with propidium iodide. At least two independent experiments were performed; in each experiment cells harvested from 3 to 4 distinct Petri dishes (PDs) were resolved by electrophoresis. 50 cells per PD were evaluated. **A**: Propidium iodide staining of HeLaS$_3$ cells. **B**: Quantification of Comet assay – values of tail length and of tail moment. Bars indicate means ± SD of at least seven PDs per experimental point. Overall *P*-values were estimated using the Kruskal–Wallis test. The *P*-values of the mean differences were determined with Dunn's multiple comparison test. Stars indicate statistical significance: $^{***}P < 0.0001$; $^{**}P = 0.001$.

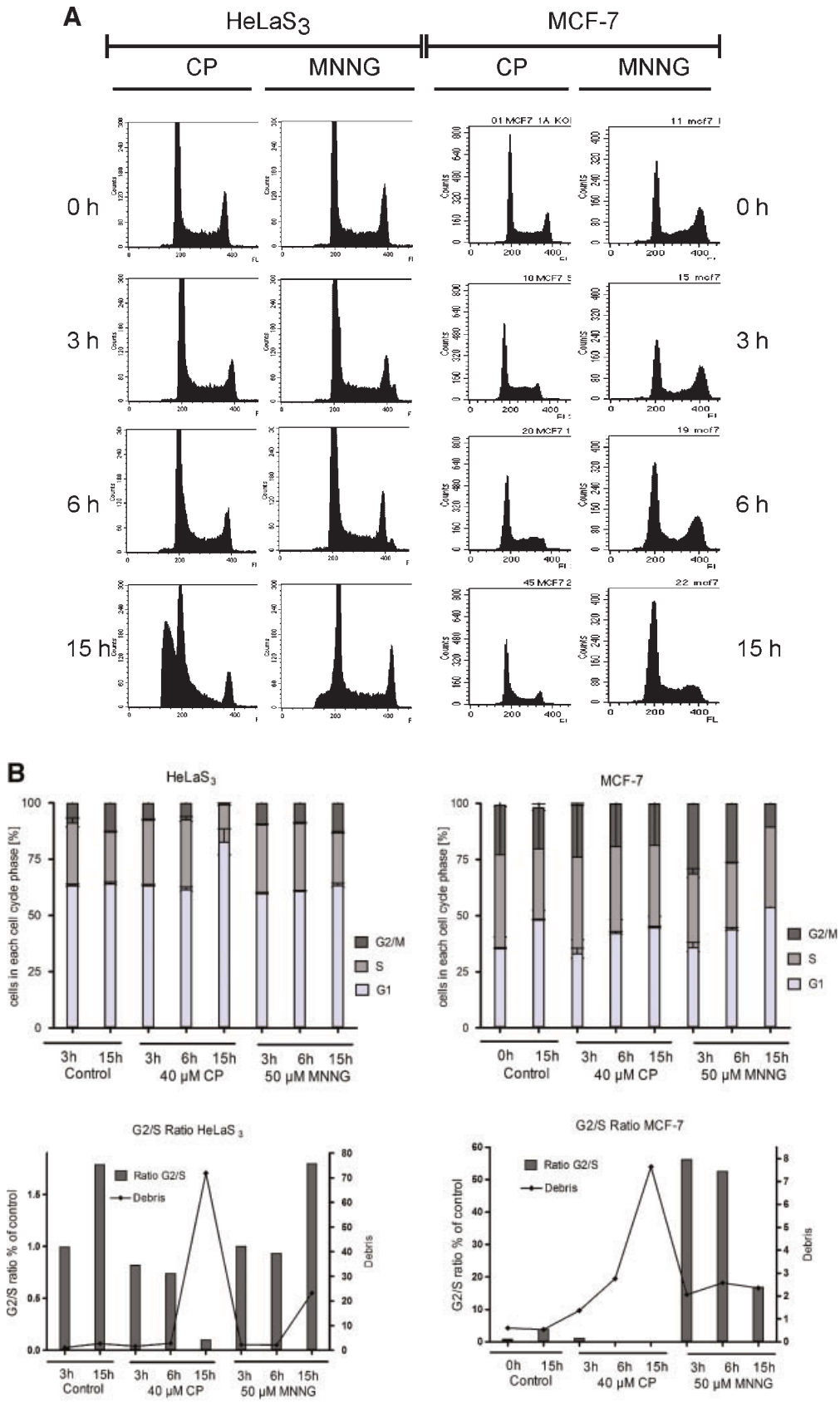
A
HeLaS3
MCF-7
CP
MNNG
CP
MNNG
0 h
3 h
6 h
15 h
B
HeLaS3
MCF-7
cells in each cell cycle phase [%]
G2/M
S
G1
3h 15h 3h 6h 15h 3h 6h 15h
Control
40 μM CP
50 μM MNNG
0h 15h 3h 6h 15h 3h 6h 15h
G2/S Ratio HeLaS 3
G2/S Ratio MCF-7
G2/S ratio % of control
Ratio G2/S
Debris

Fig. 2.

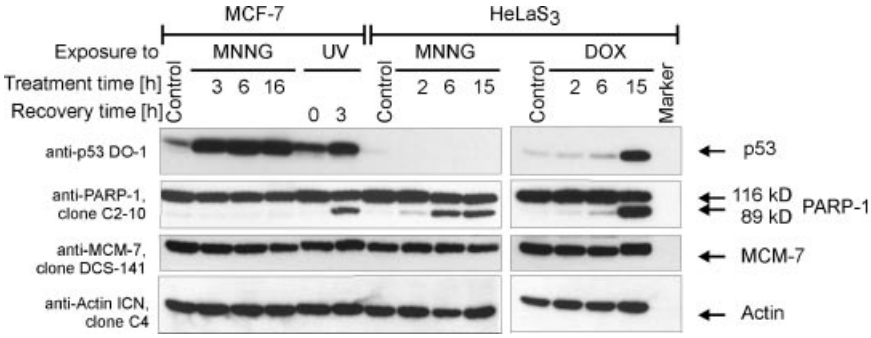

Fig. 3. Induction of p53 protein in MCF-7 cells but not in HeLaS$_3$ cells after treatment with MNNG. Whole cell lysates (WCLs) obtained from human cancer cells exposed to 50 μM MNNG or other stress stimuli for indicated periods of time were separated on 10% SDS gels and transferred electrophoretically onto a PVDF membrane. The blot was incubated with anti-p53 MAb DO-1. The membrane was sequentially incubated with anti-PARP-1, with anti-MCM-7, and then with anti-actin antibodies.

in HeLaS$_3$ and MCF-7 cells. In MNNG-treated MCF-7 cells the p53 response was strongly induced. The p53 increase was comparable with that observed upon exposure to UV radiation or treatment with DOX. Despite strong DNA-injury, p53 was not up-regulated in MNNG-treated HeLaS$_3$ cells (Fig. 3). However, other anti-cancer drugs such as DOX (Fig. 3) and CP [Wesierska-Gadek et al., 2002] activated p53 protein in a time-dependent mode. These results are consistent with our previous report that p53 can be re-activated in HeLaS$_3$ cells by CP [Wesierska-Gadek et al., 2002] but not by MNNG [Wesierska-Gadek et al., 2003] and raise the questions why MNNG does not induce p53 response in HeLaS$_3$ cells despite strong DNA injury.

Proper Signaling of DNA Damage in N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)-Treated HeLaS$_3$ Cells

The induction of p53 upon DNA damage strongly depends on the recognition of damage by the Rad1-Rad9-Hus1 protein complex and sequential induction of the signaling cascade encompassing activation of ATM or ATR kinase and then phosphorylation of effector kinases such as Chk1 and Chk2. As shown in Figure 4, Chk2 and NBS1 (also called nibrin) became phosphorylated upon exposure of HeLaS$_3$ cells to MNNG. Unlike Chk2, Chk1 was only weakly activated upon exposure of HeLaS$_3$ cells to MNNG (Fig. 5). Finally, the activation of site-specific phosphorylation of H2AX was examined. As depicted in Figure 6, γ-H2AX was more strongly accumulated in HeLaS$_3$ cells upon 15 h MNNG than after treatment with anti-cancer drugs (DOX, CP, or E). The highest increase in γ-H2AX levels did not coincide with the activation of caspases. As depicted in Figure 6, caspase-3 was activated in HeLaS$_3$ cells after CP treatment for 10–15 h. This correlates very well with the accumulation of sub-G$_1$ cells detected by flow cytometric analysis (Fig. 2).

Caffeine Inhibits Signaling of DNA Damage in N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)-Treated HeLaS$_3$ Cells

DNA strand breaks result in primarily activation of ATM kinase what in turn induces the signaling cascade. To check whether the strong activation of effector kinase upon MNNG treatment was mediated by ATM kinase, we examined the effect of MNNG alone or after pretreatment with caffeine, a known inhibitor of ATM/ATR kinases, on the activation of Chk2

Fig. 2. The effect of MNNG and CP on the distribution of human cancer cells in distinct cell cycle phases determined by flow cytometry. Exponentially growing HeLaS$_3$ and MCF-7 cells treated with 50 μM MNNG or with 40 μM CP for indicated periods of time were harvested and stained with propidium iodide. Measurement of DNA content was performed on the Becton Dickinson FACScan. Two independent experiments were performed; in each experiment at least two cultures per time point and treatment were stained and measured. A hypoploid population of cells (sub-G1) was detected only in HeLaS$_3$ cultures treated with CP for 15 h. **A**: DNA profile of HeLaS$_3$ and MCF-7 cells. **B**: Distribution of HeLaS$_3$ and MCF-7 cells in distinct cell cycle phases (upper panel) and comparison of the G$_2$/S ratios and ratio of cell debris after exposure of human cancer cells to MNNG and CP (lowe panel). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

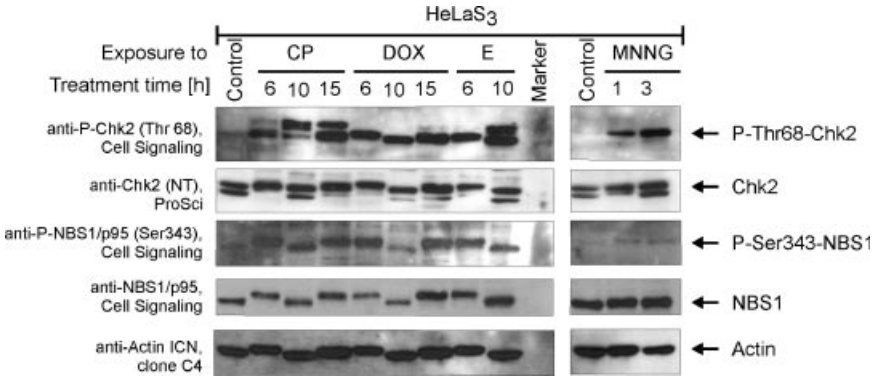

Fig. 4. Signaling of DNA injury in $HeLaS_3$ cells exposed to MNNG. WCLs from $HeLaS_3$ cells exposed to 40 μM CP, to 2 μg/ml DOX, 10 μg/ml E, or to 50 μM MNNG for indicated periods of time were resolved on 10% SDS gels and electroblotted. To detect the activated, site-specifically phosphorylated form of Chk2 kinase as well as NSB1/p95 nibrin, blots were incubated with antibodies directed against the phosphorylated form and sequentially with antibodies against total protein irrespective of their post-translational modifications. Equal protein loading was checked by incubation with anti-actin antibodies.

kinase. As shown in Figure 7, caffeine abolished the site-specific phosphorylation of Chk2 after MNNG and DOX treatment, thereby evidencing that DNA strand breaks generated in $HeLaS_3$ cells upon MNNG treatment activated ATM kinase.

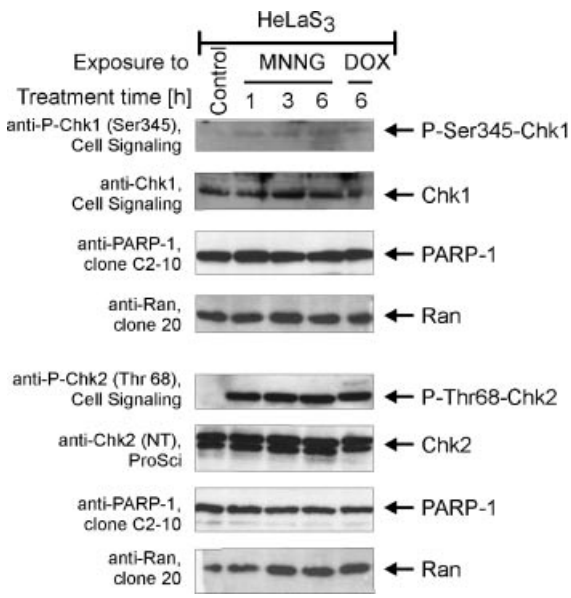

Fig. 5. Chk2 is more strongly activated than Chk1 in $HeLaS_3$ cells upon treatment with MNNG. WCLs prepared from untreated human $HeLaS_3$ cells and cells exposed to 50 μM MNNG and to 2 μg/ml DOX for indicated periods of time were separated on 10% SDS gels and transferred electrophoretically onto a PVDF membrane. The conditions of immunoblotting were the same as described in Figure 4. Blots were incubated with antibodies against phosphorylated forms of Chk1 and Chk2 and sequentially with antibodies against total proteins irrespective of their post-translational modifications. Equal protein loading was checked by incubation with anti-Ran antibodies.

Repression of E6 Contributes to the Up-Regulation of p53 in Stressed $HeLaS_3$ Cells

Finally, we addressed the question, why p53 protein was not induced in $HeLaS_3$ cells despite proper signaling of DNA injury. Since the stability of p53 protein in $HeLaS_3$ cells is primarily controlled by E6 oncoprotein, we monitored its level in $HeLaS_3$ cells exposed to different DNA damaging agents. As shown in Figure 8, the increase in p53 protein coincided with the repression of the E6 protein level. This observation confirms previous results that reactivation of p53 protein in $HeLaS_3$ cells depends on its escape from E6-mediated degradation [Wesierska-Gadek et al., 2002].

Expression of E6 in MCF-7 Cells Abolishes the Activation of p53 After N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) Generated DNA Lesions

To evidence that expression of viral E6 protein is responsible for the lack of induction of the p53 pathway in MNNG-treated $HeLaS_3$ cells, we compared the effect of the alkylating agents on parental MCF-7 cells and MCF-7 cells expressing E6 oncoprotein (Fig. 9). In parental cells MNNG even at low concentrations activated p53 protein. In E6 oncoprotein positive MCF-7 cells MNNG failed to induce p53 despite a strong DNA injury (Fig. 9). Thus, these results unequivocally demonstrate that expression of E6 oncoprotein inactivates proper p53 response to DNA damaging insults independently of cellular background.

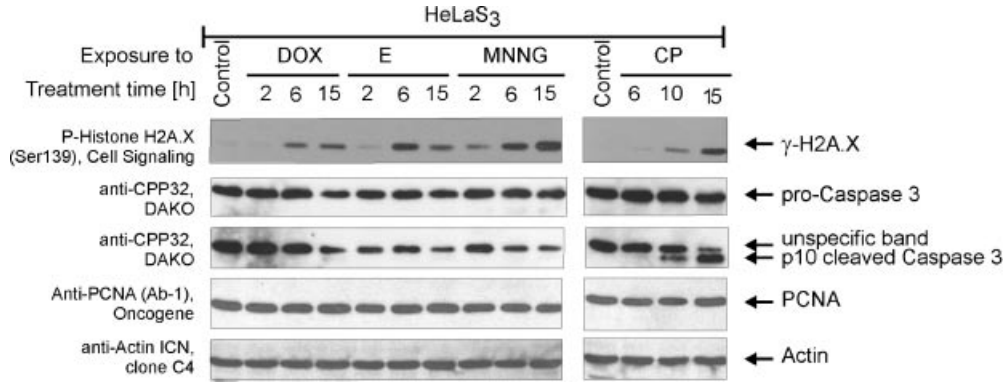

Fig. 6. Induction of γ-H2AX in HeLaS$_3$ cells treated with MNNG and different anti-cancer drugs. WCLs prepared from untreated human HeLaS$_3$ cervix cancer cells and cells exposed to 2 μg/ml DOX, to 10 μg/ml E, or to 50 μM MNNG for indicated periods of time were separated on 10 or 15% SDS gels and transferred electrophoretically onto a PVDF membrane. The blots were incubated with the anti-P-Ser139 H2AX antibody, with anti-caspase-3 antibody and with anti-PCNA antibody. The membrane was sequentially incubated with anti-actin antibodies.

DISCUSSION

The monofunctional alkylating agent MNNG is an environmental carcinogen that, upon activation, reacts preferentially with nucleic acids resulting in N-alkylation of the DNA base guanine and subsequently in DNA double strand breaks [Singer, 1975; Shulman, 1993]. Generated adducts such as O^6-alkyl-guanine are genotoxic [Eadie et al., 1984]. The mutagenicity is attributable to mispairing properties of the substituted base that might lead to point mutations and chromosomal aberrations [Kleihues and Margison, 1974]. The DNA lesions immediately induce a cellular emergency program to preserve genomic integrity [Adamson et al., 2002; Levine, 1997] that encompasses a series of surveillance pathways including cell-cycle checkpoints. Initiation of cell-cycle checkpoints arrests cell cycle progression, thereby providing time for detection and repair of damaged DNA.

Members of the Rad family of checkpoint proteins, widely expressed in all eukaryotic cells, are involved in the damage recognition. Three of these proteins of the clamp complex termed 9-1-1 namely Rad1, Rad9, and Hus1 that exhibit structural similarity to PCNA, seem to be sensors for DNA injury [Abraham, 2001; Green et al., 2000; O'Connell et al., 2000]. During the earliest stages of checkpoint activation, the damage information is transduced to

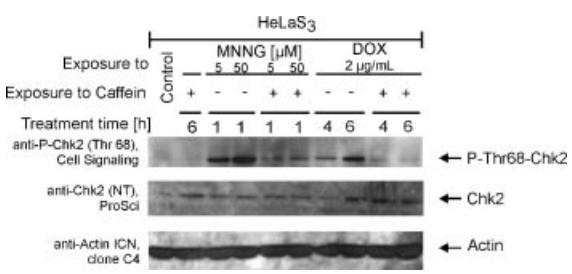

Fig. 7. Exposure of MNNG-treated HeLaS$_3$ cells to caffeine abolishes the activation of Chk2. Asynchronously growing naive or caffeine-pretreated HeLaS$_3$ cells were treated with 50 μM MNNG or with 2 μg/ml DOX for indicated periods of time and subjected to preparation of WCLs. Proteins were separated on 10 or 15% SDS gels and transferred electrophoretically onto a PVDF membrane. The blots were incubated with the anti-P-Thr68 Chk2 antibody and sequentially with anti-Chk2 antibody recognizing total protein irrespective of its phosphorylation. The membrane was sequentially incubated with anti-actin antibodies.

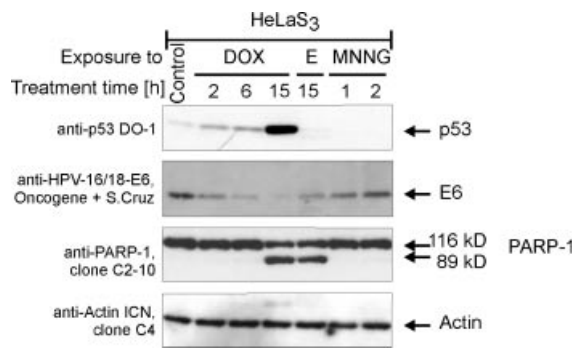

Fig. 8. Up-regulation of p53 protein in stressed HeLaS$_3$ cells coincides with the repression of E6 oncoprotein. WCLs prepared from untreated human HeLaS$_3$ cervix cancer cells and cells exposed to 2 μg/ml DOX, to 10 μg/ml E, or to 50 μM MNNG for indicated periods of time were separated on 10 or 15% SDS gels and transferred electrophoretically onto a PVDF membrane. The blots were incubated with the anti-p53 antibody DO-1 and anti-E6 antibodies. The membrane was sequentially incubated with anti-PARP-1 and then with anti-actin antibodies.

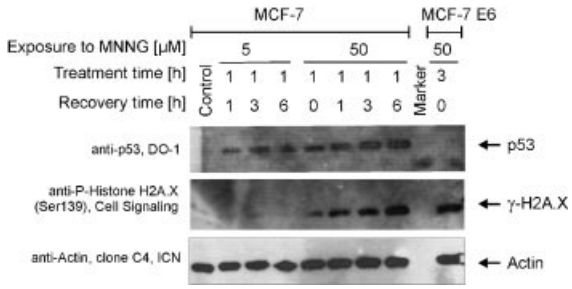

Fig. 9. Expression of viral E6 protein in MCF-7 cells abolishes the activation of p53 tumor suppressor protein in response to MNNG generated DNA lesions. Asynchronously growing MCF-7 cells or MCF-7/E6 cells were treated with 5 and 50 μM MNNG for indicated periods of time, respectively, and lysed. WCLs (30 μg protein/lane) were separated on 10 or 15% SDS gels and transferred electrophoretically onto a PVDF membrane. The blots were incubated with the anti-p53 antibody DO-1 and anti-P-Ser139 histone H2AX antibodies. The equal protein loading was checked after sequential incubation with anti-actin antibodies.

the members of the apical kinases ATM and ATR that belong to the family of phosphoinositide-3-kinase related kinases (PIKK) [Abraham, 2001; Durocher and Jackson, 2001; Shiloh, 1997]. In mammalian cells, ATM and ATR seem to be responsible for control and regulation of all known cell-cycle checkpoints, except for the mitotic spindle checkpoint activated after exposure of cells to nocodazole, a microtubule polymerization poison [Abraham, 2001; Durocher and Jackson, 2001]. Double strand DNA breakage generated in cells resting in G_1 phase induces preferentially ATM kinase which initiates a series of events, among others leading to the accumulation and activation of p53 tumor suppressor protein [Adamson et al., 2002; Banin et al., 1998; Fritsche et al., 1993].

The increase in cellular p53 levels is attributable primarily to its protein stabilization. In unstressed healthy cells wt p53 protein is extremely unstable due to the activity of its negative regulator mouse double minute 2 protein (MDM2) (in human cells designated HDM2) [Haines et al., 1994; Momand et al., 1992; Oliner et al., 1993]. The *hdm2* gene is p53-dependent. It harbors in the first intron a p53-specific regulatory element acting in cis [Zauberman et al., 1995].

HDM2, a really interesting new gene (RING) finger E3 ubiquitin ligase [Haines et al., 1994; Fuchs et al., 1998; Haupt et al., 1997; Kubbutat et al., 1997], interacts with the amino-terminal part of p53 protein and inhibits its transcriptional activity as well as targets for polyubiquitylation and subsequent proteasomal degradation [Fuchs et al., 1998; Haupt et al., 1997; Kubbutat et al., 1997]. Some amino-terminal serine residues at position 15, 18, and 20 of p53 that are targets of distinct protein kinases are critical for the interaction with HDM2. Phosphorylation of the critical serine residues releases p53 from destabilizing interaction with HDM2 that is accumulated in the nuclei [Appella and Anderson, 2001]. Moreover, after Pin1-mediated isomerization p53 becomes active as transcription factor [Zacchi et al., 2002; Zheng et al., 2002]. The main kinases targeting the site-specific phosphorylation of p53 are ATM, Chk1, and Chk2. It is worth mentioning that ATM kinase has a dual role: it can directly modify p53 at serine 15 or can induce the activating phosphorylation of Chk2 kinase. Similarly, ATR kinase can also phosphorylate p53 directly, or via activation of Chk1.

Exposure of human MCF-7 breast cancer cells and $HeLaS_3$ cervix cancer cells to the alkylating agent MNNG resulted in a strong DNA damage. A short treatment with MNNG even at low concentrations generated double strand breaks as evidenced by the Comet assay. In the light of these findings one would expect that exposure of human cancer cells expressing wt p53 protein to MNNG, would initiate a series of emergency events leading to activation of a p53 response. As expected, exposure of human MCF-7 breast cancer cells to MNNG resulted in a strong accumulation of p53 protein. After MNNG treatment for 3 h, the p53 level was highly elevated and exceeded that occurring after 3 h recovery from exposure to UV. Unlike in MCF-7 cells, in $HeLaS_3$ cells no increase in p53 could be detected despite a strong DNA damage. Interestingly, after treatment with DOX for 15 h, a strong up-regulation of p53 protein was observed, thereby evidencing that the p53 stress signaling pathway in $HeLaS_3$ cells is not impaired.

Therefore, we posed the question why strong DNA injury generated by MNNG does not induce a p53 response in $HeLaS_3$ cells. First, we explored whether in these cells the operating systems for detection and signaling of DNA damage work properly. The γ-H2AX protein was induced in $HeLaS_3$ not only by MNNG but also by different anti-cancer drugs. Surprisingly, the highest γ-H2AX levels were observed very early upon treatment with MNNG. Exposure of $HeLaS_3$ cells to MNNG led to the activation of

Chk2 kinase and nibrin (NBS1/p95) as detected by an increase in their site-specific phosphorylation. The increase in activating phosphorylation of Chk2 kinase preceded the strong induction of γ-H2AX. The phosphorylated form of histone H2AX is colocalized with ATM in complexes generated in close proximity to DNA double strand breaks [Paull et al., 2000]. It is well established that activated ATM kinase induces the phosphorylation of its downstream effectors such as Chk2, H2AX, and nibrin.

Nibrin (NBS1), the product of the gene mutated in the human chromosomal instability disorder called Nijmegen breakage syndrome (NBS) [Shiloh, 1997], is an important participant in the S-phase checkpoint that is usually induced in normal cells upon DNA damage. Nibrin is found in a complex with two other proteins involved in the maintenance of genome integrity, Mre11 and Rad50 (NMR complex) [Petrini, 1999]. ATM phosphorylates nibrin on three distinct serine residues and it seems that the specific, phosphorylated nibrin form is necessary for formation of NMR complexes. To substantiate the responsibility of ATM kinase in the cellular response to MNNG-generated DNA damage, in the next series of experiments we compared the extent of the Chk1 and Chk2 activation in $HeLaS_3$ cells. A strong phosphorylation of Chk2 at Thr68 was already induced after exposure of $HeLaS_3$ cells to MNNG for 1 h. Unlike Chk2, Chk1 was only very weakly modified at Ser345, thereby indicating that according to the previous reports [Adamson et al., 2002] MNNG induced in $HeLaS_3$ cells primarily ATM but not ATR apical kinase. Finally, the involvement of ATM kinase in the activation of damage signaling upon MNNG treatment was evidenced by pre-treatment of $HeLaS_3$ cells with caffeine, a specific inhibitor of ATM/ATR kinases [Sarkaria et al., 1998]. Pretreatment of $HeLaS_3$ cells with caffeine abolished the activating phosphorylation of Chk2 in response to MNNG and DOX. These results clearly evidence that the recognition and signaling of DNA lesions generated by MNNG or distinct anti-cancer drugs properly works in $HeLaS_3$ cells.

In the light of these findings the question appeared how to explain the obvious difference in the MNNG-mediated activation of the p53 response between human MCF-7 and $HeLaS_3$ cells? Although in both human cancer cell lines the *p53* gene is not mutated, they differ in the pathways regulating expression and functionality of its gene product. While in MCF-7 cells the stability of p53 protein is preferentially controlled by its own negative regulator HDM2 and under certain conditions by alternative proteins, among others by the homeodomain-interacting protein kinase 2 (HIPK2) [Hofmann et al., 2002; Wesierska-Gadek et al., 2007], in human cervix carcinoma $HeLaS_3$ cells, p53 is targeted by E6 protein encoded by the oncogenic HPVs [Scheffner et al., 1993; Scheffner et al., 1990]. The E6 viral oncoprotein, recruiting the cellular ubiquitin-protein ligase E6-associated protein (E6-AP) activity, targets p53 for accelerated degradation through its enhanced ubiquitylation. It has been demonstrated that in the latter a switch from HDM2 to E6-mediated degradation of p53 occurs [Hengstermann et al., 2001].

Remarkably, it has been demonstrated that E6 does not induce ubiquitylation of p53 in the same way as HDM2 in order to promote its degradation, suggesting important differences between the HDM2 and E6 effects on p53 degradation [Camus et al., 2003]. Indeed, a very recent report provides the first evidence of an E6-dependent, ubiquitin-independent, p53 degradation pathway in vivo [Camus et al., 2007].

Since some anti-cancer drugs like CP or DOX are able to up-regulate p53 protein in $HeLaS_3$ cells, the question appeared whether for reactivation of p53 expression upon stress stimuli in $HeLaS_3$ cells, besides reduction or inactivation of the p53 destabilization by the E6-mediated ubiquitylation, an additional activity, for example, down-regulation of viral E6 protein would be necessary. We have previously observed that proteasomal inhibitors did not induce the accumulation of ubiquitylated forms of p53 in human $HeLaS_3$ cervix carcinoma cells and only one out of four used inhibitors was able to increase cellular p53 [Wesierska-Gadek et al., 2002]. The monitoring of the E6 oncoprotein level in untreated control $HeLaS_3$ cells and cells exposed to MNNG and two anti-cancer drugs revealed that the down-regulation of the E6 oncoprotein levels closely correlates with the increase of cellular levels of p53 protein. Whereas DOX after longer treatment is able to repress E6, neither E nor MNNG have the capability to negatively affect its levels. Previously, the escape of wt p53 from E6-mediated degradation was reported after treatment of $HeLaS_3$ with CP [Wesierska-Gadek et al.,

2002]. These observations strongly support the assumption that for activation of the p53 pathway in stressed $HeLaS_3$ cells a repression of E6 oncoprotein is required. It seems that some anti-cancer drugs are able to block the transcription of virally encoded proteins. Interestingly, repression of virally encoded E6 protein in HPV positive cervix carcinoma cells promoted induction of apoptosis [Wesierska-Gadek et al., 2002; Butz et al., 1996]. Thus, MNNG fails to negatively affect the expression of E6 oncoprotein in $HeLaS_3$ cells. These results are supported by more recent reports [Vidal et al., 2005] and also provide explanation why a strong DNA injury generated in $HeLaS_3$ cells by MNNG did not initiate apoptosis [Wesierska-Gadek et al., 2003].

The difference in the escape from the negative regulation of p53 expression after DNA damage by endogenous HDM2 auto-regulatory feedback loop and virally encoded E6 oncoprotein was finally evidenced by comparison of the activation of the p53 pathway between the MCF-7 mother cell line and MCF-7/E6 cells. Exposure of the latter to MNNG failed to induce a p53 response despite a strong DNA damage that was detected by an increase in site-specific phosphorylation of histone H2AX. These results are in concordance with a number of recent studies in which siRNA-induced repression of E6 expression led to re-activation of the p53 pathway in HPV-positive cells [Bai et al., 2006; Courtete et al., 2007]. Recent reports have revealed that compounds of plant origin, for example, lignans from the creosote bush (3′-O-methyl-nordihydroguaiaretic acid) can repress the viral promoter responsible for E6 gene expression [Allen et al., 2007].

The induction of the p53 pathway upon exposure of human cancer cells to MNNG might also affect the cell cycle progression. Accumulation of human MCF-7 cells but not of $HeLaS_3$ in G_1 and G_2 phases of the cell cycle after MNNG treatment became evident and was at least partially attributable to the activation of the p53 pathway and to the intact checkpoints in the former.

Taken together, our results show that the signaling of DNA damage is not sufficient to induce p53 response in cells in which strong DNA lesions were generated. The possibility of the activation of a p53 response depends on the cell type and the ability to release p53 protein from its negative regulatory circuit.

ACKNOWLEDGMENTS

We thank Paul Breit for preparation of an image depicting the Comet assay.

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